The Role of Tetraspanins in *Pseudomonas aeruginosa* Adherence to Human Cells

A thesis submitted for the Degree of Doctor of Philosophy

By

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Declaration

I declare that the work presented in this thesis is the work of the candidate, with the following exceptions:

- The production of the tetraspanin EC2 fusion proteins was performed by Dr Marzieh Fanaei, Dr John Palmer and Dr Rachel Hulme.
- The immunofluorescent microscopy investigation for the effects of EC2: GST proteins on *S. aureus* adhesion to HaCaT cells was performed by Dr Jenny Ventress (Figure 4.15 A and B).

I also, declare that this thesis has not been submitted for a degree to any other university or institution.

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Abstract

Infection of host tissue is commonly initiated by adherence of pathogenic bacteria, such as *Pseudomonas aeruginosa,* to cell surface membrane proteins, sugars or lipids. One class of proteins known to play a major role in cellular processes, such as adhesion are tetraspanins, specifically CD9, CD81 and CD151. Prevention of adhesion could provide a new therapeutic strategy for treating infectious diseases. This work aims to investigate the potential of using recombinant tetraspanin extracellular domains (EC2), anti-tetraspanin antibodies and CD9 EC2-derived peptides as anti-adhesives in treating *P. aeruginosa* infections. Also, to create a new higher throughput method to measure bacterial adhesion using flow cytometry.

Expression profiles of different tetraspanins in human epithelial cell lines, A549, HEC1B and keratinocyte HaCaT cells were visualised using immunofluorescent microscopy and quantified using flow cytometry. Monolayers of adherent cells were pre-treated with the tetraspanin reagents (EC2: GST recombinant proteins, anti-tetraspanin antibodies and CD9 EC2-derived peptides), before infection with GFP-labelled *P. aeruginosa* (PAO1) for 60 minutes. Bacterial adhesion was determined by counting the number of bacteria per infected cells with fluorescence microscopy. Analysis revealed the anti-adhesive properties of these tetraspanins (anti-tetraspanin antibodies and recombinant proteins).

In conclusion, a flow cytometry method was developed but was found to be too un sensitive to measure changes in bacterial adhesion. Moreover, anti-tetraspanin antibodies, recombinant proteins and CD9 EC2- derived peptides may have the potential to inhibit adherence of *P. aeruginosa*. This work has identified a role for tetraspanins in bacteria-host cell adhesion, and the possible use of tetraspanin reagents as therapeutics for treating *P. aeruginosa* infection.

Conferences

- Poster presentation at **the 6th Europen Conference on Tetraspanins** on 18th, 19th and 20th June 2014 in Lille, France.

- Poster presentation at **SfAM Antimicrobial Conference** on 24th November 2016 in London, UK.

- Oral presentation at the 7th **Tetraspanin Conference** on 7th, 8th and 9th September 2016 in Sheffield, UK

- Poster presentation at the **9th Saudi Student Conference UK** on the 14th and 15th of February 2016 in Birmingham, UK.

- Poster presentation at the **Yorkshire Immunology Group Symposium** on 15th May 2017 in Leeds, UK.

- Poster presentation at the **16th International Conference on *Pseudomonas*** on 5th, 6th, 7th, 8th and 9th September 2017 in Liverpool, UK.

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List of Abbreviations

AHLs	N-Acyl homoserine lactones
AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CCR	Chemokine receptors
CF	Cystic fibrosis
CFU	Colony forming units
COPD	Chronic obstructive pulmonary diseases
CXCR4	CXC Chemokine Receptor 4
DAPI	4,6-Diamidino-2-phenyl-indole Hydrochloride
DMEM	Dulbecco's Modified Essential Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E1	Envelope
EC1	Extracellular domain 1
EC2	Extracellular domain 2
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EMEM	Eagle's Modified Essential Medium
EPS	Extracellular polymeric substances
ExoS	Exoenzyme S
ExoT	Exoenzyme T
ExoU	Exoenzyme U
ExoY	Exoenzyme Y
FACS	Fluorescence activated cell sorting
FCR	Fc Receptor
FCS	Foetal Calf Serum
FITC	Fluorescein isothiocyanate
FnBP	Fibronectin binding protein
FSS	Forward scatter
GFP	Green fluorescent protein
HBSS	Hank's Balanced Salt Solution
HCV	Hepatitis C virus
HEC1B	Human endometrial carcinoma-1-B
HIV	Human immunodeficiency virus
HPV	Human papilloma virus
HSPG	Heparan sulphate proteoglycans

HTLV	Human T-cell leukaemia virus
IC_{50}	50 % inhibitory concentration
IL	Interleukin
LB	Lysogeny Broth
LDH	Lactate Dehydrogenase
LEL	Large extracellular loop
LPS	Lipopolysaccharide
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OD	Optical Density
PAO1	*Pseudomonas aeruginosa* strain O1
PBS	Phosphate buffered saline
PI4KIIα	Type II phosphatidylinositol 4-kinase alpha
PI4KIIβ	Type II phosphatidylinositol 4-kinase beta
RT	Room temperature
SEL	Small extracellular loop
SEM	Standard Error of the Mean
siRNA	small interfering ribonucleic acid
SSC	Side scatter
T1SS	Type 1 secretion system
T2SS	Type 2 secretion system
T3SS	Type 3 secretion system
TEM	Tetraspanin enriched microdomains
Tfp	Type IV pilus
TLR	Toll-like receptors
TM	Transmembrane domains
UPEC	Uropathogenic *E. coli*
UTI	Urinary tract infection

CHAPTER 1: Introduction

1.1 Tetraspanins

Tetraspanins are a family of transmembrane proteins, of up to 350 amino acids, with molecular weights ranging from 20-50 kDa. Since their discovery in the early 90s in human melanoma cells (Wright and Tomlinson, 1994; Maecker *et al*., 1997), they have been found in all cell types apart from red blood cells (Boucheix *et al*., 2001). Tetraspanins are integral membrane proteins that associate with different types of proteins through which they mediate cellular processes such as fusion, adhesion, invasion, tissue differentiation and immunological responses through different molecular signalling (Hemler, 2003; Hemler, 2005; Levy and Shoham, 2005b; Tham *et al*., 2010; Hong *et al*., 2012). In humans, 33 of these proteins have been identified with varying expression in different cell types as well as in other eukaryotic organisms like protozoans, fungi and plants (Fanaei *et al*., 2011; DeSalle *et al*., 2013).

1.1.1 The structure of tetraspanin proteins

All tetraspanins have a similar structural pattern, which consists of four hydrophobic transmembrane domains (TM1-TM4) with cytoplasmic and extracellular loops (Figure 1.1). The four transmembrane domains forming two extracellular loops (EC1 with between 20 to 28 amino acids and EC2 with between 76 to 131 amino acids) and one cytoplasmic loop (Maecker *et al*., 1997; Huang *et al*., 2010). The N and C termini, typically short sequences of amino acids that may be longer in some members, are located on the cytoplasmic side of the membrane. The EC2 loop, also called the large extracellular loop (LEL), is larger than EC1 which is also referred to as short extracellular loop (SEL), and contains a constant region with 3 highly conserved α-helices and a hypervariable region with between 2 and 4 disulphide linkages that are essential for almost all tetraspanin interactions (Seigneuret *et al*., 2001; Hemler, 2005). The TM1, TM3 and TM4

domains also contain polar residues of unknown functions and a number of post translational modification sites, usually palmitoylation on cysteine residues within the transmembrane regions (Stipp *et al*., 2003; Levy and Shoham, 2005b).

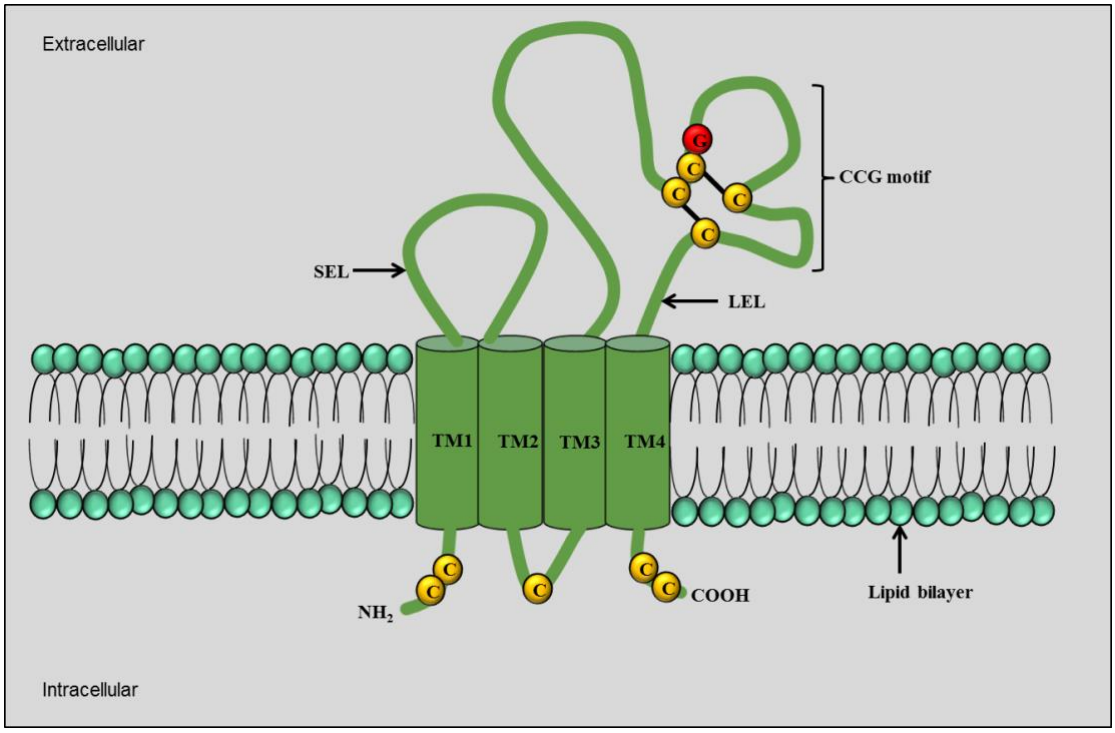

Figure 1.1 A schematic representation of tetraspanin structure.

Tetraspanins are composed of four trans-membrane (TM) domains (green cylinders) and contain conserved amino acid sequences. TM1 and TM2 flank the short extracellular loop (SEL), whereas TM3 and TM4 flank the long extracellular loop (LEL). Shown here is the conserved cysteine-cysteine-glycine (CCG) motif in the LEL, which forms disulphide bridges with additional conserved cysteine residues (C). Also highlighted here are the conserved cysteine residues in the amino (NH_2) and carboxy (COOH) terminal regions and the inner loop of the intracellular portion of the molecule. Adapted from Levy and Shoham, 2005.

1.1.2 Tetraspanins mediate protein-protein interactions and play roles in infectious diseases

There are now several pieces of evidence that tetraspanins have a major role in the lateral arrangement of cell membrane proteins (Boucheix *et al*., 2001; Hemler, 2003; Hemler, 2008; Charrin *et al*., 2009). Tetraspanins interact with different proteins including other tetraspanins in a tetraspanin-tetraspanin interaction, or with receptors and other signaling proteins to make up a functional complex at the cell surface, called a tetraspanin enriched microdomain (TEM) (Hemler, 2003; Wright *et al*., 2004; Charrin *et al*., 2009). This consequently leads to the involvement of tetraspanins in several cellular processes, including differentiation, proliferation, migration and invasion in both malignant and infectious diseases (Hemler, 2003; Wright *et al*., 2004; Zoller, 2009). New insights into tetraspanin protein-protein interactions and microdomain compositions have shown TEM to be dynamic in nature, conferring on cells the ability to adapt to their constantly evolving environment (Barreiro *et al*., 2008; Espenel *et al*., 2008). This may also explain why most tetraspanin interactions with other proteins are temporary or weak.

It is evident that the interaction between tetraspanins and their partner molecules results in the formation of functional units. For instance, CD151 (tetraspanin) interacts with integrins α6β4, α5β1 and α3β1 to regulate integrin-dependent cell migration, motility and strengthening of adhesion. This is done in part by regulating the trafficking of these integrins across the cell membrane through the modulation of endocytosis and vesicular trafficking of these CD151-associated integrins (Liu *et al*., 2007). Patients that harbour a mutation in the CD151 integrin binding domain are known to suffer skin blistering and hereditary nephritis (Karamatic Crew *et al*., 2004). CD9 and CD81 associates with CD9P-1, an immunoglobulin domain molecule, to maintain muscle regeneration ability. Mice

lacking these two tetraspanins lose the ability to regenerate muscles through the formation of huge dystrophic myofibres that are formed more rapidly in mice lacking both tetraspanins (Charrin *et al*., 2013). These studies demonstrate that dysfunction or lack of tetraspanin expression can lead to the development of various phenotypic defects. Interestingly, deficiency of the human CD53 protein has been associated with chronic infectious diseases caused by viruses, bacteria and fungi (Mollinedo *et al*., 1997). Tetraspanins functionally interact with known immune receptors including protein constituents of B cell receptor complex, MHC molecules, C type lectins (selectins) and integrins. As such, tetraspanins are able to modulate the immune system through receptor signalling and regulate proliferation of leukocytes, antigen presentation and cytokine expression.

1.1.2.1 The role of tetraspanins in viral infections

Viruses are obligate intracellular parasites, relying on the host cell machinery for survival during various stages of their life cycle in order to cause infection (Hemler, 2008). Tetraspanins have been shown to play important roles in the pathogenesis of intracellular pathogens such as viruses by enhancing the life cycle and the ability to invade and colonise host cells. For instance, some viruses use TEM to facilitate entry into host cells and to infect other cells (Dahmane *et al*., 2014). As such, tetraspanins play important role in the pathogenesis of many viruses considering the poor infectivity of viral particles.

1.1.2.1.1 Tetraspanins and HIV infection

Tetraspanins take part in the pathogenesis of HIV-1 infection, which is responsible for the development of AIDS. HIV-1 infects mainly CD4+ T-cells and also other antigen presenting cells such as macrophages and monocytes. It acts by primarily binding to CD4

on target cells to gain entry into the host cells, and subsequently binds to CCR5 or CXCR4, which are chemokine receptors (Martinez-Munoz *et al*., 2014). Afterwards, there is the internalisation of the virus by endocytosis or by cell fusion at the cell surface. This allows the virus to replicate its genetic material using the host replicating machinery. The first indication that tetraspanins play a role in the pathogenesis of HIV-1 was the discovery in H9T cells that CD63 selectively co-localises at budding structures and newly formed HIV-1 virions (Meerloo *et al*., 1992). Usually, during HIV infection, there is cell-cell adhesion or fusion between uninfected and infected cells leading to transmission of viral material and subsequent separation. These cells could otherwise form what is known as a syncytium. CD9 and CD63 have been recently shown to negatively regulate the HIV-1 induced cell-cell adhesion by inhibiting the transmission from hemifusion of the cells to opening of the pore for viral particle transfer (Symeonides *et al*., 2014). Conversely, CD63 was shown to support Env mediated cell-cell fusion through CCR5 and CD4 as CD63 siRNA treated R5X4-tropic HIV-1 strains had marked inhibition in infectivity while the MLV or VSV-pseudotyped strains expressing an NL4-Env protein did not show inhibition of infectivity (Li *et al*., 2014). This suggests that infection of non-infected cells may be cell type dependent, probably due to alternative pathways for regulating infectivity at the hemifusion stage. In addition, CXCR4 is a coreceptor like CCR5, and it is required in the alternative pathway of HIV infectivity because it allows adhesion of infected cells with new host cells when binding through CCR5 is prevented. As such, there is CCR5 or CXCR4 tropic HIV infection. Interestingly in IL-21 activated B cells, CD63 was shown to inhibit the expression of CXCR4 by increasing endocytosis of CXCR4, which is activated by the increased expression of GRK6, an endocytosis related molecule in CD4+ T cells (Yoshida *et al*., 2011).

1.1.2.1.2 Hepatitis C virus

The role of tetraspanins in the pathogenesis of viral infections has been widely documented and one of the initial findings was the identification of CD81 as a receptor for HCV (Pileri *et al.*, 1998). HCV is the major cause of liver disease and is also associated with cryoglobulinaemia, a B lymphocyte proliferative disorder. HCV has two envelope glycoproteins, E1 and E2, which are covalently linked as a dimer (E1E2) and both glycoproteins are required for virus binding and host cell invasion (Op De Beeck *et al.*, 2004). It has been demonstrated that palmitoylation of cysteine residues on CD81 plays an important role in protein-protein interactions in TEM and in association with cholesterol-rich domains of membranes. Inhibition of this modification of CD81 reduces its interaction with other tetraspanins and thus reduces infectivity of the host cell by HCV (Zhu *et al.*, 2012). It was recently demonstrated that EWI-2wint, an inhibitor of HCV during cell entry reduced the mobility of CD81 by encouraging its confinement and concentration at enriched areas, making it unavailable for HCV entry into target cells (Potel *et al.*, 2013). Thus, changes in the dynamics properties of CD81 are utilised by HCV in the presence of a natural inhibitor to favour target cell invasion.

1.1.2.1.3 Tetraspanins in other viral infections

Apart from HIV and HCV pathogenesis, tetraspanins have also been demonstrated to play key roles in several other viral infections. HTLV-1 is the causative agent of adult human T-cell leukaemia, which uses CD4+ and CD8+ T cells as its viral reservoir (Jones *et al.*, 2006). The infection caused by the virus is likely through cell-cell interaction as virions are usually of poor infectivity and tetraspanins have been shown to maintain cell adhesion and fusion in viruses (Hemler, 2003). Tetraspanins associate with laminin-binding integrins and are instantly sequestered in immunological synapses formed between

antigen presenting cells. T-cells and B-cells predominantly express CD82 as the major tetraspanin on their surface and it has been demonstrated that during HTLV-1 infection, the HTLV-1 virion through its Gag protein interacts with CD82 containing tetraspanin microdomain to facilitate sequestration of newly formed virions to sites of cell-cell interaction (Mazurov *et al*., 2006). Tetraspanins have also been shown to be involved in the pathogenesis of human papillomavirus type 16 (HPV16) which is linked to the development of cervical cancer (Cornet *et al*., 2013). HPV16 is a non-enveloped virus possessing a double stranded DNA molecule and binds to host cells via a heparan sulphate proteoglycan such as syndecan-1 (Letian and Tianyu, 2010). Prior to its internalisation via an endosome, HPV16 that is bound to the cell surface binds to CD151 moving along the plane of the cell membrane. This was demonstrated in a study by Scheffer *et al*., (2013) where CD151 depleted cells showed reduced internalisation of HPV16. In this study, it was also shown that CD151 associated integrins (α3β1 and α6β1/4) are responsible for the HPV16 infection.

1.1.2.1.4 Tetraspanins and bacterial infections

The defence mechanism of a host to bacterial infection is usually associated with bacterial surface structures and its mode of infection (Smith, 1977). Gram-negative bacteria are destroyed by complement factors or cytotoxic cells through cell wall lysis while Gram-positive bacteria or mycobacteria require specialised phagocytic cells such as neutrophils and macrophages. During infection, the activation of the humoral immune system results in antibody production by B-cells while cellular immunity is elicited by T-cells. Some bacterial species, however, have developed techniques to evade the cellular defence mechanisms of the host, some of which involve the alteration of cellular immunity and immune signalling, invasion of the host cell, and neutralisation of immune mediator

actions, most of which involve cell-cell interaction. Adhesion of bacteria to host tissues also provides resistance to mechanical methods of bacterial clearance such as mucus and tears (Pierre *et al*., 2016).

1.1.2.1.4.1 Escherichia coli (E. coli)

Uropathogenic *E. coli* (UPEC) is the major causative agent for urinary tract infections (UTIs) and the only documented direct interaction between a bacterium and tetraspanin is that between UPEC and uroplakin 1a (Svanborg and Godaly, 1997). The pathogenesis of UTI begins with the binding of UPEC to the epithelia of the bladder through the interaction between a bacterial adhesion molecule, FimH adhesin, and uroplakins on the epithelial lining of the urinary tract (Wu *et al*., 1996; Kątnik-Prastowska *et al*., 2014). Uroplakin la (Tetraspanin 21) and uroplakin lb (Tetraspanin 20), together with uroplakin 2 and 3a form a receptor complex which is assembled into a crystal-like structure covering most of the epithelia of the urinary tract (Wang *et al*., 2009). Through this structure, FimH adhesin binds to the epithelial surface to enable adhesion of UPEC. Through this mechanism, UPEC can bind to deeper layers of the bladder with the help of other tetraspanins like CD63 to remain in a quiescent state until the bladder becomes filled whereupon the bacteria become active. It has also been shown that tetraspanin CD82 plays an important role in the formation of phagosomes containing *E. coli* and *Staphylococcus* during infection (Artavanis-Tsakonas *et al*., 2011).

1.1.2.1.4.2 Listeria monocytogens (L. monocytogenes)

L. monocytogenes is a Gram-positive bacteria that is the causative agent for listeriosis (Williams *et al*., 2012) *L. monocytogenes* invades epithelial cells through interaction with E-cadherin and hepatocyte growth factor receptor, Met. Type II phosphatidylinositol 4-

kinase alpha and beta (PI4KIIα and PI4KIIβ) were both identified as required for entry of the bacteria downstream of Met through their interactions with CD81 (Pizarro-Cerda *et al*., 2007; Tham *et al*., 2010; Martinez del Hoyo *et al*., 2015). Tham *et al*. , (2010) showed that the tetraspanins CD81, CD63 and CD9 are all expressed on the cell surface and co-localise with PI4KIIα at the bacterial entry site. Through live cell imaging techniques and siRNA depletion of these tetraspanins, they confirmed that CD81 is required for internalisation of the bacteria into target cells.

1.1.2.1.4.3 Neisseria meningitidis (N. meningitidis)

N. meningitidis is a Gram-negative bacterium that resides in the nasopharynx without any symptoms; the bacteria has been reported to be present in about 15 % of adults. It is the causative agent for meningococcal disease and the main cause of bacterial meningitis (Tunbridge and Read, 2004). Through antigenic and phase variation of molecules like adhesins and invasins, the bacteria is able to bind epithelial cells which aids internalisation (Hill *et al*., 2010). It has been shown that tetraspanins play an important role in bacterial colonisation during initial stages of *N. meningitidis* infection. When cells infected with *N. meningitidis* were treated with anti-CD63, anti-CD9 and anti-CD151 antibodies, adhesion of the bacteria to target epithelial cells was greatly inhibited (Green *et al*., 2011a). CD63, CD151 and CD9 were demonstrated to be responsible for the adhesion of bacteria to target cells because incubation of the cells with antibodies against these tetraspanins (anti-CD63, anti-CD151 and anti-CD9) or incubation with recombinant proteins containing only EC2 domain of these tetraspanins resulted in similar results. Furthermore, it is becoming increasingly clear that cells treated with anti-tetraspanin antibodies experience reduced adhesion with some bacteria such as *Staphylococcus aureus*, *N. lactamica*, *E. coli* and *Streptococcus pneumonia*e (Green *et al*., 2011a; Monk

and Partridge, 2012).

1.1.2.1.4.4 Staphylococcus aureus (S. aureus)

S. aureus is a Gram-positive coccus that colonises the skin, nasal epithelium and respiratory tract. It is the causative agent of folliculitis, cellulitis and impetigo (Johnson *et al*., 2016). *S. aureus* is able to access the tissues through a break in the epidermis and once it colonises the skin, *S. aureus* causes skin and soft-tissue infections (SSTIs), such as itchiness and necrotizing fasciitis (Hersh *et al.*, 2008). Like most bacterial skin pathogens, *S. aureus* invades epithelial cells through fibronectin binding protein (FnBP) (Arrecubieta *et al*., 2008). In addition, the bacteria can also penetrate deeper into tissues from where it can pass into many areas of the body and then causes systemic infections (Blot *et al*., 2002).

As mentioned in section 1.1.2.1.3.1, anti-tetraspanin antibodies and EC2 proteins were found to be able to reduce *S. aureus* adhesion to host cells, and more recently, it was found that the short peptides derived from the EC2 domain of CD9 also could reduce *S. aureus* adhesion to epithelial cell lines, primary keratinocytes and 3D tissue engineered model of the human skin (Ventress *et al*., 2016).

1.2 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a rod-shaped, aerobic Gram-negative bacterium, and an opportunistic pathogen associated with an array of life threatening infections that occur in cystic fibrosis (CF) patients, UTIs, blood stream infections, pneumonia and surgical site infections (Fujitani *et al*., 2011; Lechtzin *et al*., 2011; Dai *et al*., 2013), as well as in immunocompromised patients (Afessa and Green, 2000). *P. aeruginosa* accounts for between 11 to 13.8 % of all known nosocomial infections and in the intensive care unit,

it is responsible for 13.2 to 22.6 % of reported nosocomial infections (Pittet *et al*., 1999; Kim *et al*., 2000; Lizioli *et al*., 2003; Erbay *et al*., 2003; Weinstein *et al*., 2005). In addition, *P. aeruginosa* accounts for up to 50 % of the mortality in patients that are mechanically ventilated (Giantsou and Manolas, 2011) and is the leading cause of morbidity in patients with diseases such as CF and chronic obstructive pulmonary diseases (COPD) (Gallego *et al*., 2014).

1.2.1 Virulence factors and disease pathogeneses of *P. aeruginosa*

P. aeruginosa produces a varying number of the virulence factors that are responsible for the pathogenesis of the clinical diseases involving *P. aeruginosa*. Analysis of the roles of different virulence factors from various animal models has increased the understanding of disease pathogenesis. It is worthy to note that the pathogenesis of *P. aeruginosa* is related to the precise crosstalk between a host of virulence factors, extending from effective colonisation and biofilm formation to tissue invasion, necrosis and or dissemination of the pathogen via the vascular system that may result in systemic or local inflammatory responses. These virulence factors are either intracellular or extracellular based on localisation and the mechanism of action, and the physical relation to the bacterium. Extracellular factors are classified according to their secretion mode into the type I, II and III secretion systems. The type I secretion system (T1SS) is an ABC-exporter system that relies on AprD, an ATP binding cassette (ABC) protein; AprE, a membrane fusion protein and AprF, an outer membrane protein (Duong *et al*., 2001). In *P. aeruginosa*, this system secretes HasAp, a haeme-binding protein required by the pathogen for haemoglobin utilisation, it also secretes AprX, which is a member of the ABC exporter system and alkaline phosphatase (Duong *et al*., 2001).

1.2.1.1 Quorum sensing regulation of virulence in *P. aeruginosa*

1.2.1.1.1 Effectors of the T2SS and the T3SS systems

Components of the quorum sensing system (discussed below) have been shown to be important in invasion and biofilm formation in *P. aeruginosa* pathogenicity (Yang *et al*., 2017). *P. aeruginosa* is known to have five secretory systems but the type II secretion system (T2SS) and the type III secretion systems (T3SS) are the major toxins producing systems. The majority of *P. aeruginosa* virulence factors are produced by the T2SS such as exotoxin-A (PA1148), alkaline protease (PA1246), the LasA protease (PA1871) and LasB elastase (PA3724). The LasA and LasB elastases are controlled by the *N*-Acyl homoserine lactones (AHL) system (see section 1.2.3.1.) and are regarded as the most important factors in acute infections. They are both involved in the degradation of elastin in human lung tissue and in the vasculature via their elastolytic activities (Galloway, 1991). The exoproteins that are produced by the T2SS elicit a pathological response when in contact with eukaryotic cells and at a distance from the site of infection using quorum sensing (Figure 1.2).

The type III secretion system (T3SS) on the other hand produces virulence factors such as the exoenzyme S (ExoS), exoenzyme T (ExoT), exoenzyme U (ExoU) and exoenzyme Y (ExoY), all of which require close contact with the target cells in order to elicit cytotoxic effects (van Delden, 2004; Juan *et al*., 2017). This system uses a transmembrane syringe-like structure, the "injectisome" to introduce toxins into mammalian cells (Quinaud *et al*., 2005; Engel and Balachandran, 2009). Of the virulence factors secreted via the T3SS pathway, ExoS and ExoT have been of most interest in *P. aeruginosa* pathogenicity. It has been reported that a significant proportion of clinical isolates produces ExoS (Feltman *et al*., 2001; Vareechon *et al*., 2017). Although both ExoS and

ExoT are not secreted by the same strain of *P. aeruginosa*, they are both involved in the development of chronic and acute infections (Shaver and Hauser, 2004). The activities of ExoS and ExoT have been elucidated in infections like keratitis and corneal ulcers and their activities in these infections are dependent on ADP ribosyltransferase expression. ExoS and ExoT have been shown to aggravate these infections by promoting neutrophil apoptosis through impairment of the pro-apoptotic processes in neutrophils. Their activities also promote bacterial survival by impairing the anti-bacterial activities of neutrophils (Sun *et al*., 2012). The delicate interplay between these virulence factors is important in the pathogenesis of *P. aeruginosa* infection and this complex interplay is still not fully understood to date.

Evidence for a possible link between the T2SS and T3SS for coordinated infectivity has been documented in the literature. In a study by Cowell *et al*., (2003), *P. aeruginosa* containing mutant lasA and/or lasB genes showed an increase in the basal levels of ExoS and ExoT, which are the T3SS proteins/toxins that inhibit the invasion of the bacteria in the host cell. The increased expression of the T3SS proteins also correlates with a reduction in invasion of the bacteria in a host cell. This suggests that LasA and LasB possibly induce invasion by repressing the expression of ExoS and ExoT expression. This repression allows the biosynthesis of different virulence factors required for host cell attachment and invasion. Although the mechanism by which LasA and LasB regulates ExoS and ExoB is currently not clear, the expression of ExoS and ExoT is controlled by ExsA, which is the transcriptional activator for ExoS and ExoT (Cowell *et al*., 2000), and as such LasA and LasB may regulate expression of these toxins through ExsA.

1.2.2 Pathogenicity of *P. aeruginosa*

Communication between cells mediated by different signalling molecules enables bacteria to trigger orchestrated responses in a way that achieves infectivity (acute or chronic) in host cells. In *P. aeruginosa*, the signalling pathway regulating infection is perhaps one of the most complex ones known to date. In most Gram-negative bacteria, especially in *P. aeruginosa*, *N*-Acyl homoserine lactones (AHLs) are the major cell to cell signalling molecules used in controlling the expression of several genes in a process known as quorum sensing (Withers *et al*., 2001). AHLs are comprised of a fatty acid of varying chain length with an amide bond linking it to a homoserine lactone unit. AHLs are synthesised by the LuxI family of proteins and they bind to LuxR family receptors (transcriptional factors) forming a complex that binds to the DNA, thus regulating the transcription of several virulence genes (Table 1.1).

Table 1.1 Some virulence factors of *P. aeruginosa*.

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PA position	Gene name	Description	References
PA4528	piLD	Motility and attachment	(Beatson *et al*., 2002, Stover *et al*., 2000)
PA0410	pill	Motility and attachment	(Köhler *et al*., 2000)
PA4554	pilYJ	Motility and attachment	(Choi *et al*., 2002)
PA0762	algU	Sigma factor	(Wagner *et al*., 2003)
PA4446	algal	Secretion of alginate	(Firoved and Deretic, 2003)
PA0765	macC	Transcription regulator	(Govan and Deretic, 1996)
PA1248	ETV	Alkaline protease	(Wilhelm *et al*., 1999)
PA3478	rhiB	Rhamnosyl transferase	(Davey *et al*., 2003)
PA3831	pepA	Leucine amino peptidase	(Govan and Deretic, 1996)
PA5112	estA	Esterase	(Govan and Deretic, 1996)
PA5449	wbp	Glycosyl transferase	(Daniels *et al*., 2002)
PA3841	exoS	Exoenzyme S	(Okuda *et al*., 2012)
PA0044	exoT	Exoenzyme T	(Aiello *et al*., 2010)
PA1871	lasA	Elastase A	(Murray *et al*., 2010)
PA3724	lasB	Elastase A	(Murray *et al*., 2010)

1.2.2.1 The Quorum Sensing System

In *P. aeruginosa*, there are two AHL systems, the Las and Rhl systems. The Las system produces *N*-3-oxo-dodecanoyl homoserine lactone (3-oxo-C_{12}-HSL) by the LasI synthase (PA1432), and *P. aeruginosa* responds to the molecule via the LasR (PA1430) transcriptional factor (Passador *et al*., 1993; Pearson *et al*., 1994). Through this system, the bacteria control interaction with host cells, acute infection and host cell damage by regulating expression of T2SS virulence factors (Gambello *et al*., 1993; Passador *et al*., 1993; Jones *et al*., 1993). The Rhl system produces *N*-butanoyl homoserine lactone (C_4-HSL) by Rhl1 synthase and senses the molecule through the RhlR transcriptional factor (PA3477). Both the Las and the Rhl systems function synergistically to produce several virulence factors like the rhamnolipids, staphylolytic proteases, exotoxin A, pyocyanin and lectins (Smith and Iglewski, 2003; Bjarnsholt and Givskov, 2007). The timely orchestration of the biosynthesis of these virulence factors is essential for *P. aeruginosa* pathogenicity. For instance, production of rhamnolipids in the later stages of biofilm formation helps to maintain the biofilm architecture (Davey *et al*., 2003).

Phospholipase C is expressed in different organisms playing diverse roles in cellular biology. For instance, in *L. monocytogenes* it enhances infectivity of the bacteria in eukaryotic hosts while the homologue of phospholipase C expressed by *Clostridium perfringens* is the main cytotoxic agent involved in the pathogenesis of gas gangrene (Awad *et al*., 1995; Marquis *et al*., 1997). *P. aeruginosa* expresses two types of phospholipase C, which are the haemolytic (PlcH) and the non-haemolytic (PlcN) homologues. Both PlcH and PlcN are secreted via the T2SS system through the outer membrane of *P. aeruginosa* and are both used to hydrolyse the phosphatidylcholine component of the host cell membrane, thus resulting in a pathogenic and inflammatory

response (Meyers *et al*., 1992). There also exists some evidence suggesting that the hydrolysis of phosphatidylcholine by phospholipase C in the lung surfactant results in aggravated lung infection (Lema *et al*., 2000). Phospholipase C and rhamnolipids are believed to work synergistically in breaking down lecithin and lipids, enhancing tissue invasion (Van Delden and Iglewski, 1998). Rhamnolipids in this process act as a bio-surfactant to break down the lipid content of membranes, giving extra access to phospholipase C to further breakdown the membrane phospholipid.

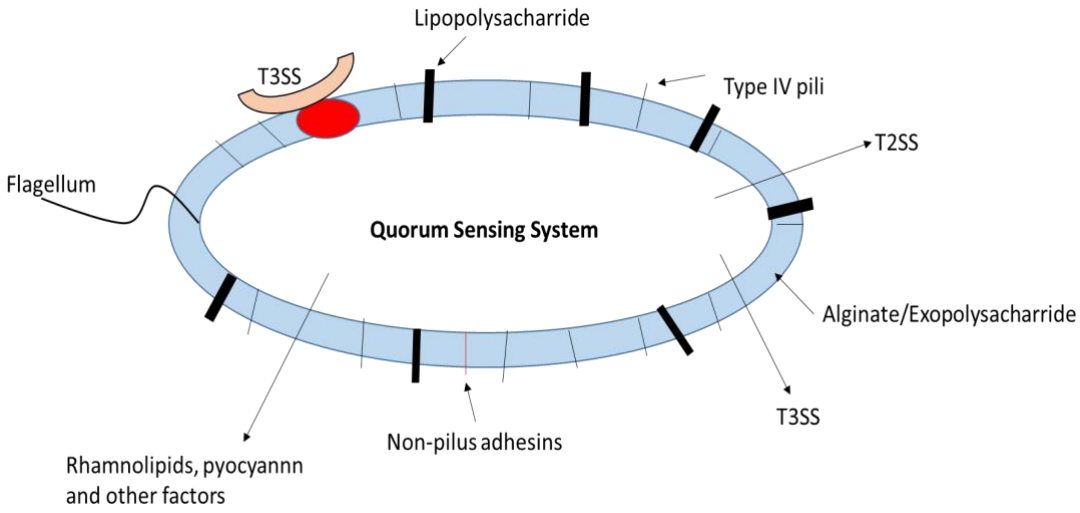

Figure 1.2 Virulence factors involved in quorum sensing in *P. aeruginosa*.
The factors involved in quorum sensing are both cell-associated (involving the type IV pili, flagella and adhesins) as well as the extracellular factors produced by T2SS (LasA and LasB elastases, alkaline protease and phospholipase C) and T3SS (ExoS, ExoT, ExoU and ExoY), and other extracellular factors like rhamnolipids and pyocyanin. The quorum sensing system is used to regulate the activities of the virulence factors. Adapted from van Delden, 2004.

1.2.2.2 Attachment and invasion of *P. aeruginosa* and disease pathogenesis

The pathogenesis of *P. aeruginosa* infections constitutes a complex and multifactorial process. Because *P. aeruginosa* is an opportunistic pathogen, it requires a break in the host's first line of defence such as damage to the mucosal lining or immune suppression as seen in HIV patients. The attachment of bacteria is a crucial initial step involving complex interactions between host cell receptors present on either the apical or basolateral surface of the epithelium and bacterial adhesins. Through the use of epithelial cells cultured as a monolayer, which reflects simple epithelial tissue, it has been demonstrated that N-glycans on the surface of epithelial cells are important molecules sufficient enough to facilitate bacterial binding and subsequent entry as well as the cytotoxicity observed at the apical surface of the polarised epithelium (Bucior *et al*., 2010). On the basolateral surface, heparan sulphate proteoglycan (HSPG) is required and sufficient to facilitate the binding. Bucior *et al*., (2010) demonstrated that on the apical surface of cells that are not completely polarised (tissue damage model), HSPGs are highly expressed and this leads to enhanced binding of *P. aeruginosa* causing tissue damage.

In *P. aeruginosa*, there are two major adhesins that have been discovered so far: the flagellum (Chi *et al*., 1991; Feldman *et al*., 1998; Engel, 2007) and the type IV pilus (Tfp) (Hospenthal *et al*., 2017). The flagellum is a polymer made up of flagellin that is encoded by the *fliC* gene, although several other genes are involved in flagellum assembly and function (Dasgupta *et al*., 2003). The functions of the flagellum include adhesion to the epithelium, swimming motility and formation of biofilm. Monomeric flagellin protein is recognised by the immune system either through its binding to Toll-like receptor 5 (TLR5) present on the cell surface or through cytosolic sensors (Feuillet *et al*., 2006; Balloy *et al*., 2007; Lightfield *et al*., 2008; Kofoed and Vance, 2011). The mechanistic

details of the interaction between flagella and host epithelium are currently lacking but the flagellum cap protein of *P. aeruginosa* strain O1 (PAO1) has been shown to bind to the Lewis^{x} oligosaccharides found in mucins (Scharfman *et al*., 2001). Since mucins are found at high levels on CF epithelial surfaces, this may explain why the bacteria colonises CF epithelium coupled to the fact that CF patients have thick and dehydrated mucus that makes it difficult for ciliary clearance of the bacteria. In a recent study, Bucior *et al*., (2012) demonstrated that Tfp is required to mediate the binding of N-glycans to the apical surface of polarised epithelium while flagella are required for binding of bacteria to the HSPGs on the basolateral surface of the polarised epithelium, thus facilitating bacterial entry. The binding of *P. aeruginosa* flagella to heparan sulphate chains on HSPGs at the basolateral surface of the airway epithelium also leads to activation of EGFR and subsequently PI3/Akt pathway, a process that has been shown to contribute to bacterial cytotoxicity (Zhang *et al*., 2004).

Tfps are appendages that are polarly localised and comprised of pilin polymers. These polymers undergo reversible assembly/disassembly, thus allowing the bacteria to undergo what is termed twitching motility, which allows the bacteria to move across solid surfaces (Mattick, 2002). Tfps also serve as receptors for phage, they facilitate the initial stage of biofilm formation and they function as adhesins to mammalian cells (Engel, 2003). Different glycosphingolipids have been identified in different studies as host receptors for Tfp mediated cell binding at the apical surface of polarised cells (Saiman and Prince, 1993; Comolli *et al*., 1999).

Tfps are among the first set of factors identified in genetic screens in the search for traits necessary for *P. aeruginosa* biofilm formation through their participation in microcolony formation and surface attachment (O'Toole and Kolter, 1998). Chiang and Burrows

(2003) demonstrated that although the Tfp adhesive function is required for biofilm formation, twitching motility also plays an important role in the biofilm architecture development. Bacterial mutants that lack the retraction ATPase PilT (a Tfp Atpase response dynamics) have been shown to form thick, undifferentiated biofilms compared to those that twitch. In a study by Klausen and colleagues, a more detailed explanation about the role of twitching in biofilm formation was provided. They cultured biofilms of non-piliated and wild type *P. aeruginosa* labelled with cyan and yellow fluorescent proteins, respectively. They observed the formation of the mushroom-like structure of mature microcolonies with the non-piliated cells forming the stalk while the wild type cells formed the umbrella above the mutants (Klausen *et al.*, 2003b; Klausen *et al.*, 2003a). The interesting finding was the distinct spatial separation between the cell types. This result suggests that twitching is used by the wild type cells to climb the mutants that are non-motile.

1.2.2.3 Biofilm formation and twitching in *P. aeruginosa*

Biofilms represent an important form of growth for bacteria serving as protection in harsh environmental conditions. Bacteria, especially *P. aeruginosa* usually exists encased as a colony in a biofilm with each bacterium attached to several others and to a surface through a structure made up of extracellular polymeric substances (EPS) (Flemming *et al.*, 2007). The EPS in *P. aeruginosa* is a biopolymer, it contains 2 types of polysaccharides, which are the capsular and the aggregative polysaccharides. The capsular polysaccharide is the major component of the protective structure on the surface of the biofilm and a prominent example is alginate, which is a high molecular weight molecule of β-1,4 linked l-guluronic and d-mannuronic acids repeats. Another example is levan, a high molecular weight molecule of β-2,6 polyfructan. The aggregative polysaccharide confers the biofilm

with structural integrity and facilitates interaction within the biofilm (Mann and Wozniak, 2012). The biofilm is formed as a mushroom-like structure attached to a surface through the stalk. A specific bacterium in this biofilm is believed to move through the stalk up to the cap through a process known as twitching motility (Mann and Wozniak, 2012). The bacteria on the cap structure are further dispersed to non-colonised sites for the formation of new biofilm (Figure 1.3). *P. aeruginosa* at high cell density has been shown to secrete EPS and through quorum sensing forms biofilms that aids resistance against antibiotics, adverse environmental conditions and the immune system (Sakuragi and Kolter, 2007; Zeng *et al*., 2011). A medical implication is that biofilm associated bacteria on catheters or surgical implants use this to their advantage in causing serious infections.

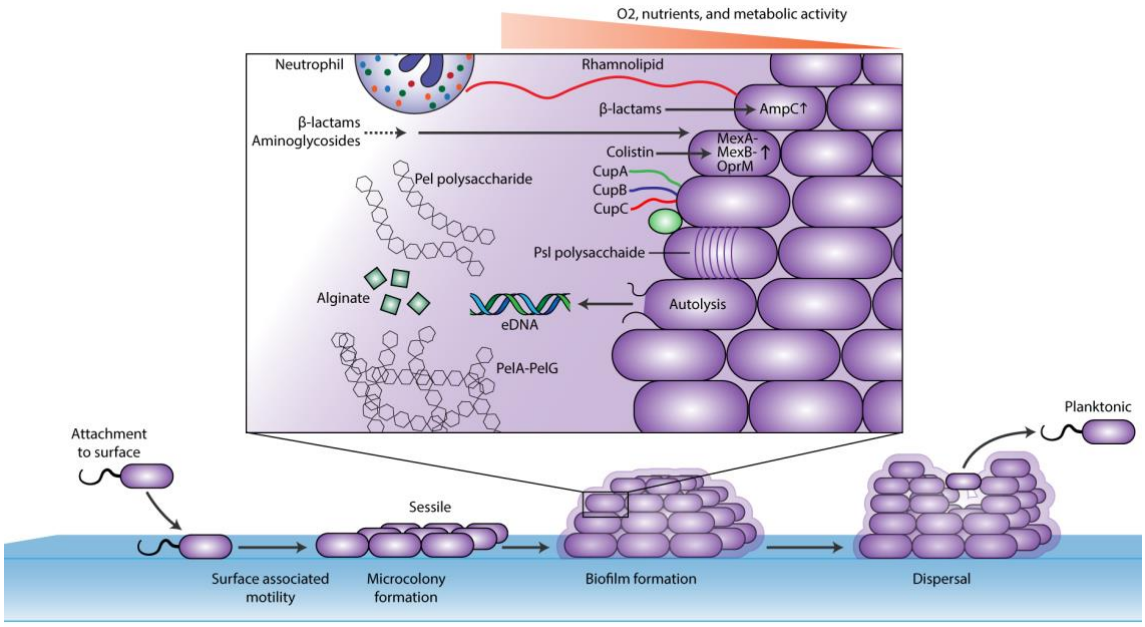

Figure 1.3 Biofilm formation and growth in *P. aeruginosa*.

The stages in biofilm formation involve bacterial attachment to host cell surface followed by growth of bacterial colonies when the structure of the biofilm begins to form. After formation of a matured biofilm, there is a detachment of cells followed by dispersal to other viable sites for attachment of new cells. Adapted from Mann and Wozniak, 2012.

1.3 Mechanisms of bacterial adhesion to host cells

In order to cause infection, the bacterial pathogens need to be in a favourable environment in the host where they can thrive. Bacteria reach their favourable environment by adhering to specific receptors on the host cell surface. Once the bacteria have adhered, they can be internalised by various methods or they can also flourish extracellularly by forming their own favourable conditions, such as a biofilm. There are various types of interactions (mechanical systems) that could be involved in the adhesion of bacteria to host cells; adhesion of bacteria to host cell involves adhesins on the bacterial surface and receptors on the host cells surface. Table 1.2 shows some examples of interactions that are involved in the adherence of bacteria to host cells and Table 1.3 presents some examples of bacteria and the adhesins they use to attach to the host cells.

Table 1.2 Types of adhesin-receptor interactions in bacterial adhesion to mucosal surfaces[a].

Type of interaction	Bacterial adhesin (example)	Receptor on epithelial cell (example)	References
Lectin-carbohydrate	Lectin (type 1 fimbriae) Capsule (hyaluronic acid of *S.pyogenes*)	Glycoprotein (uroplakin on bladder cells) Lectin (CD41 on epithelial cells)	(Wu *et al*., 1996a; Cywes *et al*., 2000)
Protein-protein	Fibronectin binding proteins (F protein of *S.pyogenes*)	Fibronectin (fibronectin on respiratory cells)	(Cue *et al*., 1998; Hanski and Caparon, 1992)
Hydrophobin-protein	Glycolipid (LTA of *S.pyogenes*) Lipid binding proteins (surface protein of *Campylobacter* spp)	Lipid receptors (fatty acid binding site in fibronectin on host cells) Membrane lipids (phospholipids and sphingolipids of cells)	(Sylvester *et al*., 1996; Szymanski and Armstrong, 1996)

[a]adaptive from Ofek, 2003.

Table 1.3 Some examples of bacterium adhesin and target surface on the host cell.

Bacterium	Adhesin	Target surface	Host cell receptor	References
Pseudomonas aeruginosa	Type IV pili	Respiratory epithelium	Asiloganglioside	(Saiman *et al*., 1990; Cacalano *et al*., 1992).
E. coli (UPEC)	Pili (PapG) Type 1 pili Subunit of pili FimH	Kidney epithelium Uroplakine of the bladder Actin cytoskeleton	Glycosphingolipids D-mannosylated receptors Mannose or tetraspanin protein uroplakin la	(Roberts *et al*., 1994; Martinez *et al*., 2000; Zhou *et al*., 2001; Sokurenko *et al*., 2008; Lai *et al*., 2013; Lillington *et al*., 2014).
(EPEC and EHEC)	T3SS		Cytoskeleton regulators	
Neisseria meningitidis	Type IV pili Opc (opacity protein c) Tfp	Vascular endothelial Nasopharyngeal epithelium Epithelium cells	Proteoglycan receptors Cell surface associated proteins CD46	(Kallstrom *et al*., 1997a; De Vries *et al*., 1998; Melican and Dumenil, 2011).
Staphylococcus aureus	Fibronectin	Skin tissue fibroblast	Fibrinogen Heat shock protein (hsp60)	(Cho *et al*., 2001; Schwarz-Linek *et al*., 2003).
Streptococcus pygenes	Sortase A	Epithelial	Fibronectin in the ECM	(Beachey, 1981; Pancholi, 1989).

1.4 Strategies in anti-adhesion therapy

It is increasingly evident that in addition to other defensive strategies, many bacteria form biofilms to resist unfavourable conditions such as antibiotic treatment and as such, there is need to employ other strategies for treatment of bacterial infections. Anti-adhesion therapy is a useful tool that can target the host or pathogen cell surface to disrupt bacterial cell attachment (the vital step of infection) in various ways (Figure 1.4).

1.4.1 Anti-adhesive agents

The development of new biological agents has allowed the treatment of several otherwise difficult to treat infectious diseases. For instance, probiotics have been widely used in anti-adhesion therapy for inflammatory diseases of the gastro-intestinal tract such as inflammatory bowel disease and ulcerative colitis mainly because of their (probiotic) presence in the intestinal flora (Guandalini, 2010). Probiotics are non-pathogenic bacteria that possess health benefits for hosts which they colonise. Several studies have shown that probiotics are effective against enteric pathogens through several mechanisms including production of bacteriocins by *Lactobacilli* with activity against Gram-positive bacteria (Cotter *et al*., 2005; Lawton *et al*., 2007), while bacteriocin produced by *Bifidobacteria* is active against Gram-positive and Gram-negative bacteria (Collado *et al*., 2005). *Lactobacillus* species have been demonstrated in previous studies to adhere to intestinal epithelial surfaces in tissue culture (Greene and Klaenhammer, 1994). *L. acidophilus* and *L. rhamnosus* were also shown in another study to adhere to host epithelial cells, thus preventing the binding of *E. coli* E2348/69 and *E. coli* O157:H7 to the host epithelial cells (Sherman *et al*., 2005).

In many systems, adhesion of the bacteria to host cells is mediated by the interaction between lectins on the bacteria and carbohydrates present on the host cell surface. Several studies have demonstrated the effective role of employing glycomimetics or sugar based inhibitors as anti-adhesives (Mulvey *et al*., 2001; Sharon, 2006). For instance, intestinal epithelium secretes mucus containing highly glycosylated mucin molecules, which act as a physical barrier against enteropathogens, thus preventing the pathogen from making contact with human intestinal epithelial cells. *P. aeruginosa* has a lectin (PA-IL), a carbohydrate binding protein, which forms dimers or tetramers that can bind sugars through an ion bridge formed with calcium at their binding sites (Cioci *et al*., 2003). The major role of these lectins is that they serve as ligands for pattern recognition receptors that are found on host cells involved in the innate immune system. The lectins expressed by *P. aeruginosa*, LecA and LecB, are produced by the Rhl system and play an important role in infection by binding to fucose or galactose as discovered in the mutagenesis studies by Winzer *et al*., (2000).

High affinity glycomimetics such as analogues of Lewis trisaccharide and multivalent glycoconjugates like galactosylated helical poly (phenylacetylene) polymers, fullerenes, and calix (4) arenes have been developed. These have been shown to inhibit the binding of lectins to sugars, thereby preventing biofilm formation as observed with the fucosylpeptide dendrimers (Bernardi *et al*., 2013). Some bacteria possess structures that aid their binding to glycosylated surfaces and these glycomimetics act to prevent or destroy the binding of bacterial structures to the glycosylated surfaces. For instance, in UPEC, the type 1 fimbriae (pili) binds glycosylated surfaces in the urinary tract and glycomimetics such as heptyl α-D-mannopyranosides have been shown to prevent biofilm formation, cell adhesion and invasion of UPECs into bladder cells by acting as a structural mimic of the natural surface structure that UPECs bind to (Wellens *et al*., 2008).

1.4.1.1 Tetaspanins as anti-adhesive molecules

Tetraspanins are expressed in virtually all cells and they mediate cell adhesion via interaction with adhesion molecules like E-cadherin and integrins (Yanez-Mo *et al*., 2001). Until recently, the only study that investigated the role of tetraspanins in preventing bacterial adhesion to host cells was done by (Green *et al*., 2011a), where they assessed the effect of structurally modified tetraspanin molecules (recombinant tetraspanin) on the interaction between pathogens and host cells (monocyte-derived macrophages, and epithelial or endothelial cells). Green *et al*., (2011a) investigated the role of tetraspanins in cell-cell adherence and in the ability of pathogens to interact and adhere to the host cells. Epithelial cells (HEC1B and DETROIT 562 cells) were either incubated with specific anti-tetraspanin antibodies or with recombinant tetraspanin extracellular domains (EC2s) or both, which inhibited the attachment of *N. meningitidis*, *E. coli*, *S. aureus* and *S. pneumonia*e to epithelial cells. Small interfering RNA (siRNA) knockdown of the tetraspanins also gave similar result by inhibiting the adherence of *N. meningitidis* to the epithelial cells. These findings provide a link between tetraspanins and the pathogen interaction with epithelial cells, and thus gives insight into the use of recombinant tetraspanin proteins as anti-adhesives in preventing attachment of pathogens to host cells. The major advantage of this technique is that the recombinant tetraspanin can be made from human proteins and they are relatively small, therefore they will not elicit an immune response. Another benefit of this technique will be the ease of clearance of the protein as it is a biological agent.

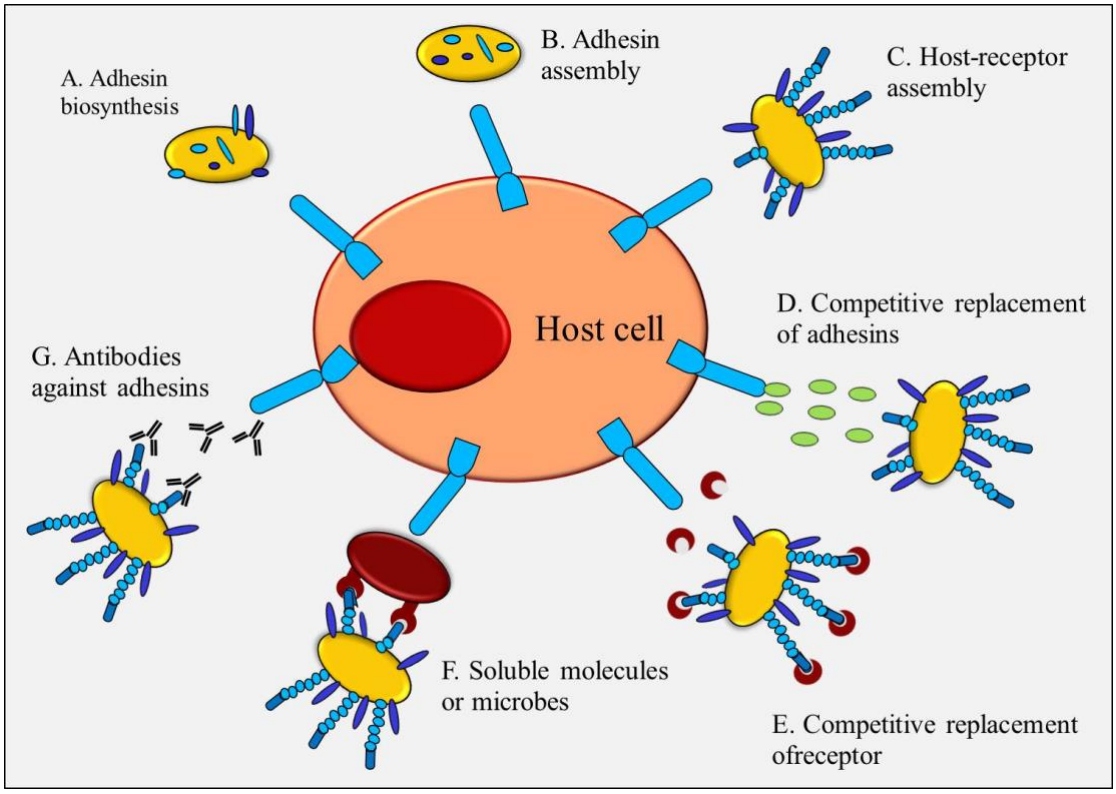

Figure 1.4 Strategies in anti-adhesion therapy.

The use of natural flora of the body to decrease the infection works by inhibiting the contact between the pathogen and the host. The normal flora can colonise specific niche and block the attachment of the pathogen to host cells. Adapted from Krachler and Orth, 2013.

1.5 Different methods used to investigate adhesion of bacteria to host cells

The first step in infection is adhesion of the pathogen to host cells. Many anti-adhesion agents that block bacterial adhesion have been identified, including probiotics, which are capable of blocking the adhesion of pathogenic bacteria to host cell. Therefore, it is important to develop techniques that can detect or measure bacterial adhesion. Currently, there are various techniques available to detect or measure bacterial adhesion. However, these techniques are not well established and there are few studies that have compared these techniques to each other. Table 1.4 presents the comparison between different methods commonly used to quantify adhesion of bacteria to cells.

Table 1.4 Methods commonly used to quantify adhesion of bacteria to cells.

Method	Advantage	Disadvantage	References
Plating (CFU)	• Counts only the viable bacteria	• The bacteria should be dissociated • Cannot discriminate bacteria in a mixed population • Not well suited for high throughput screening (HTS) • Counting of bacterial colonies may not be accurate • Laborious	(Pederson and Breed, 1940; Vesterlund *et al*., 2005; Esteban Florez *et al*., 2016).
Light Microscopy	• It can determine the pattern of bacteria bound to individual cells • Allows direct measurement of bacteria bound to the specific cellular site	• This method cannot be used when studying the adhesion of one bacterial strain • Time consuming • Cannot discriminate bacteria in a mixed population • Long process with fixation and staining	(Garrett *et al*., 2008).
Spectrophotometry	• Does not require long lab process	• Sensitivity and accuracy may be lower compared to plating or microscopic enumeration • Cannot distinguish bacteria in a mixed population • Does not differentiate between live and dead bacteria	(Que *et al*., 2012).
Electron Microscopy	• Effective for quantitating bacteria bound to structures such as tissues	• Expensive. • Sample preparation is complicated, the specimen requires to be coated with a very	(Garrett *et al*., 2008).

		thin layer of metal.	
Radiolabels	• Distinguishes bacteria in a mixed population • Sensitive to detect small number of bacteria • Easy to obtain radiolabelled bacteria • Possible to monitor the simultaneous adhesion of bacteria to the same substrata	• Safety and cost concerns	(McEldowney and Fletcher, 1987).
Bioluminescence assays	• Bioluminescence assays have 10–1000× higher sensitivity levels than do fluorescence assays like green fluorescent protein/GFP • Provides a quantitative method for the assessment of bacterial viability • Allows exact quantification of the viability of cells within complex biological systems, such as oral biofilms	• Difficult to exclude the background auto luminescence	(Ray, 2008).
Fluorescence (flow cytometry, image analysis)	• Allows examination of bacterial adhesion to immobilised cells • Allows to study invasion of bacteria into cells • Specific population of cells can be separated by size	• They may alter the surface properties of bacteria or affect the viability of bacteria	(Esteban Florez *et al*., 2016).

1.6 Application of flow cytometry in bacterial studies

Flow cytometry is a powerful tool which can be used to investigate bacterial adhesion, which is critical for the pathogenesis of extracellular pathogens. Typically, bacterial invasion of eukaryotic cells is quantified by antibiotic assays followed by dilution plating and counting of colonies, which is time consuming and laborious. This antibiotic assay is based on the recovery of viable bacterial cells after its internalisation by eukaryotic cells, therefore "it can severely underestimate the number of intracellular bacteria if intracellular killing by eukaryotic cell occurs" (Pils *et al.*, 2006a).

Flow cytometry is a potential method for investigating the adherence of bacteria to host cells. Hytönen *et al.*, (2006b) studied the adhesion of streptococcal strains and PulA deficient mutants to Squamous cell carcinoma (SCC) cells by both the conventional solid phase adhesion assays and flow cytometry, they found that with the conventional solid phase adhesion methods there was no difference in the binding of bacteria to host cells, while with the flow cytometric assay they found there was a statistically significant difference between the adhesion of streptococcal strains to the human epithelial cells.

Consequently, there has been a rapid rise in the use of the flow cytometry technique for analysing host-pathogen interactions, specifically to measure the adhesion of bacteria to host cells. Previous studies have used flow cytometry to investigate the interaction between bacteria and eukaryotic cells, as well as fungi and other eukaryotic cells (Brooks *et al.*, 2013). However, its potential application for complex studies has been underestimated. Lately, a flow cytometry-based approach was developed to analyse the adhesion of different strains of *Trichomans vaginalis* to vaginal ectocervical cells (Brooks *et al.*, 2013). A limitation of the approach in this study was the inability to quantify

adhesion, but, other researchers have described that flow cytometry can be used to detect and measure viable microbes (Pina-Vaz *et al*., 2004; Barbosa *et al*., 2008; Pina-Vaz and Rodrigues, 2010). Previously, flow cytometry has been used to study the adhesion and invasion of *S. aureus* strain 8325-4 and its invasion-deficient isogenic mutant into osteoblastic MG-63 cells (Trouillet *et al*., 2011a). In order to be analysed by flow cytometry, bacteria need to be genetically modified to express fluorescent proteins, eukaryotic cells, on the other hand, are identified using the light scattering phenomenon. However, a direct labelling method has been developed to enable improved identification of the target cells (Hara-Kaonga and Pistole, 2007).

Pils *et al*., (2006a) have previously developed a protocol, which verified the use of flow cytometry as a tool for defining the uptake of bacterial pathogens by host cell types such as epithelial cells or fibroblasts. However, in a mixed population of bacterial strains, flow cytometry was found to be unable to detect and accurately quantify the different strains using fluorescein-conjugated antibacterial antibody (Phillips and Martin, 1988).

1.6.1 Basic principles of flow cytometry

Flow cytometry is an advanced method used to measure multiple characteristics of a single cell. This technique enables the simultaneous measurement of multiple parameters such as size, granularity and volume as cells flow in suspension through a measuring device (Wilkerson, 2012). The cell suspension is mixed with the sheath fluid, the sheath fluid focuses the cell suspension, which makes cells pass in a single file towards a laser light source. The light source provides the fluorescence excitation, which strikes the moving particle passing through, leading to light scattering and fluorescence emission. The emission and light scattering are captured by a detector, which converts the information to data that can be processed and analysed.

Therefore, the data obtained gives valuable information about the biophysical, biochemical and molecular properties of particles and this approach makes flow cytometry a powerful tool for detailed and rapid analysis of a complex population of cells (Stuchly *et al*., 2012). Flow cytometry is used in numerous applications including cytokine detection, cell proliferation, cellular components and enzyme activity detection (Wlodkowic *et al*., 2013).

1.6.2 Fluorochromes and their properties

There are a restricted number of fluorescent compounds that can be used in a cell, which reduces the amount of information that can be obtained during analysis. Flexibility is achieved with the use of fluorochromes/fluoroprobes, which are fluorescent compounds attached to antibodies that re-emit light upon excitation and can be used to target specific proteins within the cell and on the surface (Davies, 2009). Fluorescent proteins such as GFP (green) and DsRed (red) were widely used as an indicator to observe gene expression by cell transformation. The fluorescent-labelled bacteria can be detected by flow cytometry, this provides an important tool to investigate bacterial pathogenicity (Valdivia *et al*., 1996).

1.6.3 Data analysis

Analysis of the flow cytometry data is critical to understanding the biological experiments. In flow cytometry, gating is used to selectively group together a population of cells with common characteristics, this allows the researcher to further investigate and quantify the selected cells with the aim of selectively showing the details of the cells of interest. This is achieved using characteristics such as forward and side scatter (FSC, SSC) of the cells to form regions. Careful selection for gating also provides the difference

between the particles and can easily eliminate dead cells and debris. FSC and SSC plots are used to separate cells by their characteristics such as size and shape in a mixed population. For example, the difference in size between white blood cells, granulocytes, lymphocytes and monocytes allows them to be separated from each other (Jahan-Tigh *et al*., 2012).

1.7 Hypothesis and aim of the project

In this study, it was hypothesised that disruption of TEM, by antibodies, recombinant tetraspanin EC2 domains or synthetic peptides (CD9 EC-derived peptides) will inhibit the attachment of *P. aeruginosa* to host cells and thus prevent infection. The aim of this study was to investigate whether tetraspanins play a role in the adhesion of *P. aeruginosa* to human epithelial cells and subsequently, to investigate the potential of using tetraspanin reagents as anti-adhesives in treating *P. aeruginosa* infections by inhibiting adherence to human epithelial cells if the tetraspanins are involved in adhesion.

In order to investigate the role of tetraspanins in the pathogenic adhesion of *P. aeruginosa* to host cells, this project involved the following processes;

i. Growth optimisation of *P. aeruginosa* followed by expression profiling of tetraspanins in human epithelial cells using flow cytometry. This involved the development of a fluorescent labelled bacteria for detecting the role of tetraspanins during the bacterial adhesion.
ii. Developing flow cytometry based assay (which could potentially replace the microscopy assay) for investigating the role of tetraspanin reagents in bacterial adhesion.
iii. The use of tetraspanin antibodies to investigate the inhibition of *P. aeruginosa*

adhesion to cells.

iv. The use of recombinant tetraspanin molecules to inhibit *P. aeruginosa* adhesion to host cells, to investigate which tetraspanins are involved in adhesion to host cells.

v. The use of tetraspanin CD9 derived peptides to inhibit *P. aeruginosa* adhesion to epithelial cells.

vi. The use of tetraspanin CD9 derived peptide 810 to investigate the production of cytokines in epithelial cells.

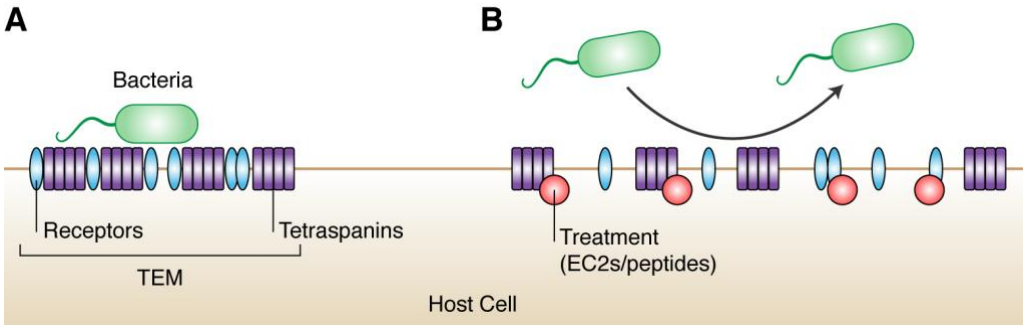

Figure 1.5 Disruption of TEM.

The treatment of host cell with recombinant EC2 proteins, antibodies, or CD9 EC2-derived peptides may disrupt TEMs. This can cause the redistribution of host cell receptors and therefore prevent the bacteria from adhering to the host cell surface. **(A)** Normal organisation of TEM and bacterial receptors on the host cell surface (without tetraspanin treatments). **(B)** After tetraspanin treatments, receptors are redistributed and the pathogens can no longer adhere to the host cells and are removed by shear pressure.

CHAPTER 2: Materials and methods

2.1 Materials

2.1.1 Reagents

Table 2.1 Reagents used in this study.

Reagents	Preparation
Dimethyl sulphoxide (DMSO)	DMSO (Sigma) was used for freezing cells by diluting 10 x in cell culture media (containing 10 % foetal calf serum).
Gentamicin	Gentamicin (Lonza) was used by diluting 0.8 mg/ml from a 10 mg/ml stock in cell culture media.
Saponin	Saponin (Sigma) was prepared in 0.05 % trypsin-PBS at a concentration of 1 % and filtered before use.
Trypsin-versene	Trypsin- versene® (Lonza) was produced by the addition of 100 ml of 10 x trypsin-versene® (EDTA) to 1 litre of Hank's balanced salt solution (HBSS). Aliquots of 10 ml were stored at -20 °C for later use.
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, USA) was used according to the manufacturer's instructions at a concentration of 0.5 mg/ml and stored at -20 °C until use.

2.1.2 Solutions

Table 2.2 Solutions used in this study.

Solutions	Preparation
Cell dissociation solution	Cell dissociation solution was purchased (CDS) (Sigma) as 1x solution. To dissociate the cells, 5 ml was added to a T75 Flask.
Hanks buffered saline solution (HBSS)	HBSS with/without Ca2+ and Mg2+ (Lonza).
Paraformaldehyde solution 4 %	4 g paraformaldehyde was dissolved in 50 ml of double distilled water (ddH_2O) containing 1 ml of 1 M solution of NaOH by heating for 60 minutes at 60 °C. After cooling, 10 ml of

	10 x PBS was added and the pH was adjusted to 7.4. The solution was diluted to 1 x with 100 ml ddH_2O, filter sterilised and stored at -20 °C.

2.1.3 Buffers

Table 2.3 Buffers used in this study.

Buffers	Preparation
Phosphate buffered saline (PBS), pH 7.3	PBS containing NaCl (140 mM), Na_2HPO_4 (8 mM), KCl (2.7 mM) and $K_2H_2PO_4$ (1.5 mM) was prepared by dissolving 10 x phosphate buffered saline (PBS), 80 g NaCl, 2 g KCl, 11.5 g Na_2HPO_4, 2 g KH_2PO_4 in 1 litre of H_2O. Aliquots were autoclaved for 15 minutes at 121 °C.
Triton X-100 (Lysis buffer)	Triton X-100 (Sigma) was prepared in PBS at 0.1 %. The solution was filter sterilised before use.
Wash buffer/dilution buffer for immunofluorescence (BBN)	BBN consisting of 0.1 % sodium azide and 0.2 % bovine serum albumin (BSA) dissolved in PBS. BBN was stored at 4 °C. This was also used as a diluent for antibodies.

2.1.4 Commercial kits used

CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) was purchased and used in accordance with the manufacturer's instructions.

2.1.5 Stains

Table 2.4 Stains used in this study.

Stains	Preparation
DAPI staining solution	Staining solution was prepared by mixing 0.1 % Triton X-100, 0.02 % sodium dodecyl sulphate and 0.5 µg /ml of 4, 6-diamidino-2-phenyl-indole hydrochloride (DAPI) in PBS. The solution was stored at 4-8 °C.
Vectashield mounting medium	Vectashield mounting medium (Vector Laboratories Inc., USA). The ready-to-use mounting medium was stored at 4 °C.

2.1.6 Antibodies

2.1.6.1 Primary antibodies

Table 2.5 Primary antibodies used.

Antibody	Target antigen	Specificity	Concentration used	Source
Mouse anti- human CD9 (602.29)	CD9	Human	10 μg/ml	In house
Mouse anti human (H5C6)	CD63	Human	10 μg/ml	In house
Mouse anti human CD81	CD81	Human	10 μg/ml	Ancell product number:302-820
Mouse anti- human CD151 (14.A2)	CD151	Human	10 μg/ml	In house
Isotype-mouse IgG 1		Human	10 μg/ml	BioLegend Cat:400124

2.1.6.2 Secondary antibody

Anti-Mouse IgG-FITC, FITC conjugates produced in goat (Sigma), Cat number F-2653.

2.1.7 Tetraspanin EC2 : GST protein

The transformation was done using a modified protocol from Higginbottom *et al*., (2003) using two bacterial expression strains: *E. coli* Rosetta-gami B (DE3) pLysS (Novagen) and *E. coli* Shuffle T7 Express lysY (New England Biolabs) by co-workers Dr Marzieh Faneaei and Dr John Palmer.

2.1.8 Tetraspanin derived peptides

Solid phase synthesis with Fmoc chemistry (Houghten, 1985) was used to synthesise the EC2 region of tetraspanin CD9 (Tetraspanin 29), the majority of the peptides synthesised

were 14-15 amino acids long. They were purchased from GenScript in lyophilised form. They were prepared to the desired stock solution using ddH_2O.

Table 2.6 CD9 EC2 domain: peptide sequences.

Peptide	Description	Amino acids sequence
800	15 amino acids long, second central part of the large loop of EC2.	EPQRETLKAIHYALN
800 (Scrambled)	Scrambled control consisting of 15 amino acids, second central part of the large loop of EC2.	AYPHLERNLQEIAKT
810	14 amino acids long, part of the second variable sub-loop of EC2.	GPKKDVLETFTVKS
810 (Scrambled)	Scrambled control consisting of 14 amino acids, part of the second variable sub-loop of EC2.	TSKEKLVGPDTKVF
8001	14 amino acids long, stalk part of the large loop of EC2.	SHKDEVIKEVQEFY
8001 (Scrambled)	Scrambled control consisting of 14 amino acids, stalk part of the large loop of EC2.	EEVKKFESQHDIYV
8002	13 amino acids long, first central part of the large loop of EC2.	KDTYNKLKTKDEP
8002 (Scrambled)	Scrambled control consisting of 13 amino acids, first central part of the large loop of EC2.	NEKDPKKTLDKTY
8003	14 amino acids long, part of the first variable sub-loop of EC2.	GLAGGVGQFISDIS
8003 (Scrambled)	Scrambled control consisting of 14 amino acids, part of the first variable sub-loop of EC2.	IDSGFVGQIASGLE
8004	11 amino acids long, latter stalk part of the large loop of EC2.	PDAIKEVFDNK
8004 (Scrambled)	Scrambled control consisting of 11 amino acids, latter stalk part of the large loop of EC2.	AFPDNKDIVEK
8005	Extended peptide 8001 to include entire helix	SHKDEVIKEVQEFYKDTYNKLKT KDEPQRETLKAIHYALN
8005 (Scrambled)	Scrambled control for extended peptide 8001	THDAEKKNPINDLKKEVLERVK QKTYESTHFADLYQIEYK
800 Cap	Peptide 800 capped with Asp (D)	DEPQRETLKAIHYALN
800 Cap (Scrambled)	Scrambled control for peptide 800 capped with Asp (D)	QEALKYNRAETPLDIH

2.1.9 Bacteriology work

2.1.9.1 Media

Table 2.7 Bacteriology media.

Media	Preparation
LB (Lysogeny Broth)	10 g tryptone, 5 g yeast extract and 10 g sodium chloride were added to 1 litre distilled water and autoclaved at 121 ℃.
LB agar	10 g tryptone, 5 g yeast extract, 10 g sodium chloride and 15 g agar were added to 1 litre distilled water and autoclaved at 121 ℃.

2.1.9.2 Antibiotics

Chloramphenicol

100 µg of chloramphenicol was dissolved in 2 ml ethanol (Fisher Scientific) and stored at -20 ℃.

2.1.9.3 Glycerol stock

Glycerol was purchased (Sigma), 700 µl of overnight bacterial culture was added to 300 µl of glycerol and kept in -80 ℃ freezer until required.

2.1.9.4 Plasmids encoding fluorescent proteins

The PIN25 (GFP) plasmid and PIN29 (DsRed) plasmid were obtained from Dr Mark Thomas (Department of Infection, Immunity and Cardiovascular Disease, Medical School, University of Sheffield) (Vergunst *et al*., 2010).

2.1.9.5 Bacterial strains used in this study

- (PAO1) Wild type *Pseudomonas aeruginosa*; wound isolate (Holloway, 1955).
- (SH1000) *Staphylococcus aureus* is derived from RN4220 and contains a chloramphenicol resistance pSK5487 plasmid expressing GFP (Herbert *et al*., 2010).

PAO1 and SH1000 were grown in LB broth media containing 50 µg/ml and 10 µg/ml of chloramphenicol, respectively. Bacteria were maintained on LB agar plates at 4 °C (for short-term storage) and in 15 % glycerol-LB broth at -80 °C (for long-term storage).

2.1.10 Cell line culture and media used

2.1.10.1 Media and sera

Dulbecco's modified essential medium (DMEM)

For infection assays, DMEM-high glucose (Lonza) was made by adding 50 ml FCS and 250 µl chloramphenicol (50 µg/ml for PAO1 and 10 µg/ml for SH1000), and stored at 5 °C.

Eagle's modified essential medium (EMEM)

For infection assays, EMEM + 1 % L-glutamine (Lonza) was made by adding 50 ml FCS and 250 µl chloramphenicol (50 µg/ml), and stored at 5 °C.

Foetal calf serum (FCS)

Foetal calf serum was purchased (Source Bioscience, UK). It was further heat inactivated (HI-FCS) by incubation at 56 °C for 20 minutes, and then frozen in 50 ml aliquots until use.

Bovine serum albumin

Bovine serum albumin (First Link Ltd., UK) at 30 % was diluted in cell culture media to a concentration of 0.05 %.

2.1.10.2 Cell lines

Adenocarcinoma human alveolar basal epithelial cells (A549) (Lieber *et al.*, 1976), human keratinocytes (HaCaT) (Boukamp *et al.*, 1988), and human endometrial adenocarcinoma epithelial cells (HEC1B) (Kuramoto *et al.*, 1972), were used throughout

this study. These cell lines were kindly provided by Dr Lynda Partridge (University of Sheffield) from the in-house culture collection cell bank.

2.2 Methods

2.2.1 Tissue culture methods

2.2.1.1 General sub-culture technique

At confluence, culture media from the cells was carefully decanted using aspiration and the cells were gently rinsed with sterile PBS solution twice. After decanting the PBS solution by aspiration, cells were detached from the flask using the appropriate reagents depending on the cell type and incubated at 37 ℃ with 5 % CO_2 in a humidified incubator for about 5 minutes. The cells were removed from the incubator and slightly agitated by gently shaking the flask to completely detach the cells. The cells were re-suspended in appropriate amount of culture medium and the number of viable cells was counted using a haemocytometer prior to sub-culturing. The cells were then diluted with culture media depending on cell growth rate into new flasks and incubated at 37 ℃ with 5 % CO_2.

2.2.1.2 Cell freezing

The cells were detached from the flask as described before in section 1.10.8, centrifuged at 200 xg for 5 minutes and re-suspended in 1 ml of the freezing mixture (10 % DMSO and 90 % FCS). The cells were initially frozen at -80 ^{o}C for 24 hours and then transferred to liquid nitrogen vapour for long-term storage.

2.2.1.3 Cells thawing

The cells were transferred to 9 ml of growth media without FCS and centrifuged for 5 minutes. Then, the cell pellets were re-suspended into fresh media with FCS and transferred to a T25 flask, and allowed to grow at 37 ℃ with 5 % CO_2 for 24 hours.

2.2.1.4 The live/dead fixable cell stain

The live/dead fixable cell stain was supplied by Invitrogen. The stain was prepared by adding 100 µl DMSO to the vial of reactive dye, and then the dye was diluted in PBS to the required volume. Cells were centrifuged and the pellet re-suspended in 200 µl of the dye and incubated for 15 minutes at room temperature (in the dark). The purpose of this method was to evaluate the viability of human cells by flow cytometry. These tests are based on the reaction of a fluorescent dye with cellular amines. The reactive dye can only penetrate the compromised membranes of necrotic cells and react with free amines both in the interior and on the cell surface, resulting in intense fluorescent staining. In comparison, only the cell-surface amines of viable cells are accessible to react with the dye, causing in relatively dim staining (Figure 2.1).

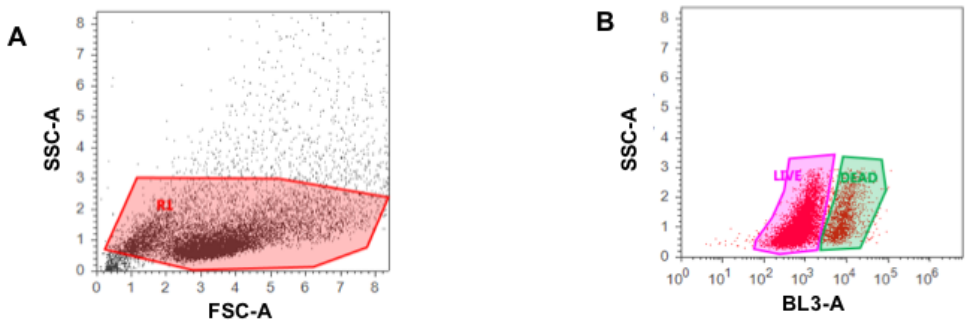

Figure 2.1 Flow cytometry analysis of cells stained with live/dead stain.

(A) The gate for all cells (live + dead). **(B)** Live cells (left) and dead (right). Analysis was done using the Attune FACS machine.

2.2.1.5 Labelling of plasma membrane antigens (immunofluorescence assay in tubes)

Immunofluorescence assays were performed on live cells and all cell manipulations were carried out at 4 ℃ to prevent internalisation, capping and shedding of antigens. For each test/assay, 0.5 to 1.0 x 10^6 cells of high viability (90 %) were used; the cells were harvested after culturing and washed twice in cold wash buffer (BSS containing 0.2 % BSA, 0.1 % sodium azide i.e. BBN). Total cells (0.5 to 1.0 x 10^6 cells) were re-suspended in the wash buffer and 1 ml aliquots were added to round bottom polystyrene tubes, (Elkay 12 × 75 mm, Cat. No. 000-2052-001) and centrifuged at 400 xg for 5 minutes. The supernatant was carefully aspirated using Pasteur pipette or micropipette and 50 µl of mouse polyclonal antibody (MAb), diluted in wash buffer as required, was added to the cell pellet. The tube was gently vortexed for few seconds and the content was incubated on ice for 45-60 minutes. The cells were washed as previously described and subjected to fluorescence-activated cell sorting (FACS) LSR II analysis. Primary antibodies were unlabelled while secondary antibodies were labelled. For FACS, the cells were re-suspended with 0.3 ml of wash buffer and were kept on ice until analysis or fixed in 0.3 ml of 2 % paraformaldehyde to be kept for up to 48 hours.

2.2.1.6 Immunofluorescence on cells grown on LabTech chamber slides (indirect staining)

Cells were harvested as described above (section 2.1.5) using trypsin/versene and the viable cells were counted. Cells were re-suspended in culture medium at approximately 7 x 10^4 cells/ml and 0.5 ml aliquots of the re-suspended cells were added into a LabTech chamber slide and incubated overnight at 37 ℃ with 5 % CO_2. The slides were examined the following day to check the cell density. The cells were washed again with 0.5 ml BSS by processing one slide at a time to avoid drying of the cells in chambers. The cells in

each chamber were fixed and permeabilised with 0.5 ml acetone and incubated at room temperature for 5 minutes. The acetone was removed from each chamber by aspiration and the chambers were gently rinsed by immersing the slides in PBS, and then flicked to remove the acetone. The slides were then placed in a tank containing fresh PBS and washed for 10 minutes with stirring. The slides were removed from the PBS tank, drained, and 100 µl of primary antibody was added to cover the entire surface of the slide and incubated for 30 minutes at RT in dark under humid conditions. The slides were rinsed with PBS for about 10 minutes, the third time and drained, followed by addition of 100 µl of secondary antibody labelled with FITC as quickly as possible. Then the slides were incubated at RT in the dark under humid conditions for 30 minutes. Next, the slides were washed again as above and were removed from the chambers and mounted on a plastic chamber compartment according to manufacturer's instructions as described above. The slides were stored in the dark at 4 ℃ until fluorescent microscopic analysis.

2.2.2 Bacteriology methods

2.2.2.1 Bacterial culture techniques

Solid culture technique for viable bacteria

Viable bacterial cells were stored at -80 ℃ in 50 % glycerol in LB media. When needed, aliquots of the bacterial stocks were streaked using a sterile metal loop and transferred onto solid LB agar containing chloramphenicol at 50 µg/ml. The plates were incubated in a humidified incubator at 37 ℃ to allow growth of individual colonies. Bacteria were propagated on fresh solid media on a daily basis and it was assumed that each individual colony developed from one viable bacterium. Several individual colonies were used to streak fresh solid agar plates so as to avoid phase variability of surface structures, especially with the use of the *Pseudomonas* genus.

Broth culture technique for viable bacteria

LB with 50 µg/ml chloramphenicol was used for *Pseudomonas*: 8-12 individual colonies of *Pseudomonas* were picked from the overnight solid agar culture plate using a sterile loop and each colony was used to inoculate 10 ml of fresh LB broth in a 25 ml universal tube. The cultures were incubated with agitation (using a plate mixer) for 2 hours at 37 °C. The absorbance of the culture was measured at OD_{600nm} and the number of viable bacteria were counted to ensure the approximate CFU/ml can be calculated for each dilution that would be required in the subsequent experiments.

2.2.2.2 Bacterial growth curve

To estimate the number of viable bacteria in culture, a tenfold serial dilution of the bacterial culture was made and each dilution was plated on LB agar. The agar plates were quartered to accommodate four dilutions per plate. Triplicates of 10 µl aliquots of the serial dilutions were inoculated on each quarter, thus avoiding coalescence. The plates were air dried in a class II microbiological safety cabinet prior to incubation in a humidified environment at 37 °C. For each dilution, using the triplicates, the mean bacterial count in CFU/ml (colony forming units) was calculated using the **Equation (2.1),** and the numbers were adjusted for volume and dilution.

Equation (2.1) Colony forming units /ml=

$$\text{number of colonies x } \frac{1}{\text{volume plated (ml)}} \text{x dilution factor}$$

2.2.2.3 Labelling of *Pseudomonas* with fluorescent protein encoding plasmids (PIN25 & PIN29)

P. aeruginosa strains were inoculated in 6 ml of LB broth. The cultures were left to incubate overnight at 37 ℃ whilst shaking. The overnight cultures were divided into four 1.5 ml micro-centrifuge tubes. The tubes were centrifuged at 16,000 xg for 2 minutes at room temperature (RT) and the cells were harvested by decanting the broth. The pellet was gently re-suspended in 1 ml of sterile 300 mM sucrose solution using a micropipette. The re-suspended pellets were centrifuged at 16,000 xg for 1 minute and the supernatant was discarded. This step was repeated with the centrifugation step completed for 2 minutes. Cell pellets from the four samples were re-suspended to make a total volume of 100 µl with 300 mM sucrose solution by sequentially re-suspending each pellet in the same 100 µl volume. 500 ng of vector DNA was mixed with 100 µl of *P. aeruginosa* bacterial cells and transferred into a 2 mm-gap width electroporation cuvette. Pulse was applied using the automated settings for the bacteria *P. aeruginosa* in the Bio-Rad gene pulser (25 µF, 200 Ω, 2.5 kV). Afterwards, the electroporated cuvette was opened in a microbiological safety cabinet and 1 ml of LB broth was added to the content. The culture was then transferred into a sterile universal tube and incubated at 37 ℃ for 2 hours whilst shaking. The cells were plated onto selection plates after incubation. The selection marker was chloramphenicol, which was used at 50 µg/ml.

2.2.3 Preparing fluorescent labelled- *P. aeruginosa* for infection assays

Fluorescent labelled- *P. aeruginosa* were prepared before each experiment by streaking onto LB agar from bacterial stock to isolate single colonies. Overnight liquid cultures were then prepared by inoculating one bacterial colony into 10 ml LB broth with 50 µg/ml chloramphenicol, the bacteria were allowed to grow in a shaking incubator (120 rpm) for 16-18 hours at 37 ℃. After incubation, the overnight culture was centrifuged for 10

minutes at 4,500 xg. The bacterial pellet was washed with PBS twice and then suspended in 5 ml of appropriate cell culture media according to cell line used in the experiment. The OD_{600} was determined to be diluted to OD_{600} of 0.5, CFU/ml of $5x10^8$.

2.2.4 Microscopic detection of fluorescent labelled -*P. aeruginosa* adherence to the host cell

2.2.4.1 Adhesion assay

Cells were seeded in a 24 well plate at a density of 1.5×10^5 cells/ml on 12 mm coverslips and allowed to grow overnight at 37 ºC with 5 % CO_2. The next day, the medium was removed and wells washed once with PBS using a Pasteur pipette. Cells were then infected with the bacteria at a range of MOIs (5, 10, 15, 20, 25, 30, 35 and 40) and incubated at 37 ºC with 5 % CO_2 for 60 minutes. Cells were then washed twice with PBS.

2.2.4.2 Staining protocol for infected cells

2 % paraformaldehyde was used to fix the infected cells for no less than 30 minutes. Once the paraformaldehyde was removed, the cells were washed twice with PBS, then 400 µl solution of PBS containing 0.1 % Triton X-100, 0.02 % sodium dodecyl sulphate and 0.5 µg ml-1 DAPI was added to each well and left in the dark at room temperature for 12 minutes. A single drop of DAPI hard mounting media was placed on the slide before the coverslip was placed with cells facing DAPI. Later, cellular DNA was visualised using the UV wavelength light on a fluorescent microscope at a maximum excitation wavelength of 368 nm and a maximum emission wavelength of 461 nm.

2.2.4.3 Analysis of infected cell slides

An Olympus fluorescence microscope (BX61) at 1000 x magnification (oil-immersion lens) was used in a random field to count 100 cells, with bacterial adhesion calculated as shown in **Equations (2.2a-c)**.

Equations (2.2)

$$\textbf{a). Percentage infected=} \frac{\text{Infected cells}}{\text{Number of cells}} \text{x}100$$

$$\textbf{b). Number of bacteria per 100 cells=} 100\text{x} \frac{\text{Number of bacteria}}{\text{Number of cells}}$$

$$\textbf{c). Bacteria per infected cells=} \frac{\text{Number of bacteria}}{\text{Number of infected cells}}$$

2.2.5 Flow cytometric detection of fluorescent labelled -*P. aeruginosa* adherence to the host cell

The cells were washed as described above (section 2.2.3) and seeded into a 96-well plate at 1.0 x 10^5 cells/well in 100 µl of growth medium. Additional cells were retained for gating of the flow cytometer. The 96-well plate was centrifuged at 400 xg for 5 minutes and the media in the plate was aspirated. The cells were re-suspended in PBS by pipetting up and down. Next, the cells were centrifuged as before and the PBS aspirated. 100 µl of bacteria in media was added to the cells at a range of MOIs and incubated at 37 ºC for 60 minutes. Then, the plate was centrifuged at 400 xg for 5 minutes, the media was aspirated, and the cells were rinsed with PBS as before. The PBS was aspirated and the cells were fixed in paraformaldehyde for 30 minutes at 4 °C. Prior to FACS analysis, the cells were re-suspended gently by pipetting up and down for a few times. Attune FACS machine

was used (488 nm Laser, 530/30 BL1) at the flow cytometry core service at the Medical School, The University of Sheffield.

2.2.6 Effect of tetraspanin reagents on GFP labelled -*P. aeruginosa* adherence to the host cell

The cells were seeded as described above. Cells were incubated in presence or absence of anti-tetraspanin antibodies / isotype control at 10 µl/ml, or EC2: GST proteins / GST control at 500 nM, or CD9-derived peptides at 20 nM for 30 minutes. Cells were then washed with PBS and infected as described (section 2.2.4 or 2.2.5).

2.2.7 Cytokine measurements

Cells were seeded at 1 x 10^5 cells/well (1 ml volume) in a 24-well plate and incubated at 37 °C with 5 % CO_2 for 24 hours. An overnight culture of *P. aeruginosa* was prepared at 37 °C. The overnight culture was centrifuged at 4,500 xg for 10 minutes at 4 °C, and diluted to OD_{600} of 0.5 in DMEM (+ 1 % FBS + 50 µg/ml chloramphenicol). Cells were washed twice by pipetting with PBS and 250 µl of bacterial suspension was added at MOI of 35 and incubated at 37 °C with 5 % CO_2 for 60 minutes. After incubation, cells were gently washed with 1 ml of PBS (2 x) to remove any non-adherent bacteria. After washing, 250 µl of DMEM containing 250 µg/ml gentamicin and 1 % FBS was added, gentamicin was added to prevent further growth of extracellular bacteria. Cells were incubated for three-time points (60 minutes, 6 hours and 18 hours). After incubation, the supernatants were collected at each time point and centrifuged at 16,000 xg to pellet the bacteria, and the clear supernatant was transferred to fresh tubes and stored at -80 °C until analysis by Cytometric Bead Arrays (CBA). CBAs were run by the flow cytometry core facility at the Medical School, the University of Sheffield according to the protocol by BD Biosciences. In principle, beads are coated with antibodies against a particular

cytokine and added to the sample. The beads are then rinsed to remove any unspecific binding and a secondary fluorescent-tagged antibody is added to allow detection of the beads using flow cytometry. Samples were analysed for the following cytokines: IL-6 and IL-8. These cytokines were selected for investigation as they are released during the initial stages of infection (Sadikot *et al*., 2005).

2.2.8 Assessment of cytotoxicity using lactate dehydrogenase release

The following method was taken from (Issa, 2016). The CytoTox 96® non-radioactive cytotoxicity assay (Promega, UK) was used to measure the level of lactate dehydrogenase (LDH) in the supernatant, an indicator of cytoplasmic membrane damage, LDH is released when the integrity of the membrane is compromised. 1 x 10^4 cells/well were seeded in a 96-well plate and incubated at 37 °C with 5 % CO_2 for 24 hours to achieve a 70-80 % confluent monolayer. Monolayers were infected with bacteria at MOI of 10 for 60 minutes at the incubator. After incubation, the supernatants collected from the plate wells were transferred to a fresh 96-well plate at 50 µl/well. The supernatants were mixed with the CytoTox 96® substrate at a ratio of 1:1, and incubated at room temperature for 30 minutes. The reaction was then terminated by the addition of 50 µl 'stop' solution (provided with the kit). Absorbance was measured at 490 nm using the Thermo Scientific Varioskan plate reader. Maximum LDH release was determined as the amount released by total lysis of uninfected cells with 1 % Triton^{I} X-100 (Sigma Aldrich, UK). Percentage cytotoxicity was calculated using the **Equation (2.3).**

Equation (2.3) Percentage cytotoxicity =

$$\frac{\text{LDH release in treated cells}}{\text{Maximum LDH release}} \times 100$$

2.2.9 GFP-labelled *P. aeruginosa* internalisation assay

2.2.9.1 Determining the optimum multiplicity of infection (MOI) for internalisation assay

The following method was taken from (Ali, 2016). In order to investigate the ability of GFP-labelled *P. aeruginosa* to internalise into A549 cells, a modified protocol by Schaible *et al.*, (2013) was used. A549 cells were seeded in a 6 well plate for 48 hours at 37 ℃ with 5 % CO_2. Adherent cells were washed twice with 2 ml of PBS before incubation with GFP-labelled *P. aeruginosa* at a range of MOIs (1, 10 and 50) at 37 ℃ with 5 % CO_2 for 60 minutes to allow the bacteria to adhere to epithelial cells. Internalised bacteria were quantified by exposing A549 cells to gentamicin in order to kill the extracellular bacteria. The antibiotic was prepared in DMEM media at a concentration of 800 μg/ml, and A549 cells were treated with 1 ml gentamicin for 60 minutes at 37 ℃ with 5 % CO_2. After incubation with the antibiotic, cells were washed twice with 2 ml of PBS and treated with a lysis solution consisting of 1 % saponin (Sigma Aldrich, UK) in trypsin-versene (the sensitivity of GFP-labelled *P. aeruginosa* to gentamicin and the lysis solution was also examined). A 1 ml aliquot of saponin solution was added to each well and the A549 cells were incubated for 20 minutes at 37 ℃ with 5 % CO_2, allowing permeabilisation of the A549 cells and recovery of internalised bacteria. 100 μl aliquots were then spread onto LB plates. Next, the agar plates were incubated at 37 ℃ with 5 % CO_2 for 24 hours before the bacterial colonies were counted. Each assay contained two replicate wells. The assay was performed three times.

2.2.9.2 The effect of CD9 EC2-derived peptides on GFP-labelled *P. aeruginosa* internalisation

A549 cells were seeded in a 6 well plate for 48 hours at 37 °C with 5 % CO_2. Adherent cells were washed twice with 2 ml of PBS before incubation with the selected CD9 EC2-derived peptides for 30 minutes at 200 nM, cells were then infected with the bacteria at MOI of 10 for 60 minutes to allow the bacteria to adhere to epithelial cells. Internalised bacteria were quantified as described in section 2.2.9.1.

2.2.10 Assessment of peptides cytotoxicity

2.2.10.1 Effect of peptides on bacterial viability (ATP assay)

The following method was taken from (Issa, 2016). Adenosine triphosphate (ATP) production was measured using the BacTiter-GloTM Assay (Promega, UK). Cultures of *P. aeruginosa* were exposed to peptides at 200 nM at the mid-exponential phase (OD_{600} of ~0.5). At each time point, (0 hours, 2 hours and 24 hours) the bacterial culture was diluted 1:1000 in LB broth and an aliquot (50 µl) was transferred to a NuncTM white opaque 96-well plate (Thermo Scientific, UK). The diluted culture was then mixed with an equal volume of BacTiter- GloTM reagent. The mixture was shaken at 120 rpm for the time point (2 hours, 24 hours) and incubated at room temperature for 2 minutes. Luminescence, proportional to the amount of ATP present, was measured as relative light units (RLU) using the Thermo Scientific Varioskan.

2.2.10.2 Effect of peptides on human cell viability (MTT assay)

The following method was taken from (Issa, 2016). The MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay (Promega) was used to determine the toxicity of peptides at 500 nM concentration against the A549 cells. The assay depends on mitochondrial activity, which is represented by the change in the dye from yellow

tetrazolium MTT into intracellular purple formazan crystals by metabolically active cells (Berridge *et al*., 2005). 1 x 10^4 cells/well were seeded in a 96-well plate and incubated at 37 °C with 5 % CO_2 for 16 hours to achieve a 70-80 % confluent monolayer. Monolayers were incubated with tetraspanin derived peptides at 500 nM in media for 30 minutes. The peptides were then removed and the monolayers washed with PBS. Then, 100 µl of 0.5 mg/ml tetrazolian MTT dye prepared in FBS free media was added. Cells were incubated at 37 °C with 5 % CO_2 for 4 hours in the dark to allow the formation of formazan crystals. After incubation, media was removed and cells washed with PBS. Formazan crystals were liquefied in 100 µl DMSO at room temperature with gentle agitation for 60 minutes. Absorbance was measured at 540 nm with a reference measurement at 690 nm, using a Thermo Scientific Varioskan plate reader. **Equation (2.4)** was used to calculate the percentage viability

Equation (2.4) $$\text{Percentage viability} = \frac{\text{the absorbance of treated cells}}{\text{absorbance of untreated cells}} \times 100$$

2.2.11 Statistical analyses

Microsoft Excel and ImageJ were used to analyse the data. Graph Pad Prism 7 was used for the statistical analysis. Statistical test chosen is specified in the appropriate figure legend. Data were analysed using One-way analysis of variance (ANOVA) unless otherwise specified. In all cases reported N numbers refer to separate experiments, and all experiments were done at least three times unless listed otherwise.

CHAPTER 3: Tetraspanin expression levels in different human cells

3.1 Introduction

Tetraspanins are a superfamily of membrane proteins expressed in many eukaryotic cells, and in humans, 33 members have been recognised (Boucheix and Rubinstein, 2001). Tetraspanins associate with other tetraspanin members or cell membrane proteins or lipids to form tetraspanin enriched microdomains (TEM) (Hemler, 2003; Wright *et al*., 2004). TEM have been shown to mediate many important functions, such as adhesion, fusion and invasion (Fanaei *et al*., 2011). In order to investigate the role of tetraspanins in bacterial adhesion, it was important to determine the expression level of each tetraspanin in all the cell lines used in this study.

3.1.1 Tetraspanin distribution

Tetraspanins mediate different functions in different cells and tissues, this is reflected by the varied distribution in different cells, with each cell having a spectrum of tetraspanins that participate in the generation of specific functional microdomains (TEMs) (Levy and Shoham, 2005b). The production and subsequent trafficking of tetraspanins to cellular and organellar membranes is a well-regulated process that safeguards proper function. Though some tetraspanins appear to be distributed in all cell types (e.g. CD151 and CD9), others are relatively cell-type specific, for example, the uroplakins, that are found primarily in the epithelium of the urinary bladder (Xie *et al*., 2006).

The aim of this chapter:

- To investigate the expression level of selected tetraspanins in the three cell lines used for bacterial adhesion studies. This knowledge will help to determine which tetraspanin reagents are likely to be effective.

3.2 Results

Immunofluorescence microscopy and flow cytometry were used for screening, section 2.2.1.5 and 2.2.1.6, the three cell lines used in this study included: A549, a human lung carcinoma epithelial cell line (Lieber, Smith *et al*., 1976), HaCaT, a human keratinocyte cell line (Boukamp, Petrussevska *et al*., 1988) and HEC1B, a human endometrial epithelial cell line (Kuramoto, Tamura *et al*., 1972).

3.2.1 Microscopic visualisation of tetraspanin expression in A549 cells

In order to determine the expression of tetraspanins (CD9, CD63, CD81 and CD151) in A549 cells, immunofluorescence microscopy was performed using indirect staining of the cell lines seeded on chamber slides, as described in section 2.2.1.6. Visualisation of the target tetraspanin expression in permeabilised A549 epithelial cells showed high expression of CD9, CD63, and CD81, compared to the control (isotype); however, the expression of CD151 was low in these cells. The images in Figure 3.1 are a demonstration of the levels of these tetraspanins in A549 cells.

3.2.2 Flow cytometry analysis of tetraspanin expression in A549 cells

The quantitative evaluation of tetraspanin cell surface expression in the A549 cell line was performed using the LSRII FACS machine, as described in section 2.2.1.5. The cell population was gated depending on the FSC and SSC parameters (Figure 3.1. A and B). Figure 3.1 C shows the median fluorescence intensity (MFI) of A549 epithelial cells, the result revealed high expression of CD9, CD63 and CD81, compared to the control (isotype), whilst the expression of CD151 was lower than the other tetraspanins. The immunofluorescence microscopy analysis of tetraspanin expression in A549 cells was consistent with the data obtained by flow cytometry.

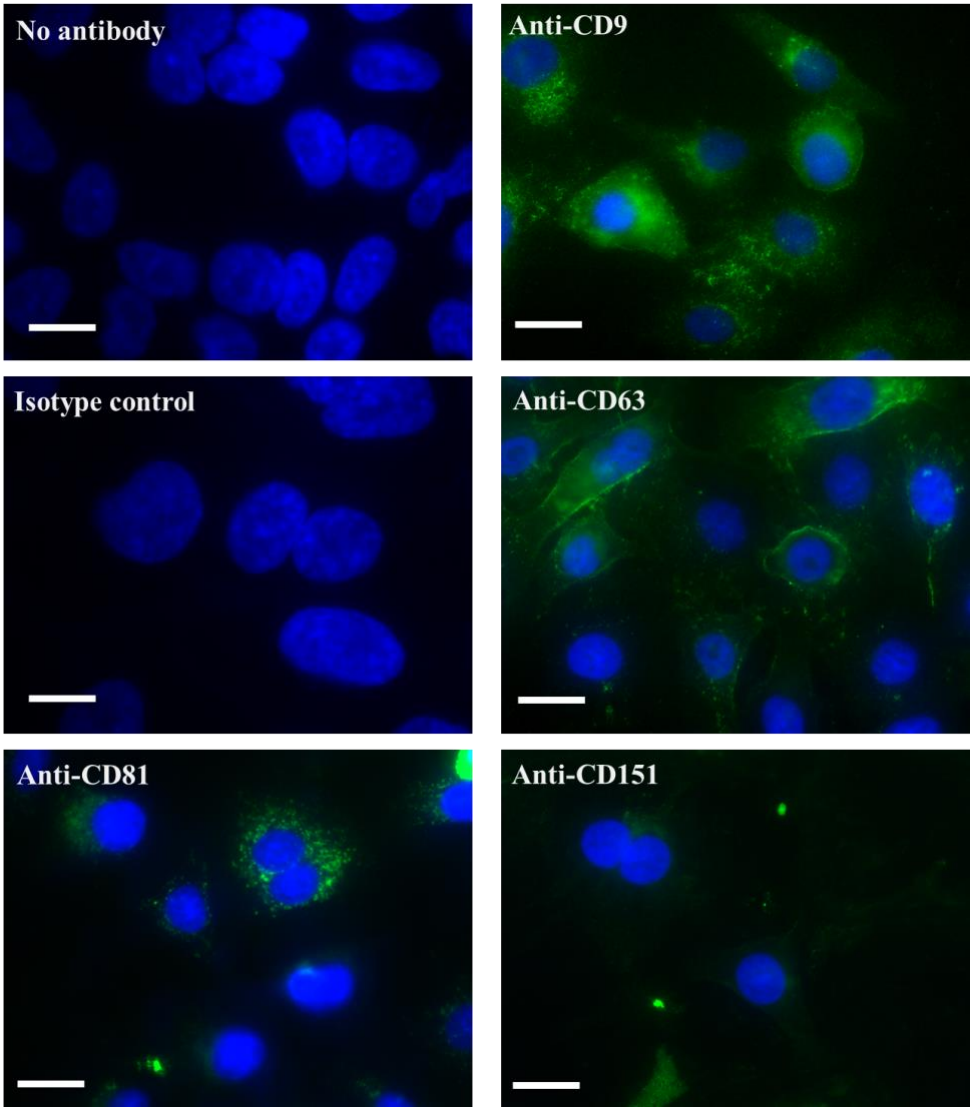

Figure 3.1 Analysis of selected tetraspanin expression in A549 cells using immunofluorescence microscopy.

A549 cells were cultured on chamber slides and fixed. Nuclei were counter-stained with DAPI. The primary monoclonal antibodies (mAbs); JC1 (isotype control), anti-CD9, anti-CD63, anti-CD81 and anti-CD151 were used at 10 μg/ml, this was followed by incubation with FITC conjugated anti-mouse IgG. The images were obtained with an Olympus fluorescent microscope by using the 100 x objective, scale bar represents 7 μm.

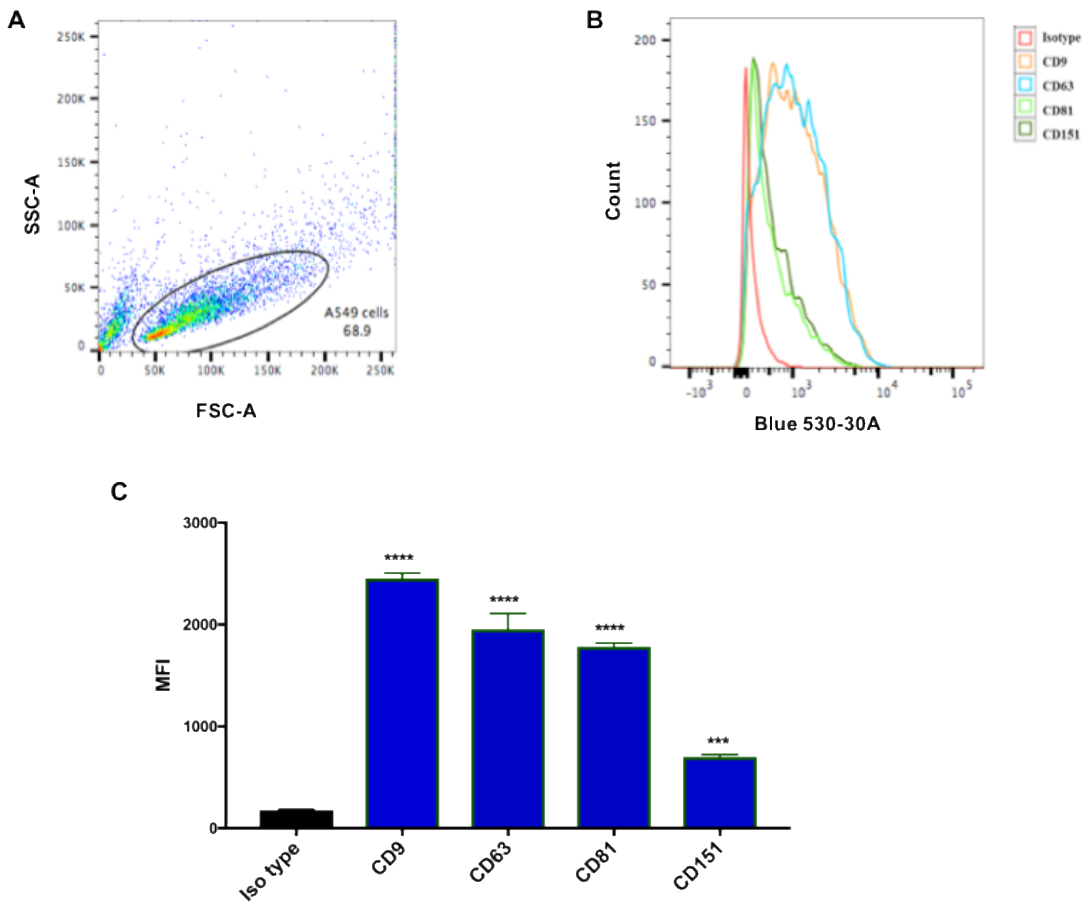

Figure 3.2 Analysis of selected tetraspanin expression in A549 cells using flow cytometry.

The primary monoclonal antibodies (mAbs); JC1 (isotype control), anti-CD9, anti-CD63, anti-CD81 and anti-CD151 were used at 10 µg/ml, this was followed by incubation with FITC conjugated anti-mouse IgG. (**A**) The dot plot shows A549 cells gated according to forward scatter (FSC) and side scatter (SSC). (**B**) The histogram plot shows tetraspanin expression levels compared to the isotype control. (**C**) Median fluorescent intensity (MFI), error bars describe the standard error, N= 4, mean ± SEM. Data were analysed by One-way ANOVA with Dennett's multiple comparison test, ****$P < 0.0001$, ***$P < 0.001$.

3.2.3 Microscopic visualisation of tetraspanin expression in HaCaT cells

Screening of tetraspanin expression on the HaCaT keratinocyte cells was performed as described above 3.2.1. The results revealed high expression of tetraspanins CD9 and CD63, while the expression of tetraspanin CD81 was lower but detectable. However, CD151 was poorly expressed in these cells, with staining similar to that of the isotype control (Figure 3.2).

3.2.4 Flow cytometry analysis of tetraspanin expression in HaCaT cells

Analysis by flow cytometry showed a different pattern of tetraspanin expression compared to immunofluorescent microscopy. High expression of CD81 was observed in terms of median fluorescent intensity with relatively low expression of CD9 and CD151 and little expression of CD63, compared to the isotype control (Figure 3.4).

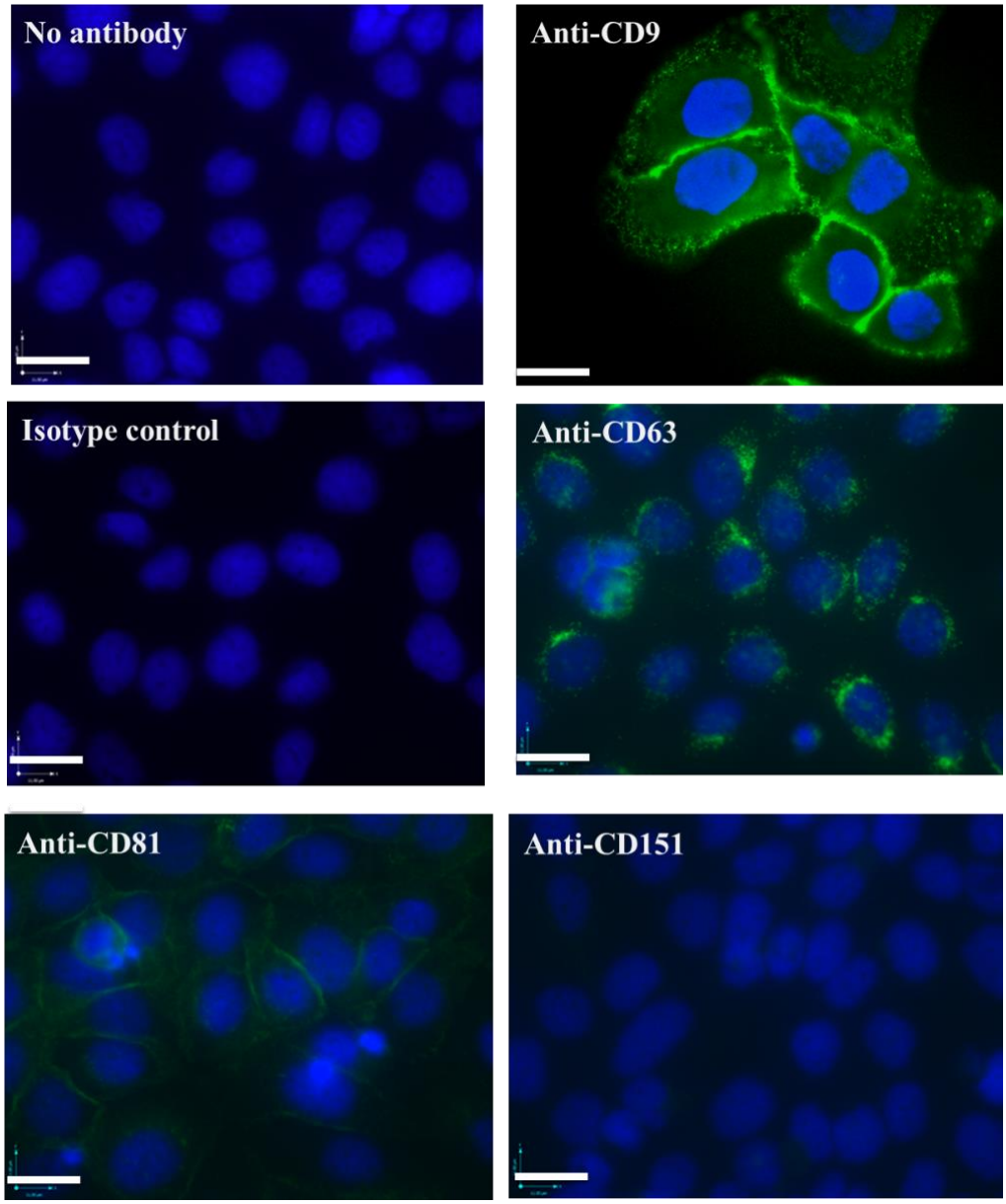

Figure 3.3 Analysis of selected tetraspanin expression in HaCaT cells using immunofluorescence microscopy.

HaCaT cells were cultured on chamber slides and fixed. Nuclei were counter-stained with DAPI. The primary monoclonal antibodies (mAbs); JC1 (isotype control), anti-CD9, anti-CD63, anti-CD81 and anti-CD151 were used at 10 µg/ml, this was followed by incubation with FITC conjugated anti-mouse IgG. The images were obtained with an Olympus fluorescent microscope by using the 100 x objective, scale bar represents 7 µm.

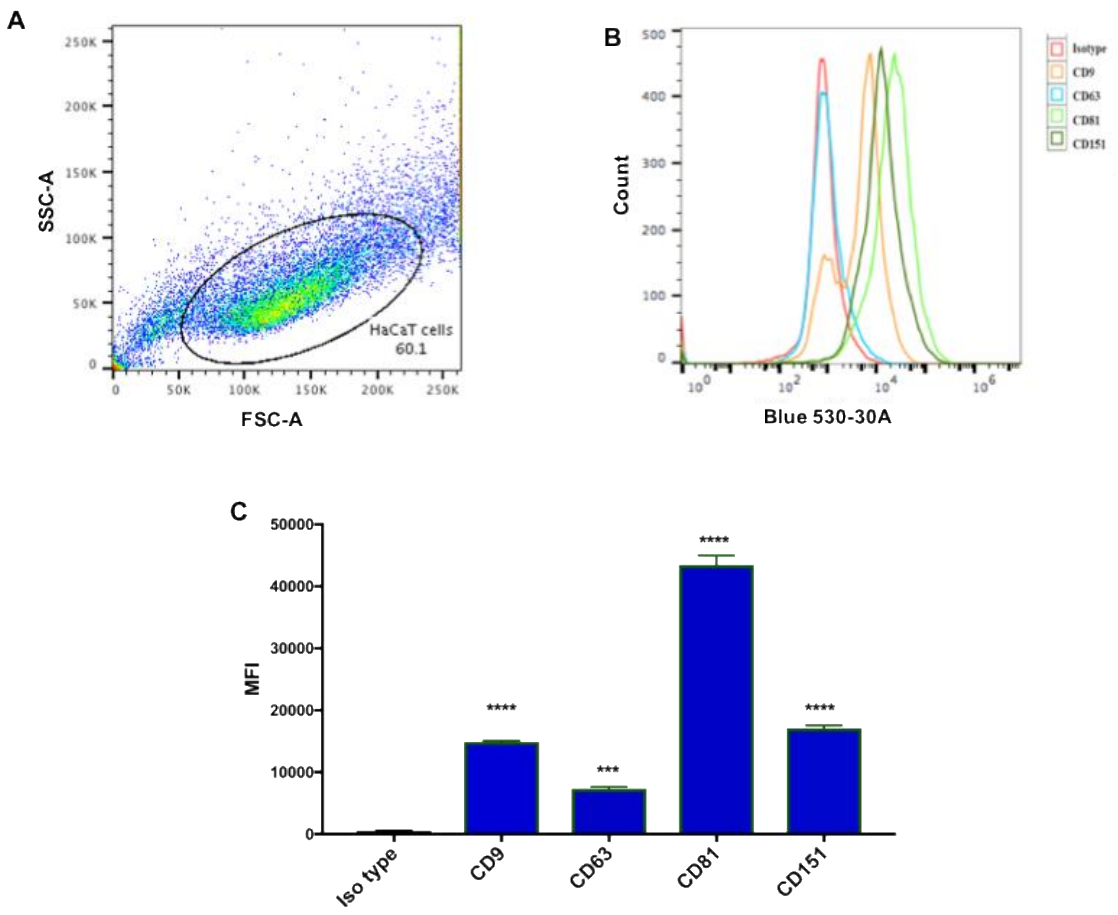

Figure 3.4 Analysis of selected tetraspanin expression in HaCaT cells using flow cytometry.

The primary monoclonal antibodies (mAbs); JC1 (isotype control), anti-CD9, anti-CD63, anti-CD81 and anti-CD151 were used at 10 µg/ml, this was followed by incubation with FITC conjugated anti-mouse IgG. (**A**) The dot plot shows HaCaT cells gated according to forward scatter (FSC) and side scatter (SSC). (**B**) The histogram plot shows tetraspanin expression levels compared to the isotype control. (**C**) Median fluorescent intensity (MFI), error bars describe the standard error, N= 4, mean ± SEM. Data were analysed by One-way ANOVA with Dennett's multiple comparison test, ****$P < 0.0001$, ***$P < 0.001$.

3.2.5 Microscopic visualisation of tetraspanin expression in HEC1B cells

Microscopic visualisation of permeabilised HEC1B cells showed a high expression of CD9 on the plasma membrane compared to the isotype control. In contrast, low expression was detected for CD63 compared to the tetraspanin CD9. Besides, CD151 displayed the smallest amount of fluorescence compared to the other tetraspanins (Figure 3.5).

3.2.6 Flow cytometry analysis of tetraspanin expression in HEC1B cells

Flow cytometry analysis revealed a high expression level of CD9 in HEC1B cells, this would indicate that these cells are a reasonable model for studying tetraspanin function in a human epithelial cell. However, CD63 and CD151 were found to be expressed at very low levels (Figure 3.6).

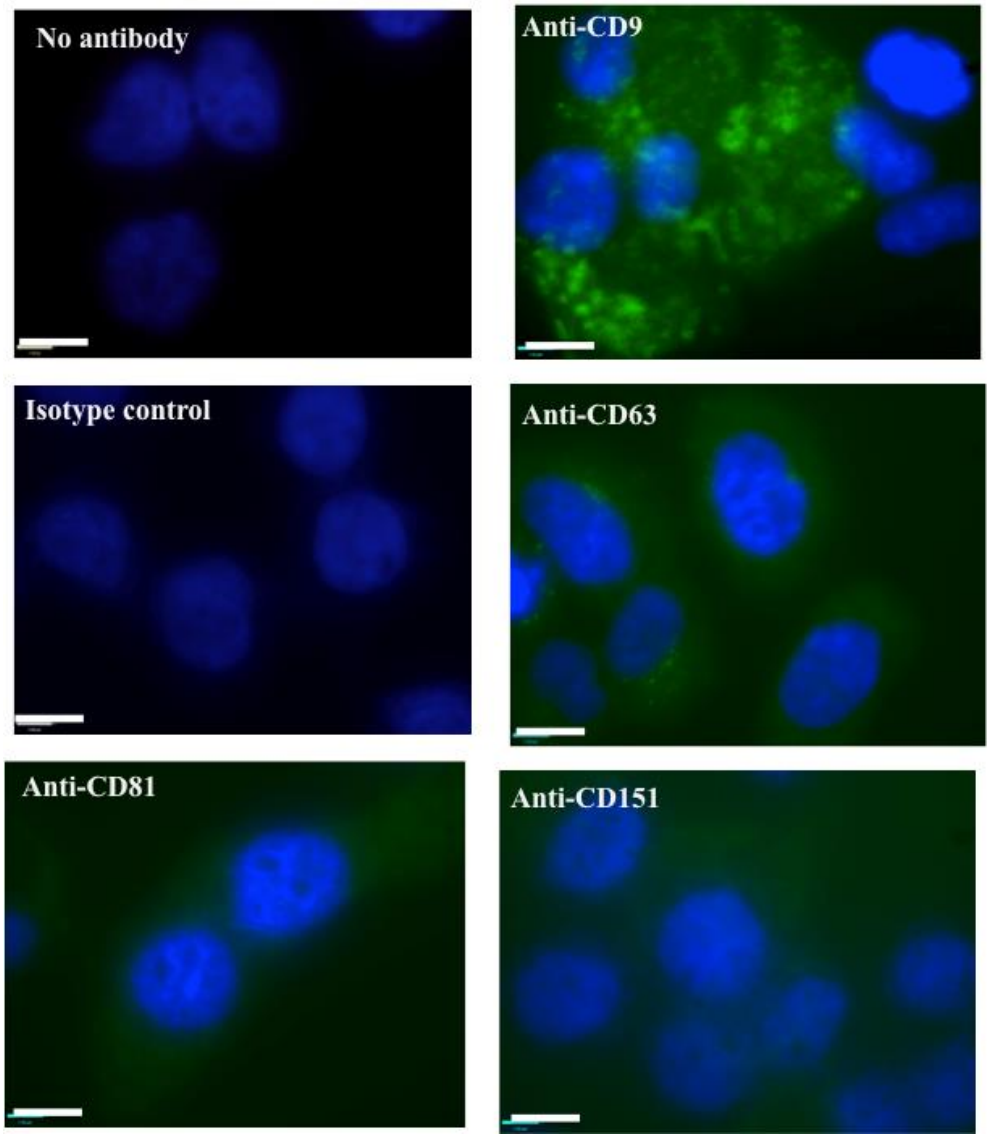

Figure 3.5 Analysis of selected tetraspanin expression in HEC1B cells using immunofluorescence microscopy.

HEC1B cells were cultured on chamber slides and fixed. Nuclei were counter-stained with DAPI. The primary monoclonal antibodies (mAbs); JC1 (isotype control), anti-CD9, anti-CD63, anti-CD81 and anti-CD151 were used at 10 µg/ml, this was followed by incubation with FITC conjugated anti-mouse IgG. The images were obtained with an Olympus fluorescent microscope by using the 100 x objective, scale bar represents 7 µm.

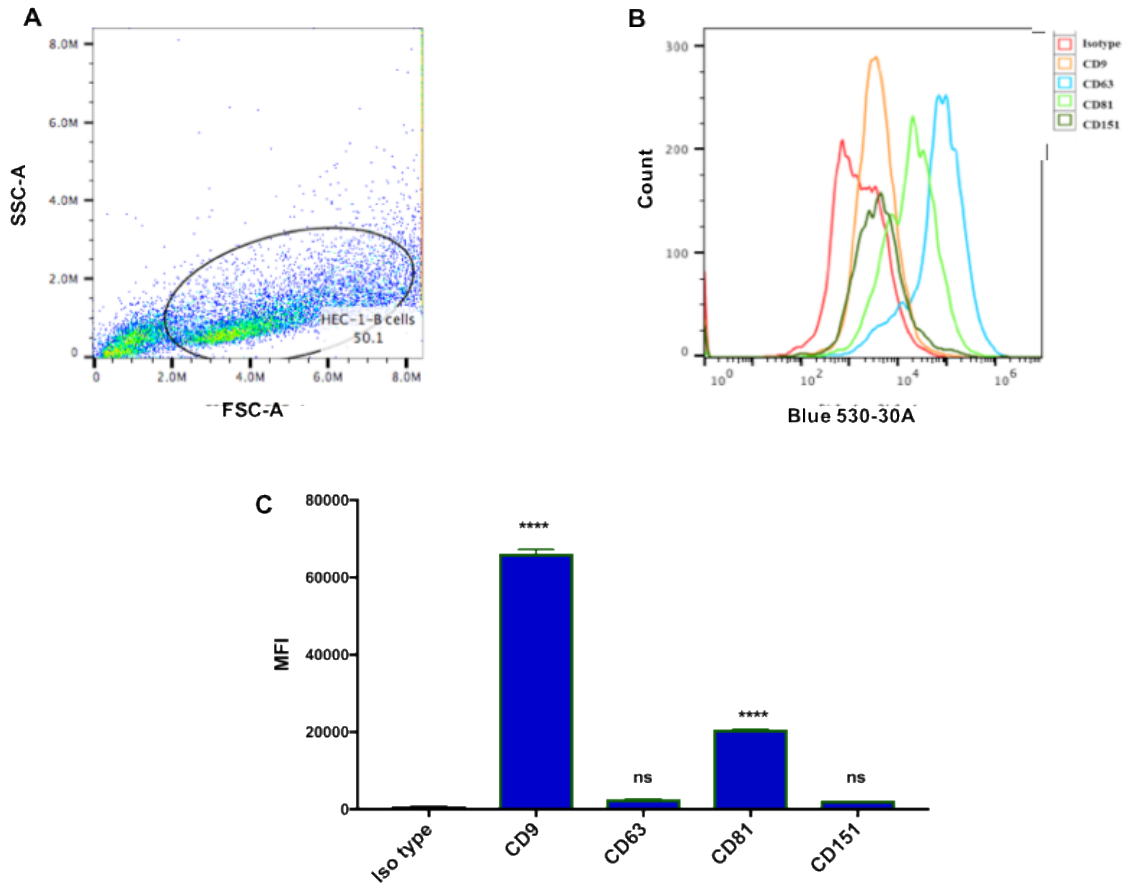

Figure 3.6 Analysis of selected tetraspanin expression in HEC1B cells using flow cytometry.

The primary monoclonal antibodies (mAbs); JC1 (isotype control), anti-CD9, anti-CD63, anti-CD81 and anti-CD151were used at 10 µg/ml, this was followed by incubation with FITC conjugated anti-mouse IgG. (**A**) The dot plot shows HEC1B cells gated according to forward scatter (FSC) and side scatter (SSC). (**B)** The histogram plot shows tetraspanin expression levels compared to the isotype control. (**C**) Median fluorescent intensity (MFI), error bars describe the standard error, N= 4, mean ± SEM. Data were analysed by One-way ANOVA with Dennett's multiple comparison test, ****$P < 0.0001$, ns denotes not significant.

3.3 Discussion

Tetraspanins including CD9, CD63, CD81, and CD151 are associated with cellular adhesion, signal transduction, migration and cell differentiation (Woegerbauer *et al.*, 2010). The aim of this chapter was to identify and quantify the expression of these tetraspanins in three cell lines A549 cells, HaCaT cells and HEC1B cells. Immunofluorescence staining and flow cytometry analysis of the three cell lines revealed that the tetraspanin CD9 was expressed at high levels in all cell lines under investigation compared to the isotype control cells, this makes all these cell lines appropriate models for studying the effect of tetraspanin CD9 EC2-derived peptides in *P. aeruginosa* adhesion. In addition, some tetraspanins such as CD63 and CD151 showed some variation between the three cell lines under study. Moreover, the expression levels of the tetraspanins in A549 and HEC1B cells were found to be similar with both immunofluorescent microscopy and flow cytometry, whereas the HaCaT cells showed different patterns of expression between the two methods.

CD63 or Tetraspanin 30 was the first described tetraspanin (Pols and Klumperman, 2009) and it is mainly linked with the membranes of intracellular vesicles (Petersen *et al.*, 2011). CD63 plays an important role in intracellular transport of other proteins (Berditchevski and Odintsova, 2007). CD151 or Tetraspanin 24 is well known for its role in enhancing cell motility and metastasis of cancer cells (Ashman *et al.*, 1991). In this study, the expression of these tetraspanins appeared to vary considerably; the A549 epithelial cell line revealed high expression levels, the HaCaT keratinocyte cell line revealed only an intermediate level expression, and the expression was found to be very low in the HEC1B cell line. This implies that a different distribution may produce a specific tetraspanin subset for each cell line allowing the formation of cell-type specific TEMs and that

explains the different function of the tetraspanin in each cell line as proposed by a number of groups (Levy and Shoham, 2005a).

Finally, CD81 or Tetraspanin 28 has been found to interact with immunoglobulin superfamily and is involved in many physiological functions (Levy, 2014; Clark *et al*., 2001). In this study, all cell lines were found to express a detectable level of CD81 but it was very high in the HaCaT cells.

To summarise, the expression of tetraspanins was variable in the three cell lines tested, showing different localisation patterns. A549 cells and HEC1B cells showed a high level of expression of CD9, and this could potentially make them suitable target cells for CD9 EC2-derived peptides therapies (chapter 6). Furthermore, chapter 5 will investigate the effect of recombinant tetraspanin EC2: GST proteins and anti-tetraspanin antibodies on *P. aeruginosa* adhesion to our tested cell lines.

CHAPTER 4: Use of flow cytometry to improve throughput in bacterial adhesion assays

4.1 Introduction

Flow cytometry is considered to be a useful tool in studying bacterial host binding and bacterial pathogenicity (Valdivia and Falkow, 1998; Pils *et al.*, 2006b). It is a rapid method for investigating bacterial adhesion and invasion. For example, some flow cytometers (such as Attune) can analyse samples in a 96-well format. Moreover, it allows the use of genetically or chemically tagged bacteria (Agerer *et al.*, 2004; DeLoid *et al.*, 2009; Trouillet *et al.*, 2011b). However, sometimes the use of modified bacteria may introduce artefacts when studying adhesion and invasion (Trouillet *et al.*, 2011b). The purpose of this chapter is to investigate the usefulness of fluorescence microscopy as a replacement of manual counting by developing a flow cytometric method that can rapidly detect the adhesion of *P. aeruginosa* to a host cell. In order to achieve this aim, a fluorescent-labelled *P. aeruginosa* (PAO1) was constructed and characterised.

The aims of this chapter:

- To generate a fluorescent *P. aeruginosa* (green and red) by genetic modification.
- To determine the optimum MOI for infection.
- To develop a flow cytometric method (Attune FACS machine) that can detect the disruption of *P. aeruginosa* adhesion to host cells using tetraspanin reagents.

4.2 Results

4.2.1 Construction of GFP and DsRed expressing *P. aeruginosa*

The plasmids PIN25 (green) and PIN29 (red) were introduced into the *P. aeruginosa* strain PAO1 by electroporation. Firstly, we determined if there was any change in bacterial growth in the presence of the new plasmid. Bacterial growth was investigated by the measurement of the OD at 600 nm and CFU/ml count (section 2.2.2.2). No significant changes were observed between the wild type and transformed bacteria (Figure 4.1). Secondly, *P. aeruginosa* was found to be successfully labelled with both plasmids (PIN25 and PIN29) as shown in Figure.4.2. There was a ~100-fold increase in the fluorescence of GFP-labelled *P. aeruginosa* and DsRed-labelled *P. aeruginosa* compared to the unlabelled *P. aeruginosa* (Figure 4.2). Finally, attachment of the fluorescent and non-fluorescent bacteria to A549 cells was analysed by flow cytometry (Figures 4.3) and the percentage of positive fluorescent bacteria compared with non-transformed wild type bacteria was measured using flow cytometry, and compared to the non-transformed bacteria (Figures 4.4). Moreover, the fluorescent bacteria were clearly visualised using fluorescence microscopy as shown in Figure 4.5.

The fluorescent bacteria were clearly detectable using both methods. However, GFP-labelled *P. aeruginosa* was used through this study as the DsRed-labelled *P. aeruginosa* was found to be more susceptible to photo bleaching.

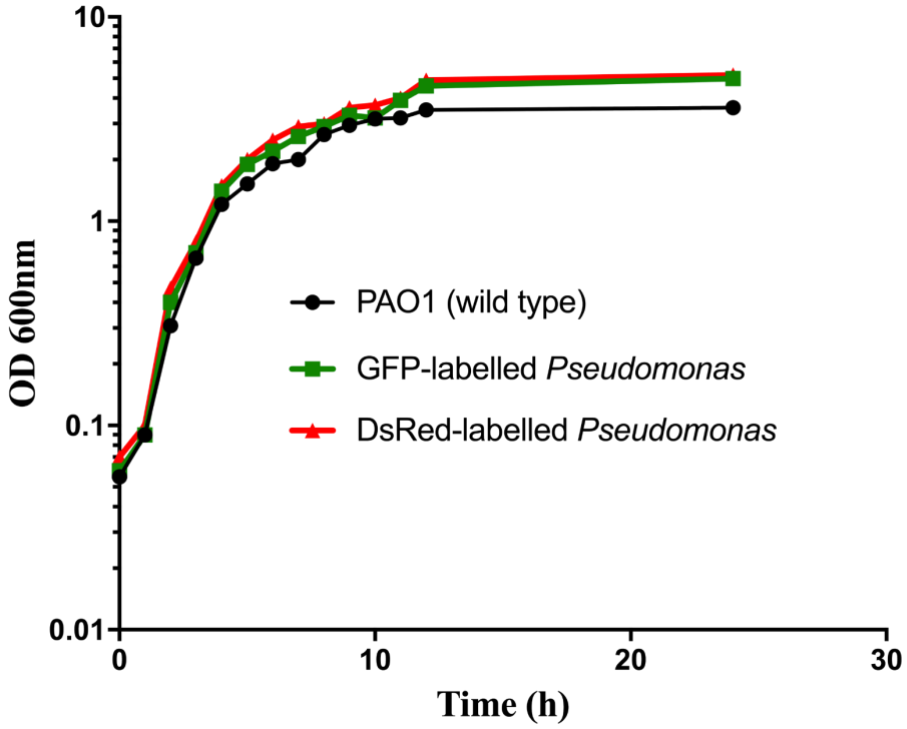

Figure 4.1 Growth curve of GFP-labelled *P. aeruginosa* and DsRed-labelled *P. aeruginosa* over time in LB medium.

Overnight cultures of GFP-labelled *P. aeruginosa* and DsRed-labelled *P. aeruginosa* were diluted to an OD_{600} of 0.05 and grown in LB medium at 37°C for 24 hours, with agitation at 120 rpm. OD was measured at 600 nm, N= 3.

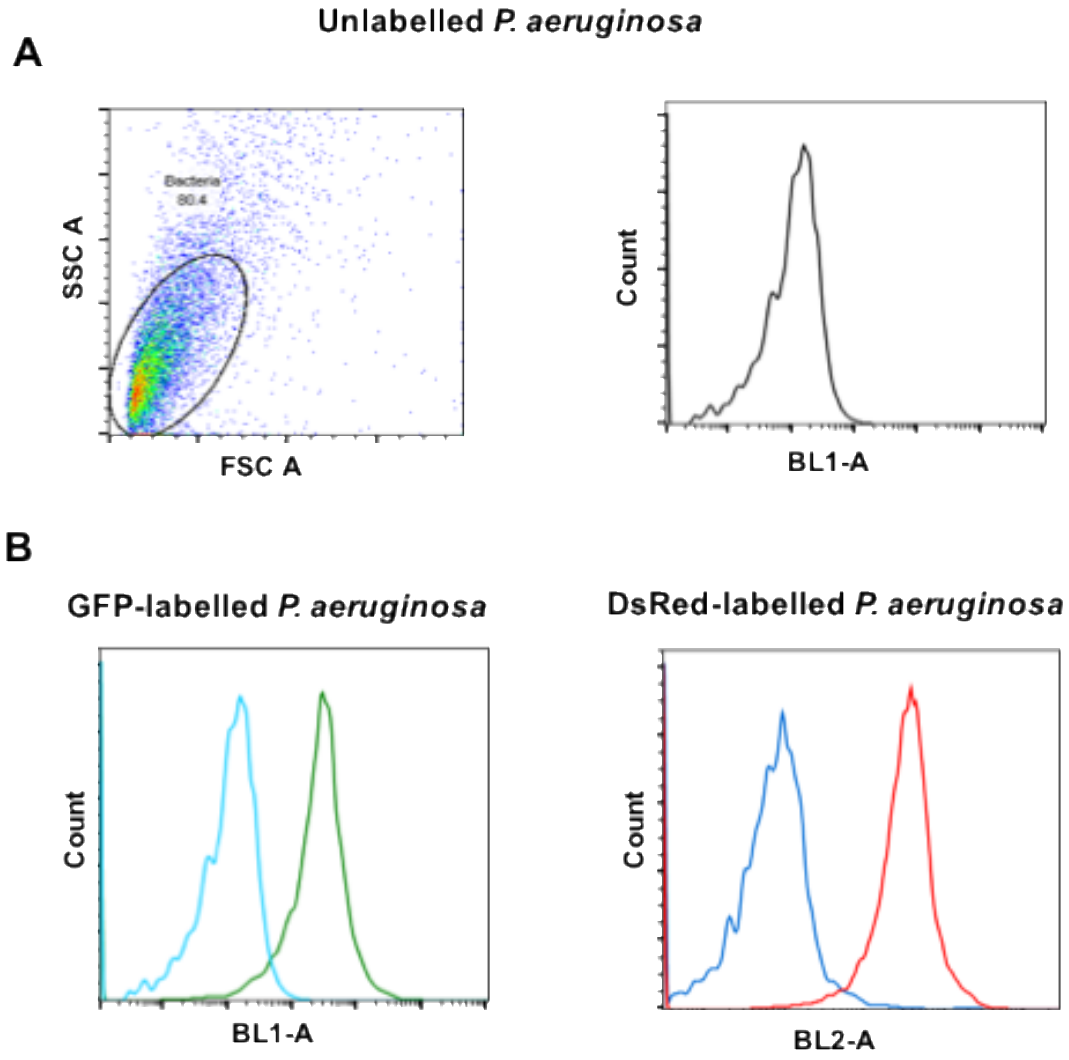

Figure 4.2 Flow cytometric analysis of labelled GFP and DsRed labelled *P. aeruginosa.*

(**A**) Distribution of unlabelled bacteria in the dot plot display of forward scatter (FCS) vs side scatter (SSC) (left panel) and frequency plot (right panel). (**B**) Labelled bacteria frequency plots for GFP (left panel) and DsRed (right panel). The efficiency of the labelling was analysed by measuring the fluorescence of the bacteria on channel BL1 for GFP (green) and BL2 for DsRed (red). The labelling of the bacteria is seen as a shift of the bacteria along the BL1 and BL2 axes in the frequency plots. The data were analysed by the Attune FACS machine.

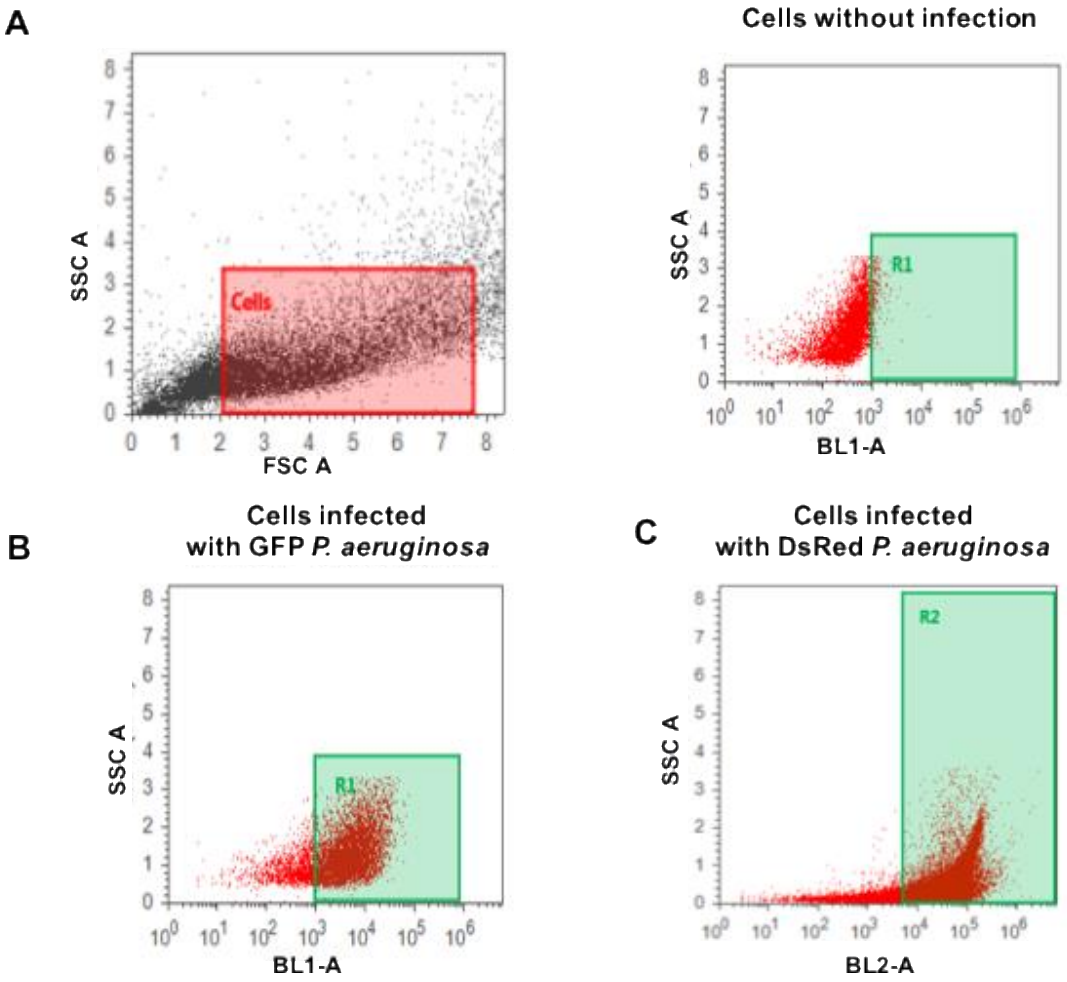

Figure 4.3 Quantifying the adhesion of fluorescent GFP and DsRed-labelled *P. aeruginosa* to A549 cells by flow cytometry.

Analysis of the green (PIN25) and red (PIN29) transformed *P. aeruginosa* by flow cytometry using Attune FACS machine. (**A**) Dot plot showing cells without infection and (**B**) cells infected with GFP-labelled *P. aeruginosa* using the filter BL-1 to detect GFP (530/30 nm), and (**C**) cells infected with DsRed-labelled *P. aeruginosa* using the filter BL-2 to detect DsRed (574/26 nm).

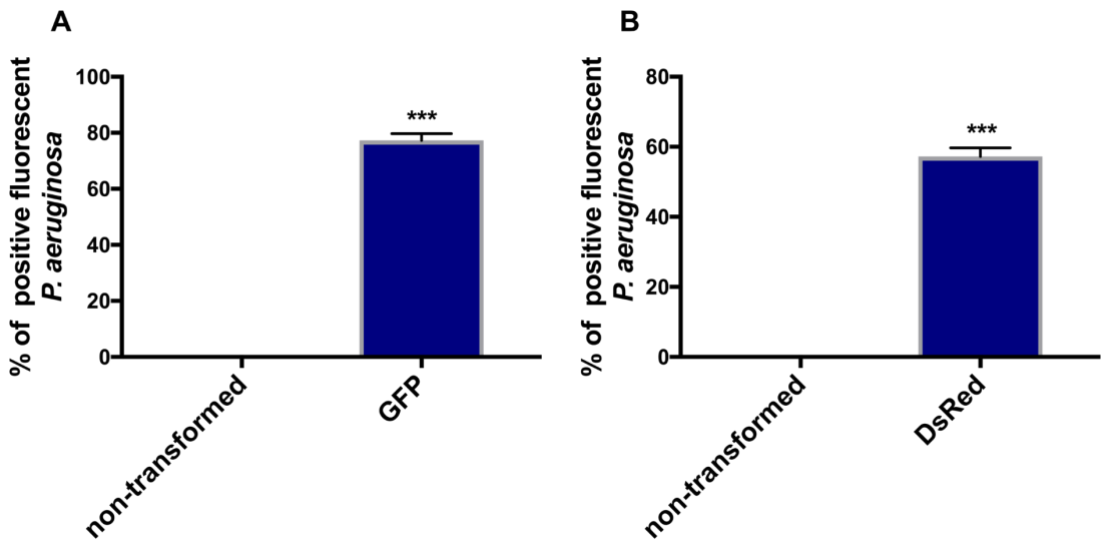

Figure 4.4 Flow cytometric analysis of fluorescent GFP and DsRed-labelled *P. aeruginosa*.

The percentage of positive fluorescent bacteria compared with non-transformed wild type was measured using flow cytometry and compared to the non-transformed bacteria. (**A**) The percentage of GFP-labelled *P. aeruginosa.* (**B**) The percentage of DsRed-labelled *P. aeruginosa.* Data were analysed using One-way ANOVA test, ***$P \leq 0.001$, N= 3. Results are mean ± standard error of the mean (SEM).

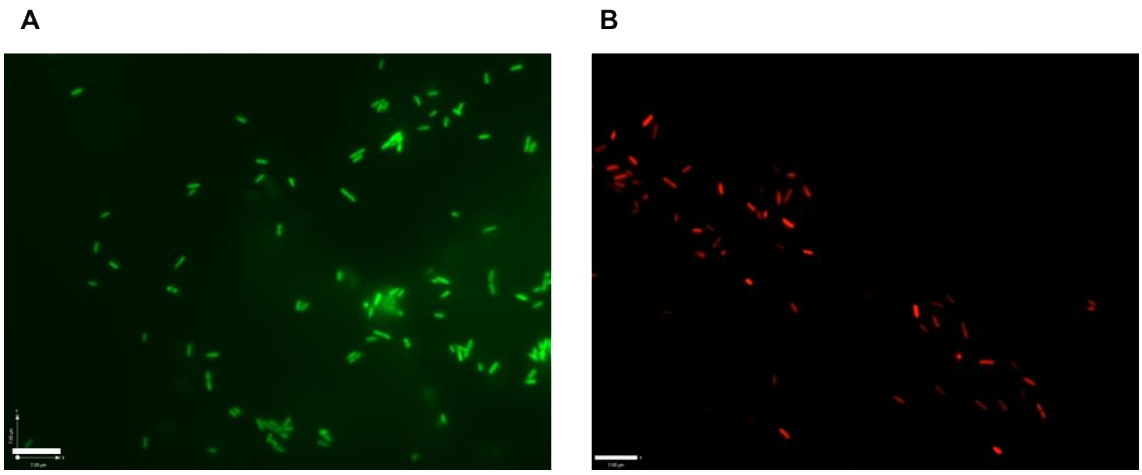

Figure 4.5 Immunofluorescence images of fluorescent labelled *P. aeruginosa* transformed with GFP and DsRed plasmid.

P. aeruginosa were cultured and prepared for microscopic imaging (section 2.2.3). (**A**) Transformed GFP-labelled *P. aeruginosa* (green) visualised using FITC filter and (**B**) Transformed DsRed-labelled *P. aeruginosa* (red) visualised using the Texas red filter. Images were obtained by fluorescence microscopy using the 100 x objective, the scale bar represents 7 µm.

4.2.2 Optimising the multiplicity of infection (MOI)

In order to study the adhesion of *P. aeruginosa* to the host cell, a range of MOIs were tested to determine the optimum MOI that gives 50 % maximal fluorescence of infected cells. GFP-labelled *P. aeruginosa* was used to infect the A549 cell line at serial bacterial dilutions and the degree of fluorescence obtained in flow cytometry was plotted in order to determine the optimal MOI (Figure 4.6). MOI of 35 was found to be the optimum MOI for flow cytometry infection (Figure 4.7). This optimisation was carried out for HaCaT cells and HEC1B cells using a similar method (data not shown).

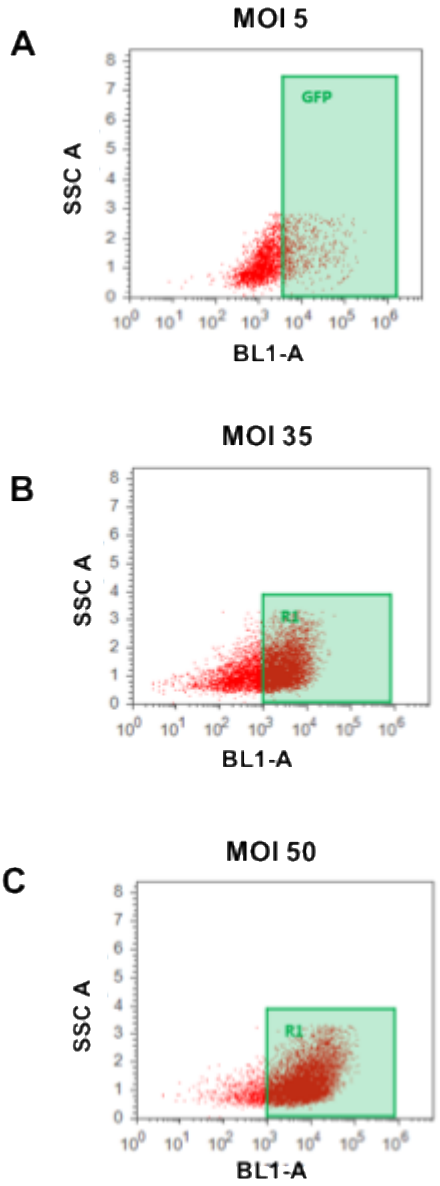

Figure 4.6 Flow cytometric analysis of A549 cells infected with GFP-labelled *P. aeruginosa* at MOIs of 5, 35 and 50.

A549 cells were incubated with GFP-labelled *P. aeruginosa* for 60 minutes at a range of MOIs followed by fixation and flow cytometry analysis using the Attune FACS machine, N= 4. The dot plots show region R1 showing positively fluorescent GFP-labelled *P. aeruginosa*. (**A**) MOI 5:1, (**B**) MOI 35:1 and (**C**) MOI 50:1.

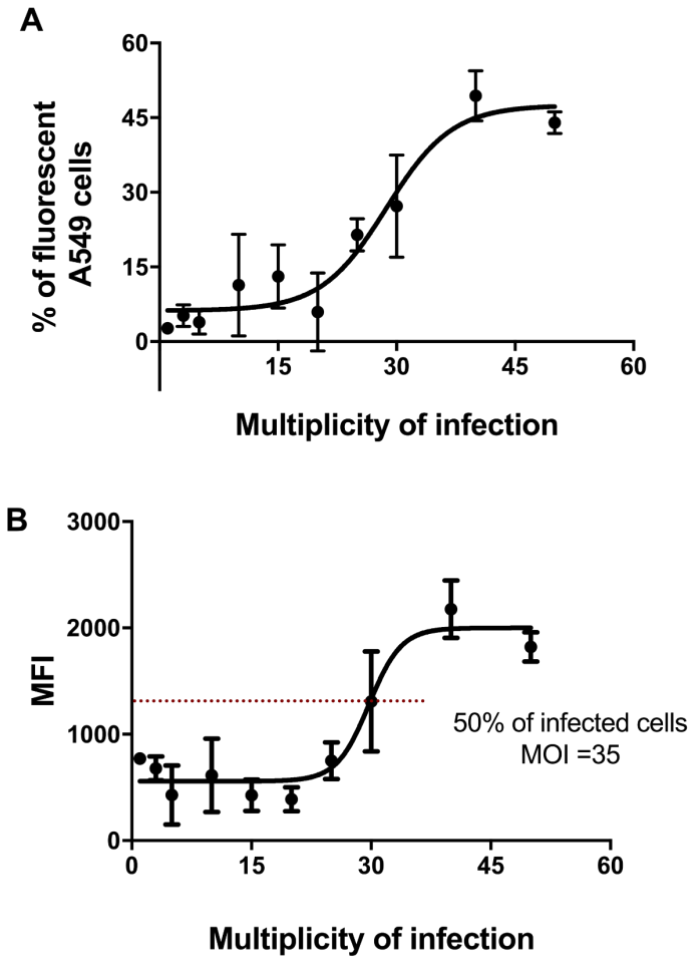

Figure 4.7 Flow cytometric analysis of optimum MOI on the attachment of GFP-labelled *P. aeruginosa* to A549 cells.

A549 cells were incubated with GFP-labelled *P. aeruginosa* for 60 minutes at a range of MOIs followed by fixation and flow cytometry analysis. (**A**) The percentage of positive fluorescent A549 cells. (**B**) The median fluorescence intensity (MFI). A549 cells were analysed using Attune FACS machine, N= 4.

4.2.3 The effect of tetraspanin treatments on GFP-labelled *P. aeruginosa* adhesion analysed by flow cytometry

The results in section 4.3.1 demonstrated the potential of using flow cytometry for detection of bacterial binding instead of using the conventional immunofluorescent microscopy method. Flow cytometry is a faster method of detection that can screen a large number of cells associated with bacteria, whereas microscopy requires manual counting of cells, thus it takes longer time and a lesser number of cells with associated bacteria are counted.

GFP-labelled *P. aeruginosa* were prepared for infection as previously described (section 2.2.3). The main difference in the procedure afterwards was that cells were harvested, subjected to tetraspanin treatments in a 96-well plate and then infected with the bacteria (section 2.2.5). Optimisation experiments were carried out for each cell line. Interestingly, the overall optimisation results showed that a higher MOI was needed to give a sufficient number of infected cells for the host cell lines to be detected by flow cytometry (section 4.3.2). Tetraspanin treatments were then screened using this method.

All data were normalised to the mean of the control (isotype for anti-tetraspanin antibodies / GST for EC2 proteins treatment) to compensate for the effect of the variation between the experiments.

4.2.3.1 The effects of anti-tetraspanin antibodies on GFP-labelled *P. aeruginosa* adherence to A549 cells

The A549 epithelial cell line was used because the A549 cells offer a useful epithelial cell model for the investigation of *P. aeruginosa* infection (Ramphal *et al.*, 1991; Hawdon *et al.*, 2010; Ahmed *et al.*, 2014). After treatment with anti-tetraspanin antibodies - anti-CD9, anti-CD63, anti-CD81 and anti-CD151, or isotype control at a concentration of 10

μg /ml for 30 minutes, the A549 cells were infected with GFP-labelled *P. aeruginosa* at MOI of 35 for 60 minutes using the flow cytometry protocol for infection as described in detail in section 2.2.5. The isotype control antibody was used to determine a possible effect of nonspecific binding of the antibody. The antibody was used at 10 μg /ml because this concentration is known to give saturating binding (Ali, 2016; Elgawidi, 2016). The cells were analysed using the Attune FACS machine and 10,000 events were acquired. The cells were gated depending on forward and side scatter (FSC and SSC respectively), and for detecting cells associated with bacteria, the blue 488 laser (BL-1) was used to distinguish the FITC signal (Figure 4.8 A), which was clearly above the auto-fluorescent background signals from the cells. The percentage of positive fluorescent cells reveals the cells with binding bacteria whilst the MFI shows the intensity of bacterial infection.

The flow cytometry method showed a slight inhibition of GFP-labelled *P. aeruginosa* adhesion to A549 cells pre-treated with the anti-CD9 antibody (Figure 4.8 B). However, no significant reduction in the percentage of infection rate was seen in cells pre-treated with anti-CD81, anti-CD63 and anti-CD151 antibodies compared to the isotype control cells (Figure 4.9 A.). Moreover, a similar pattern was seen in terms of median fluorescent intensity (Figure 4.9 B).

4.2.3.2 The effects of EC2: GST proteins on GFP-labelled *P. aeruginosa* adherence to A549 cells

The flow cytometry method was used to investigate the effect of EC2: GST proteins on adhesion of GFP-labelled *P. aeruginosa* to A549 cells. After treatment with EC2: GST proteins - CD9, CD63, CD81 and CD151, or GST control at a aconcentration of 500 nM for 30 minutes, the cells were infected with GFP-labelled *P. aeruginosa* at MOI of 35 for 60 minutes using the flow cytometry protocol for infection as described in detail in section 2.2.5, similar to the antibodies treatment method. A minor significant change in the

percentage of fluorescent A549 cells was observed when cells were pre-treated with CD81 compared to the GST control (Figure 4.10 A). While, no significant effect was observed when cells were pre-treated with CD9, CD63 and CD151 compared to the GST control (Figure 4.10 A). Also, a significant reduction was observed in the median fluorescent intensity of GFP-labelled *P. aeruginosa* adhesion in the cells that were pre-treated with CD81 prior to infection compared to the GST control cells (Figure 4.10 B). However, no significant effect was observed when the cells were pre-treated with CD9, CD63 and CD151 compared to the GST control cells (Figure 4.10 B).

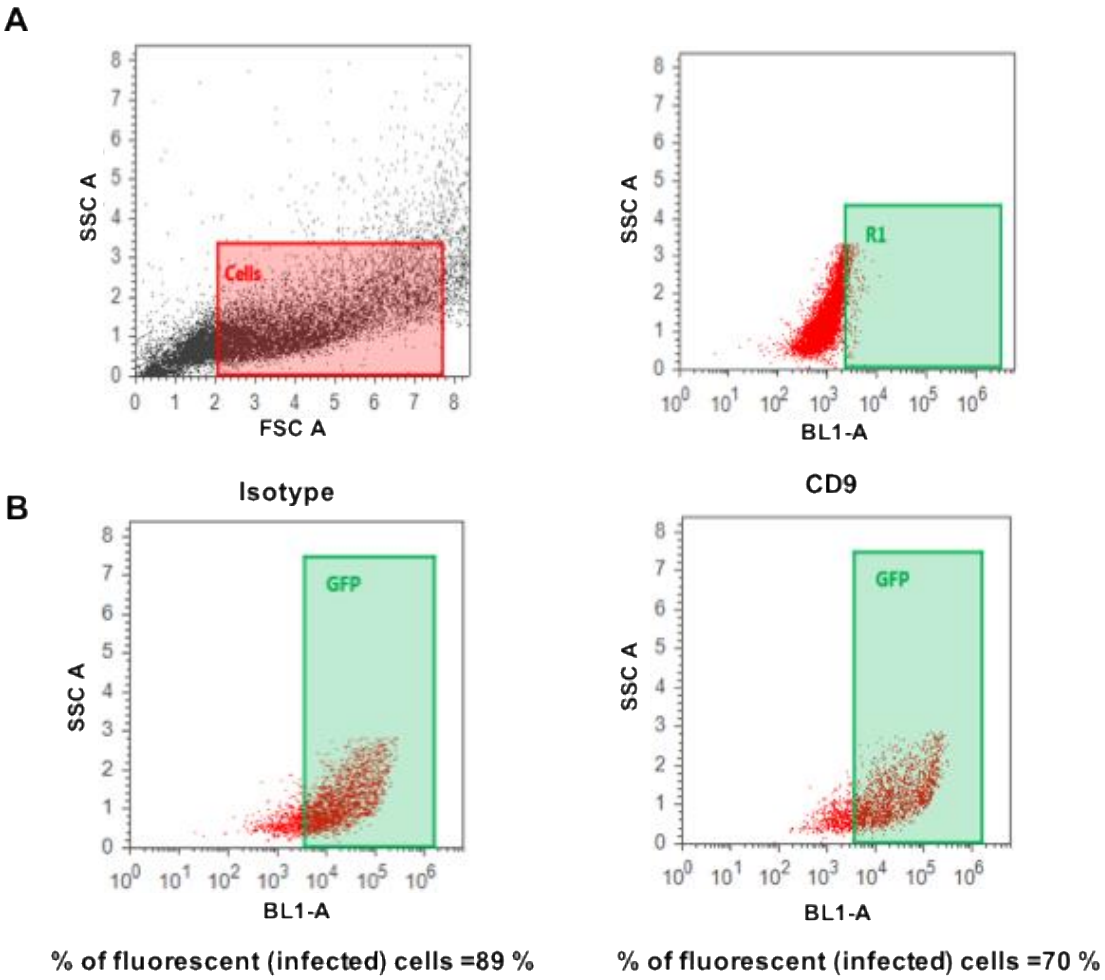

Figure 4.8 The flow cytometric detection of the effects of anti-CD9 antibody on GFP-labelled *P. aeruginosa* adherence to A549 cells.

A549 cells were pre-treated with anti-tetraspanin antibodies - anti-CD9 or isotype control at 10 µg /ml for 30 minutes before infection with GFP-labelled *P. aeruginosa* as described in 2.2.5. The analysis was done using the Attune FACS machine. (**A)** Dot plot analysis showing the gated population of A549 cells analysed based on forward and side scatter, with the region R1 showing the percentage of positively fluorescent cells. (**B**) The percentage of A549 cells after treatment with isotype control (left) or anti-CD9 (right) followed by infection with GFP-labelled *P. aeruginosa*.

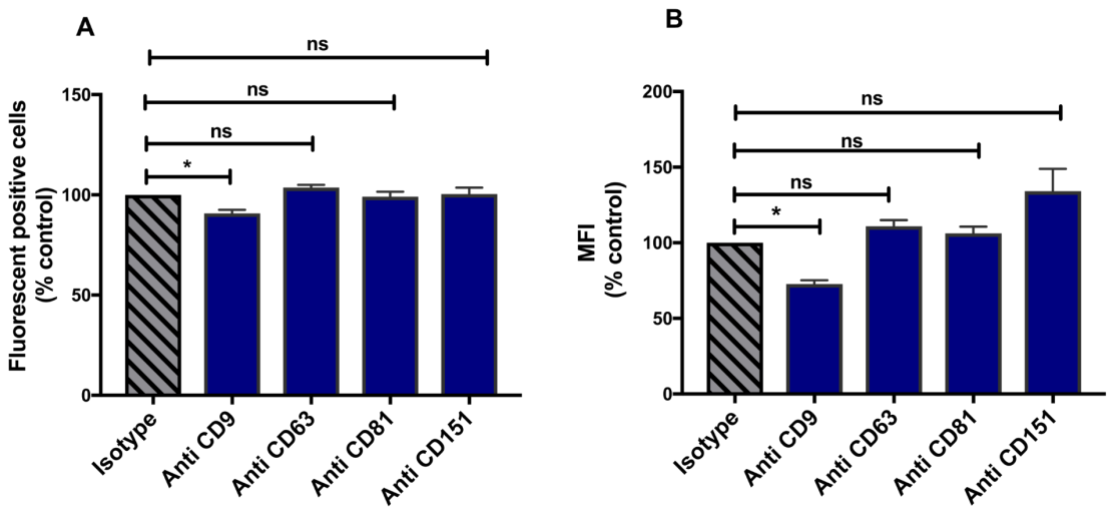

Figure 4.9 The flow cytometric detection of the effects of anti-tetraspanin antibodies on GFP-labelled *P. aeruginosa* adherence to A549 cells.

A549 cells were pre-treated with anti-tetraspanin antibodies - anti-CD9, anti-CD63, anti-CD81 and anti-CD151, or isotype control at 10 µg /ml for 30 minutes before infection with GFP-labelled *P. aeruginosa* as described in 2.2.5. The analysis was done using the Attune FACS machine. (**A)** The percentage of A549 cells infected with GFP-labelled *P. aeruginosa* normalised to 100 % isotype control. (**B**) The median fluorescent intensity (MFI) normalised to 100 % isotype control. Results are mean ± standard error of the mean (SEM), N= 4. Data were analysed using One-way ANOVA (vs. isotype control), *$P \leq 0.1$ and ns is non-significant.

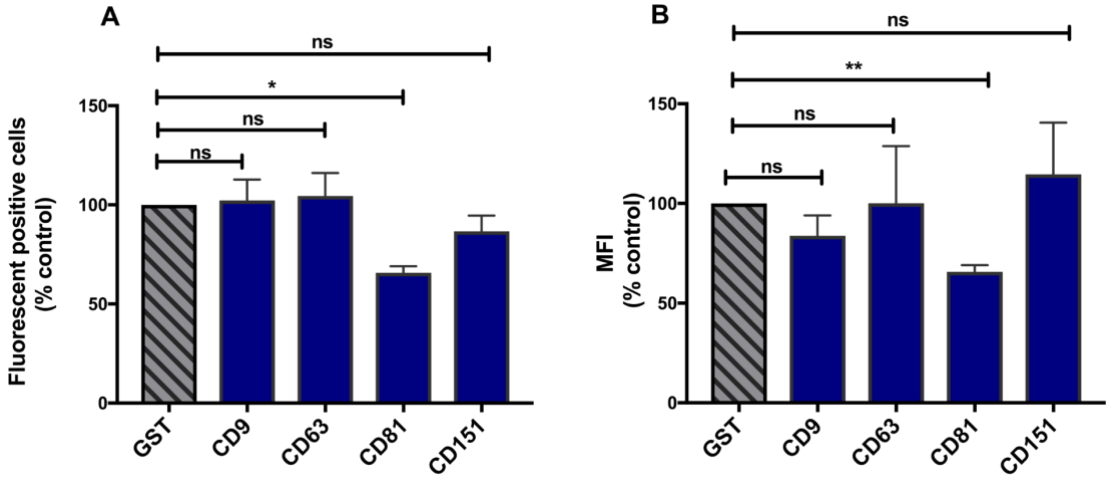

Figure 4.10 The flow cytometric detection of the effects of EC2: GST proteins on GFP-labelled *P. aeruginosa* adherence to A549 cells.

A549 cells were pre-treated with EC2: GST proteins - CD9, CD63, CD81 and CD151, or GST control at 500 nM for 30 minutes before infection with GFP-labelled *P. aeruginosa* as described in 2.2.5. The analysis was done using the Attune FACS machine. (**A)** The percentage of A549 cells infected with GFP-labelled *P. aeruginosa* normalised to 100 % GST control. (**B**) The median fluorescent intensity normalised to 100 % GST control. Results are mean ± standard error of the mean (SEM), N= 4. Data were analysed using One-way ANOVA (vs. GST control), $^{**}P \leq 0.01$, $^{*}P \leq 0.1$ and ns are non-significant.

4.2.3.3 The effects of anti-tetraspanin antibodies on GFP-labelled *P. aeruginosa* adherence to HaCaT cells

The HaCaT keratinocyte cell line was also investigated because the HaCaT cells are considered a model for *P. aeruginosa* infection (Lee *et al.*, 2016). From the initial optimising experiments, MOI of 35 for 60 minutes was chosen (data not shown). HaCaT cells pre-treated with anti-CD9 showed a significant reduction of GFP-labelled *P. aeruginosa* adhesion compared to the isotype control cells (Figure 4.11 A). While, no significant reduction was seen in the percentage of cells pre-treated with anti-CD63, anti-CD81 and anti-CD151 compared to the isotype control cells (Figure 4.11 A). In addition, in terms of median fluorescent intensity, similar patterns were observed (Figure 4.11 B).

4.2.3.4 The effects of EC2: GST proteins on GFP-labelled *P. aeruginosa* adherence to HaCaT cells

The effects of EC2: GST proteins were also investigated in HaCaT cells. Here, CD9 reduced the bacterial association of cells infected with the GFP-labelled *P. aeruginosa* whilst no significant effects were noticed with any other EC2: GST protein treatments compared to the GST control cells (Figure 4.12 A). Additionally, similar patterns were observed in terms of the median fluorescent intensity (Figure 4.12 B).

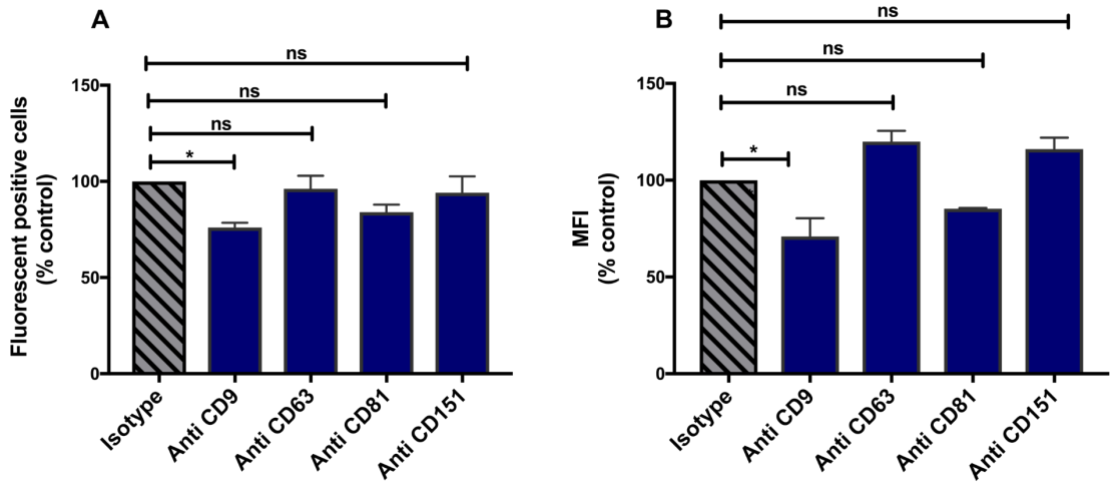

Figure 4.11 The flow cytometric detection of the effects of anti-tetraspanin antibodies on GFP-labelled *P. aeruginosa* adherence to HaCaT cells.

HaCaT cells were pre-treated with anti-tetraspanin antibodies - anti-CD9, anti-CD63, anti-CD81 and anti-CD151, or isotype control at 10 µg /ml for 30 minutes before infection with GFP-labelled *P. aeruginosa* as described in 2.2.5. The analysis was done using the Attune FACS machine. (**A)** The percentage of HaCaT cells infected with GFP-labelled *P. aeruginosa* normalised to 100 % isotype control. (**B**) The median fluorescent intensity normalised to 100 % isotype control. Results are mean ± standard error of the mean (SEM), N= 4. Data were analysed using One-way ANOVA (vs. isotype control), *$P \leq 0.1$ and ns is non-significant.

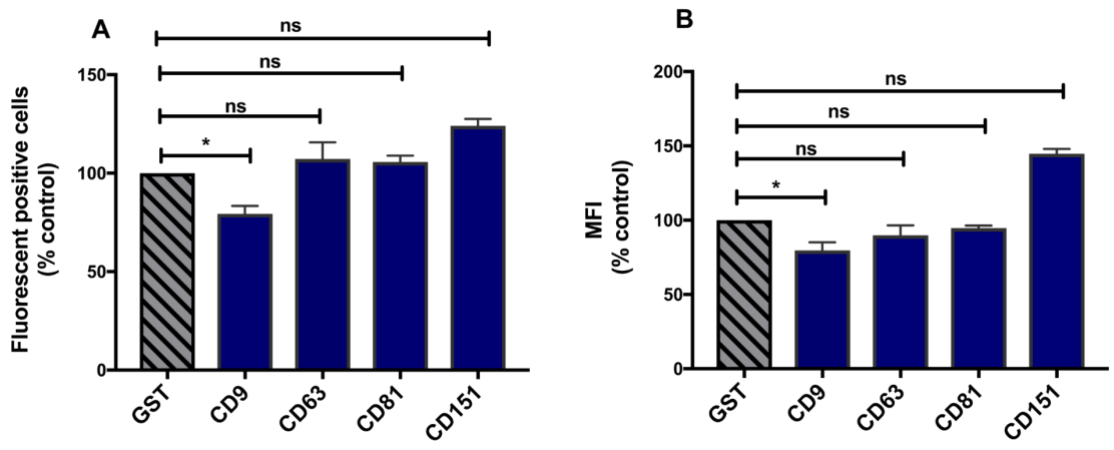

Figure 4.12 The flow cytometric detection of the effects of EC2: GST proteins on GFP-labelled *P. aeruginosa* adherence to HaCaT cells.

HaCaT cells were pre-treated with EC2: GST proteins - CD9, CD63, CD81 and CD151, or GST control at 500 nM for 30 minutes before infection with GFP-labelled *P. aeruginosa* as described in 2.2.5. The analysis was done using the Attune FACS machine. (**A)** The percentage of HaCaT cells infected with GFP-labelled *P. aeruginosa* normalised to 100 % GST control. (**B**) The median fluorescent intensity normalised to 100 % GST control. Results are mean ± standard error of the mean (SEM), N= 4. Data were analysed using One-way ANOVA (vs. GST control), *$P \leq 0.1$ and ns is non-significant.

4.2.3.5 The effects of anti-tetraspanin antibodies on GFP-labelled *P. aeruginosa* adherence to HEC1B cells

Alongside with A549 cells and HaCaT cells, another epithelial cell line was used. The HEC1B cell line is a common model for Gram-negative bacteria infection studies (Miller and Falkow, 1988; Spence *et al*., 1997; Spence and Clark, 2000). Additionally, in the previous chapter 3, the HEC1B cells were found to have a high expression level of tetraspanins CD9 and CD81, and this makes them a useful cell model for investigation. Optimisation experiments indicated that an MOI of 35 and an infection time of 60 minutes were suitable (data not shown). As previously (section 4.3.3.1), HEC1B cells were pre-treated with anti tetraspanins antibodies prior to infection. As shown in Figure 4.13, none of the anti-tetraspanin antibodies tested reduced adhesion of the cells infected with GFP-labelled *P. aeruginosa* either in the percentage of fluorescent cells (Figure 4.13 A) or as a median fluorescent intensity (Figure 4.13 B).

4.2.3.6 The effects of EC2: GST proteins on GFP-labelled *P. aeruginosa* adherence to HEC1B cells

The effects of EC2: GST proteins were investigated as described previously (2.2.5). None of the EC2: GST proteins affected the percentage of GFP-labelled *P. aeruginosa* adhesion to pre-treated HEC1B cells (Figure 4.14 A). Moreover, no significant changes in the median fluorescent intensity were observed (Figure 4.14 B).

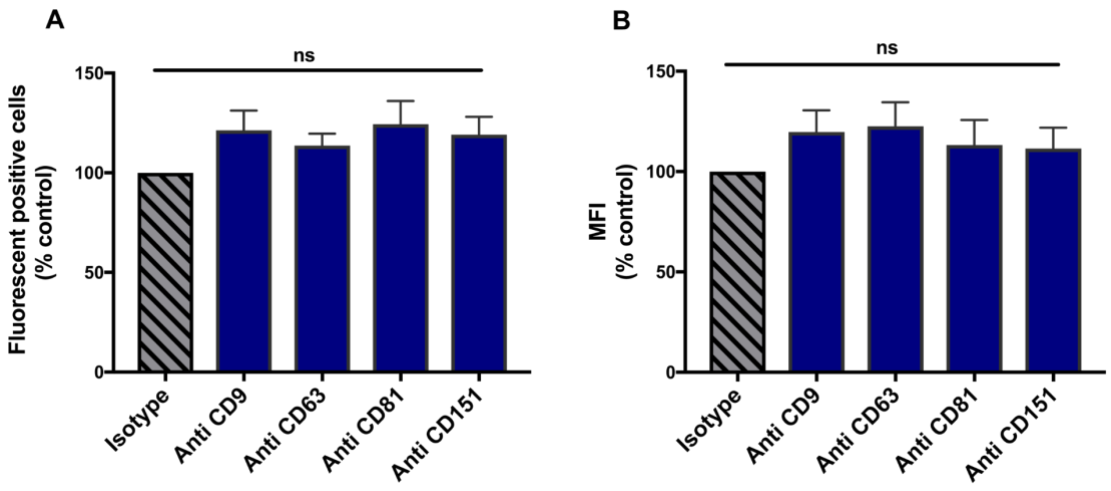

Figure 4.13 The flow cytometric detection of the effects of anti-tetraspanin antibodies on GFP-labelled *P. aeruginosa* adherence to HEC1B cells.

HEC1B cells were pre-treated with anti-tetraspanin antibodies - anti-CD9, anti-CD63, anti-CD81 and anti-CD151, or isotype control at 10 µg /ml for 30 minutes before infection with GFP-labelled *P. aeruginosa* as described in section 2.2.5. The analysis was done using the Attune FACS machine. (**A)** The percentage of HEC1B cells infected with GFP-labelled *P. aeruginosa* normalised to 100 % isotype control. (**B**) The median fluorescent intensity normalised to 100 % isotype control. Results are mean ± standard error of the mean (SEM), N= 4. Data were analysed using One-way ANOVA (vs. isotype control), ns is non-significant.

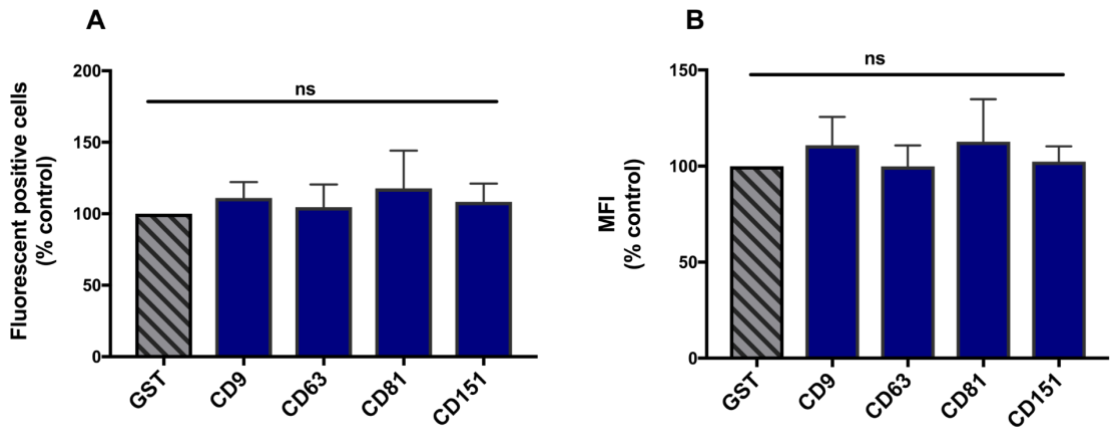

Figure 4.14 The flow cytometric detection of the effects of EC2: GST proteins on GFP-labelled *P. aeruginosa* adherence to HEC1B cells.

HEC1B cells were pre-treated with EC2: GST proteins - CD9, CD63, CD81 and CD151, or GST control at 500 nM for 30 minutes before infection with GFP-labelled *P. aeruginosa* as described in 2.2.5. The analysis was done using the Attune FACS machine. (**A)** The percentage of HEC1B cells infected with GFP-labelled *P. aeruginosa* normalised to 100 % GST control. (**B**) The median fluorescent intensity normalised to 100 % GST control. Results are mean ± standard error of the mean (SEM), N= 4. Data were analysed using One-way ANOVA (vs. GST control), ns is non-significant.

4.2.4 Comparison between microscopy and flow cytometry

The flow cytometry method was shown to be able to detect bacterial infection. A comparison study was then carried out between flow cytometry and the classical method (immunofluorescent microscopy) using a positive control (*S. aureus*). This was done to assess the suitability of flow cytometry as an alternative and high-throughput method of detecting bacterial adhesion. Data was acquired by a previous PhD student from our group (Ventress *et al*., 2016) who investigated the effects of EC2: GST proteins on *S. aureus* adhesion to HaCaT cells by immunofluorescent microscopy. Briefly, cells were incubated with EC2: GST proteins or GST control at 500 nM for 30 minutes prior to the introduction of the GFP-labelled *S. aureus* for 60 minutes. Fluorescent microscopy examination demonstrated that HaCaT cells pre-treated with EC2: GST proteins CD9 and CD81 significantly reduced the percentage of HaCaT cells with adherent bacteria by 88 % and 90 % respectively (Figure 4.15 A). Moreover, fluorescent microscopy analysis showed that pre-treatment with CD9 and CD81 reduced (to 43 % and 42 % respectively) the number of adherent bacteria per 100 cells vs GST control cells (Figure 4.15 B). On another hand, the use of flow cytometry method for the same pathogen and cell line showed different results. There was no significant reduction found in terms of the percentage of fluorescent cells pre-treated with CD9 and CD81 EC2: GST proteins (Figure 4.15 C). Furthermore, no significant reduction was seen in the median fluorescent intensity of cells pre-treated with CD9 and there was only a small but significant reduction in cells pre-treated with CD81 (18 %) of adherent bacteria vs GST control cells (Figure 4.15 D).

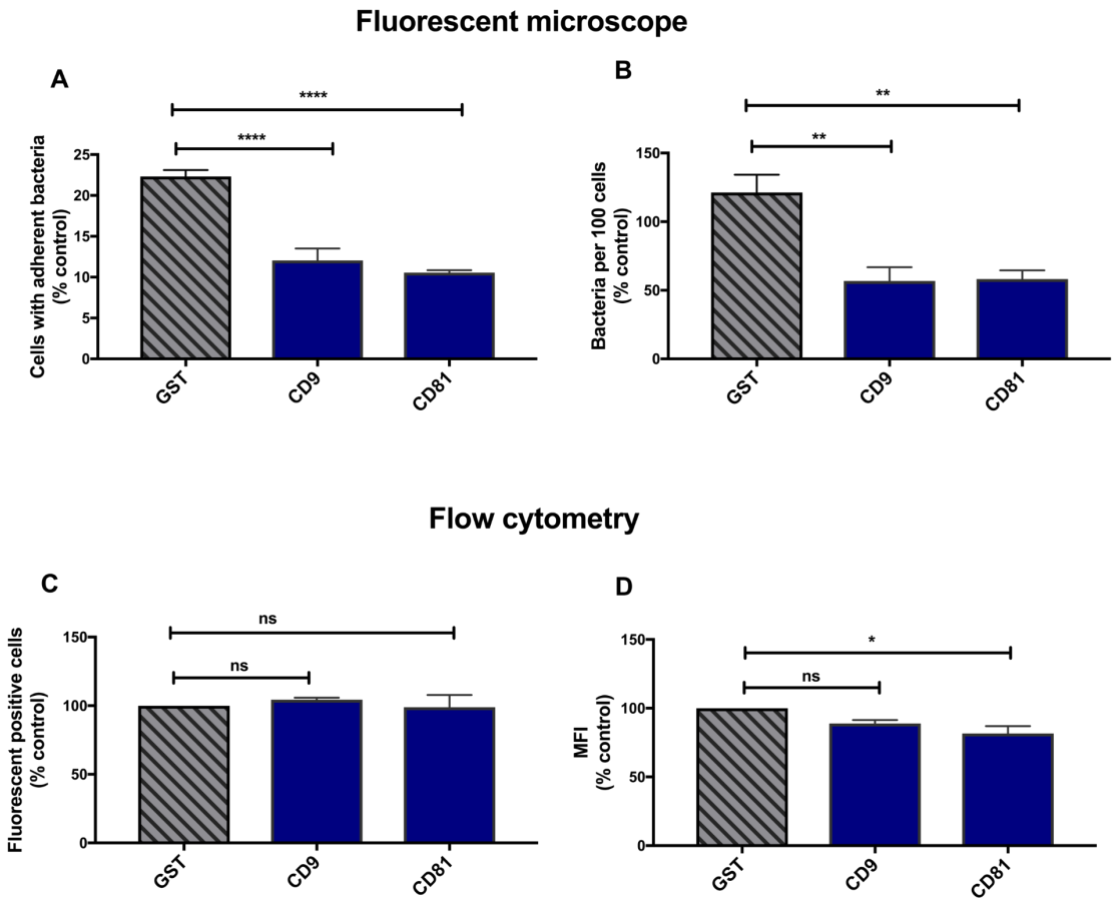

Figure 4.15 The effect of EC2: GST fusion proteins on GFP-labelled *S. aureus* adherence to HaCaT cells using fluorescent microscopy and flow cytometry.

HaCaT cells were pre-treated with EC2: GST proteins at 500 nM for 30 minutes before the infection with GFP-labelled *S. aureus* for 60 minutes. (**A**) The percentage of cells with adherent bacteria. (**B**) The percentage of change in bacteria per 100 cells, (**A and B**) were measured using fluorescent microscopy, N= 5. (**C**) The percentage of positive fluorescent cells. (**D**) The median fluorescent intensity (MFI) normalised to (100) GST control, (**C and D**) adhesion measured by flow cytometry, N=4. Data were analysed using One way ANOVA with Sidak's multiple comparisons test (vs. GST control), ***P* ≤ 0.05, ****P* ≤ 0.01, *****P* ≤ 0.001, ******P* ≤ 0.0001 and ns is non-significant.

4.3 Discussion

The aim of this chapter was to develop a rapid method for detecting bacterial adhesion, which could then be used to investigate the effects of tetraspanin treatments on the adhesion of *P. aeruginosa* to the host cell. *P. aeruginosa* was successfully labelled with both plasmids (PIN25 and PIN29) and the fluorescent-labelled *P. aeruginosa* were detected by flow cytometry. The role of tetraspanin treatments in the adhesion of GFP-labelled *P. aeruginosa* to the host cell was then investigated using flow cytometry. Furthermore, the comparison between flow cytometry method and fluorescent microscopy was also investigated.

Flow cytometry is a technique that can potentially be used to produce faster quantitative results, particularly if a 96-well plate can be used to input samples. The flow cytometer detects both extracellular and intracellular bacteria that are associated with the host cell (i.e. infected cell). This is due to the fact that data acquisition is triggered by the host cells, which are selectively identified based upon their side and forward light scatter properties. Therefore, a cell is recognised as either non-fluorescent (not infected) or with a variable strength of the fluorescence signal based on the number of adherent or internalised fluorescent bacteria. Thus, flow cytometry could give an estimate of the total number of extracellular and intracellular labelled bacteria.

HaCaT cells treated with EC2: GST proteins prior to infection with GFP-labelled *S. aureus* showed different results between the flow cytometry method and the fluorescent microscopy method. Using fluorescent microscopy revealed there was a significant inhibition of GFP-labelled *S. aureus* adhesion when HaCaT cells were pretreated with the EC2: GST proteins CD9 and CD81, while, flow cytometry showed no significant effect of the EC2: GST proteins on GFP-labelled *S. aureus* adhesion to HaCaT cells.

Additionally, using flow cytometry analysis to investigate the effect of tetraspanins treatment on the adhesion of GFP-labelled *P. aeruginosa* to the host cells showed different results compared with the investigation by fluorescent microscopy (chapter 5). A slight reduction in the number of GFP-labelled *P. aeruginosa* attached to the host cells was found by flow cytometry when the cells were pre-treated with anti-tetraspanin antibodies or EC2: GST proteins. For example; in the A549 and HaCaT cells, the pre-treatment with tetraspanin CD9 resulted in a slight inhibition of the GFP-labelled *P. aeruginosa* adhesion, and no significant inhibition was seen when HEC1B cells were pre-treated with any of the tetraspanins under investigation. Whereas, fluorescent microscopy showed a significant effect of the tetraspanin CD9 on the adhesion of GFP-labelled *P. aeruginosa* to A549, HaCaT and HEC1B cells. Moreover, recent work by our group to investigate the effect of tetraspanin CD9 derived peptides on reducing GFP-labelled *S. aureus* adherence to HaCaT cells using flow cytometry showed no significant effect of any of the peptides tested, whereas with fluorescent microscopy a significant effect was seen to inhibited GFP-labelled *S. aureus* adherence to HaCaT cells (unpublished observations by our group). Also, previous work by our group investigated the effect of tetraspanin CD9 peptides treatment on GFP-labelled *S. aureus* adhesion to HaCaT cells by flow cytometry and fluorescent microscopy. No significant effect was observed on GFP-labelled *S. aureus* adherence to pre-treated HaCaT cells by flow cytometry, whereas a significant effect was seen by fluorescent microscopy (Ventress, 2016).

This indicates that flow cytometry might not be a suitable/ sensitive method to measure changes in fluorescent labelled bacterial adhesion. A possible reason for this observation is that in flow cytometry a higher MOI was required to achieve the infection that in turn could be detected by the GFP fluorescent signal. The use of such high MOIs may have altered the efficiency of antibody/EC2 treatments (Ventress, 2016). In addition, the

mechanical stress of the assay is likely to have removed some of the adherent bacteria or maybe the overlap between the bacterial analysis with the background fluorescent and the host cell (Grootaert *et al*., 2011). This result is consistent with the previous findings by Vesterlund *et al*., (2005), who found that a high signal-to-background ratio, particularly when the cell surface is auto fluorescent, results in low sensitivity in detection of the adherent bacteria by flow cytometry. Another reason could be the difficulty of distinguishing between the live and dead bacteria adherent to a monolayer of differentiated cell lines (Grootaert *et al*., 2011).

Commonly used methods to detect the majority of the pathogenic microbes may involve microscopical counting, plating (Entis *et al*., 2001), Q-PCR, rediolabelling of the bacteria, or the enzyme-linked immunosorbent assay (ELISA) (Bernet *et al*., 1994; Ouwehand *et al*., 2001). However, it is important to note that these methods of detection require the bacteria to be viable and culturable, and this limits the detection to a specific physiological state (Harkins and Harrigan, 2004). These methods may lead to false negative results, or to the number of pathogens within the given sample may be underestimated (Vora *et al*., 2004; Lazcka *et al*., 2007). They cannot provide the required information regarding the pathogenicity or the virulence of the pathogen identified.

Therefore, efforts have been made to develop a rapid flow cytometry -based method, which can rapidly detect a lower concentration of the pathogens (Hammes *et al*., 2008; Riyaz-Ul-Hassan *et al*., 2008; Lautenschlager *et al*., 2013).

Infectious pathogens of concern for flow cytometry analysis include viruses, for example rotavirus (Kandasamy *et al*., 2016) and the human immunodeficiency virus type 1 (Venereo-Sanchez *et al*., 2017). Bacteria such as *Salmonella* (Wahid *et al*., 2016), *S. aureus* (Kennedy and Wilkinson, 2017; Meng *et al*., 2017), fungi such as *Aspergillus* and

Candida (Yun and Lee, 2017) are also target for flow cytometry. However, many issues may complicate the identification of the target microorganism such as the difficulties to distinguish between some particles as inorganic debris and the microorganism spores. Thus, flow cytometry assay could be a potentially sensitive, rapid and suitable method for immediate detection of pathological agents (multiplex analysis) (Zahavy *et al.*, 2012). Flow cytometry analysis offers investigators the ability to obtain many data for an individual cell (Winson and Davey, 2000). This method generates data about cell viability, physiological status or period of the growth cycle. Furthermore, flow cytometers can analyse thousands of cells/events per second.

A number of publications have reported the increasing uses of flow cytometry as an alternative method for detection of pathogenic microorganisms (Berney *et al.*, 2006; Cronin and Wilkinson, 2007; Allegra *et al.*, 2008; Jarzembowski *et al.*, 2008; Ryumae *et al.*, 2010; Nocker *et al.*, 2011). GFP has been widely used as a fluorescent indicator in investigating bacterial interaction with host cells in both imaging and quantification studies (Sabuquillo *et al.*, 2017). Many previous studies have used flow cytometry detection to measure bacterial infection, for example, GFP-expressing *P. fluorescens* (Tombolini *et al.*, 1997) and genetically labelled *L. monocytogenes* (Raybourne *et al.*, 2001). The use of fluorescent-labelled bacteria enables reproducible enumeration of internalised and adherent bacteria (Vesterlund *et al.*, 2005). Furthermore, flow cytometry has many uses, it has been recently approved for the analysis of bacterial attachment to host cells (Hytönen *et al.*, 2006a). In addition, Sethman *et al.*, (2002) have used the flow cytometric assay to assign functions to individual surface molecules through distinct phases of adhesion. In another study, flow cytometry was used to investigate the immune response against intracellular bacteria (Karanikas *et al.*, 2000). These examples show that flow cytometry can be used for a variety of research investigations.

The main limitation in analysing bacteria using flow cytometry is that many of their natural characteristics (such as shape, size and DNA content) differ depending on the growth conditions used, or the sources from which the bacteria were taken (Winson and Davey, 2000). Therefore, in order to produce consistent data, strict reproducibility of the conditions is required. Moreover, in some investigations a specific type of flow cytometry is required, such as in the study of bacteria that generate an aerosol, the flow cytometry has to have a filter to control this concern and the biosafety levels required will differ with the type of sorting being carried out (Schmid *et al*., 2007). Additionally, bacteria are much smaller than human cells; therefore, the analysis of pathogenic bacteria poses specific challenges for flow cytometry. Furthermore, if the flow cytometry machine is not specifically designed for microbial investigation, it is necessary to determine whether it is capable of such analysis and then optimise its performance (Harkins and Harrigan, 2004).

In flow cytometry, using the two-parameter histogram side-scatter and forward scatter, the fluorescence and ratio information allows the user to identify the location of the labelled pathogens on a two-parameter histogram. Using flow cytometry to analyse bacterial population is common, however, to develop and adjust flow cytometers to the detection of a specific bacterial pathogen is still uncommon.

In summary, fluorescent-labelled *P. aeruginosa* were constructed and a flow cytometry-based assay was developed for monitoring fluorescent-labelled *P. aeruginosa* infection. However, the fluorescent detection of flow cytometry is weaker than the microscopy assay. This could be due to the low fluorescence of intracellular fluorescent -expressing bacteria, or because of the use of GFP fluorescent labelling which may introduce a bias when measuring fine differences in adhesion (Trouillet *et al*., 2011b). Moreover, higher

MOI was required for flow cytometry and that may have affected the assay. Due to this, thereafter in this work, the fluorescent microscopy method was used to investigate the role of tetraspanin treatments on GFP-labelled *P. aeruginosa* adhesion to the host cells.

CHAPTER 5: The effect of tetraspanin EC2: GST proteins and anti-tetraspanin antibodies on *P. aeruginosa* adhesion to human cell

5.1 Introduction

Infection of host tissues is commonly initiated by adherence of bacteria to membrane proteins, sugars or lipids on the cell surface. However, for full functionality, membrane proteins are usually organised into microdomains, such as TEM. Tetraspanins such as CD9, CD81, CD63 and CD151 are a class of proteins known to play a major role in TEM organisation and thus they are potential targets for the inhibition of bacterial adhesion to host cells.

Monoclonal antibodies (mAbs) targeting cell surface proteins are potent tools that have been largely used in diagnostic, therapeutic and research studies. Previously, anti-tetraspanin antibodies have been used to identify the functions of various tetrasapnins (Tachibana and Hemler, 1999; Kaji *et al*., 2000).

The dependence of multiple bacterial target molecules on tetraspanins, our proteins of interest, suggests that tetraspanin reagents could be useful as therapeutic strategies to alter these relationships in order to prevent or treat bacterial infections, and this hypothesis is explored in this chapter.

The aims of this chapter:

- To determine the effects of tetraspanin reagents on *P. aeruginosa* adhesion to human cells.
- To assess the effect of bacterial lipopolysaccharide (LPS) on the functional activity of the recombinant EC2 proteins treatment.
- To explore the ability of anti-tetraspanin antibodies to protect A549 and HEC1B cells against *P. aeruginosa*-induced cytotoxicity using the LDH assay.

5.2 Results

5.2.1 The effect of anti-tetraspanin antibodies on GFP-labelled *P. aeruginosa* adhesion to host cells

Flow cytometry was shown in Chapter 4 to be a weak method for detection of inhibitors of bacterial attachment, therefore only immunofluorescent microscopy was used to investigate the effect of tetraspanin treatments on GFP-labelled *P. aeruginosa* adhesion to the host cell through this study. In order to investigate the effect of anti tetraspanin antibodies on GFP-labelled *P. aeruginosa,* the human cells were incubated with anti-tetraspanin antibodies or the isotype control prior to the introduction of GFP-labelled *P. aeruginosa*.

5.2.1.1 Optimising the conditions for GFP-labelled *P. aeruginosa* infection assay

The following method was taken from (Ali, 2016). Tetraspanins expression in the cell lines used in this study has been described previously (chapter 3). Other initial experiments included studying the GFP-labelled *P. aeruginosa* growth curve to find the log phase that would be used. Different multiplicities of infection (MOIs) and infection times were examined to find the most suitable ones. A growth curve was plotted by growing GFP-labelled *P. aeruginosa* in LB broth and measuring both optical density (OD_{600nm}) and viable bacterial counts (colony forming unit - CFU) during different time periods (1 - 24 hours). This experiment was repeated three times and the mid-log phase was chosen from the average to be used for the GFP-labelled *P. aeruginosa* infection assay ($OD_{600nm} \approx 0.5$, time ≈ 60 minutes and CFU≈ 5×10^8) (data not shown). For further validation, the CFU was measured separately for each experiment. To optimise the MOI and infection time, the various human cell lines were infected at different MOIs (5, 10, 20, 30 and 50) and for different times (30, 40, 60 and 90 minutes). The human cells were

fixed, stained and counted (section 2.2.4). High MOIs and longer incubation times gave high infection rates and high numbers of bacteria that made counting very difficult, whereas shorter times gave low rates of infection with less bacterial internalisation. The experiments revealed that for the three cell lines used, an MOI of 10 and infection time of 60 minutes were the most appropriate (optimisation data not shown).

5.2.1.2 The effect of anti-tetraspanin antibodies on GFP-labelled *P. aeruginosa* adhesion to A549 epithelial cells

In all the experiments, it is possible that the anti-tetraspanin antibodies used to block the surface tetraspanins antigens will bind non-specifically with Fc receptors (FcR) that are expressed by these cells. Therefore, an appropriate isotype control was included to take account of the nonspecific binding of anti-tetraspanin antibodies to FcR. The effect of anti-tetraspanin antibodies on infection of A549 cells with GFP-labelled *P. aeruginosa* was assessed using saturating concentrations of antibody (10µg/ml) (section 2.2.6). The antibody effects were measured by three parameters: the percentage of cells with associated bacteria, the change in the number of bacteria per 100 cells and the change in number of bacteria per infected cells calculated as described in section 2.2.4.3 Immunofluorescence microscopy of A549 cells infected with GFP-labelled *P. aeruginosa* was used for counting. After 60 minutes of infection, the percentage of cells associated with GFP-labelled *P. aeruginosa* was significantly reduced after pre-treatment with anti-CD151 (50 %), anti-CD9 (48 %), anti-CD63 (42 %) and anti-CD81 (30 %), when compared to the isotype control (Figure 5.1 A). Moreover, a significant reduction was observed in terms of the number of GFP-labelled *P. aeruginosa* associated with epithelial cells pre-treated with anti-CD9 (62 %), anti-CD81 (54 %), anti-CD151 (42 %) and anti-CD63 (34 %) antibodies compared to the isotype control cells as shown in Figure 5.1 B.

Also, there was no significant reduction in the number of GFP-labelled *P. aeruginosa* per infected cell, compared to the isotype control see Figure 5.1 C.

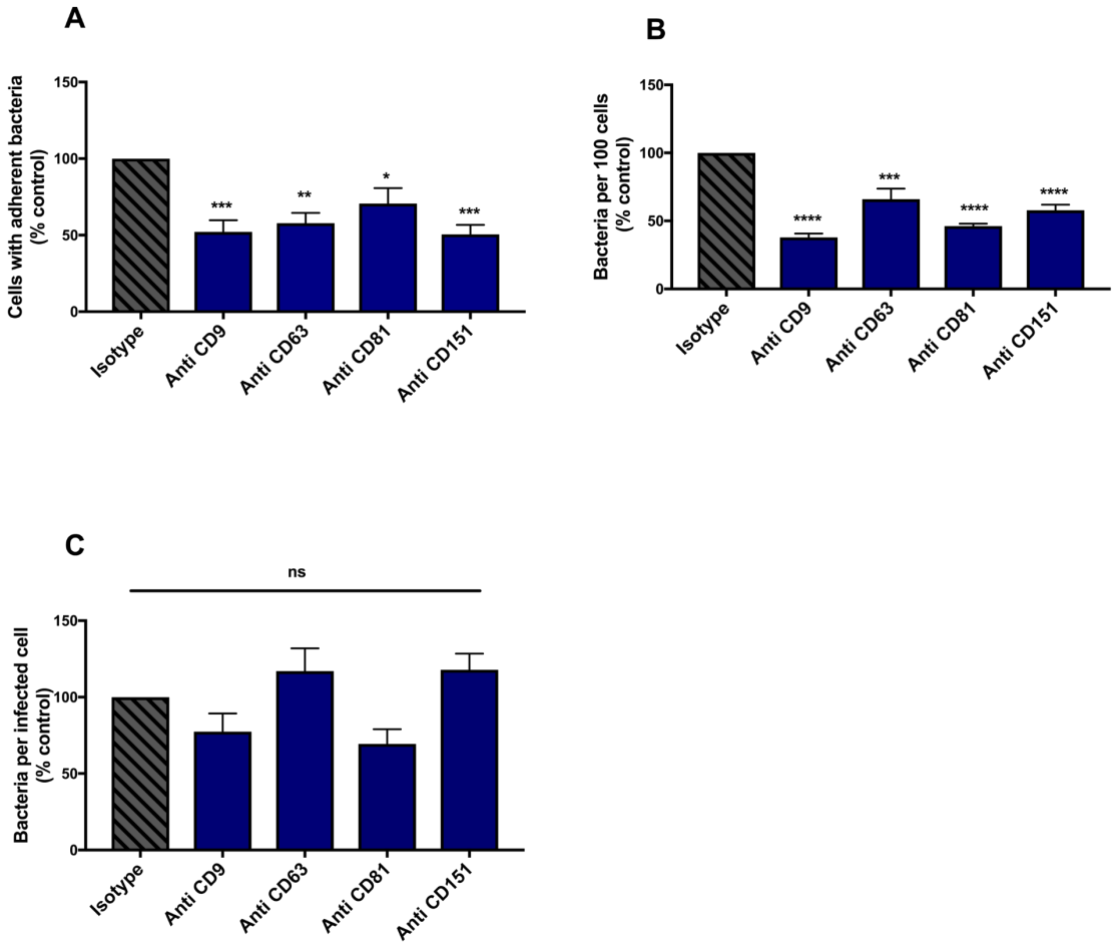

Figure 5.1 The effect of anti-tetraspanin antibodies on GFP-labelled *P. aeruginosa* adherence to A549 epithelial cells.

A549 cells were pre-treated with anti-tetraspanin antibodies (10 µg/ml) or the isotype control antibody for 30 minutes. GFP-labelled *P. aeruginosa* was incubated with adherent cells at MOI of 10 for 60 minutes, the cells were fixed, stained, then visualised using fluorescence microscopy and the number of infected cells was counted. (**A**) The percentage of cells with adherent bacteria. (**B**) The change in the number of bacteria per 100 cells. (**C**) The change in number of bacteria per infected cells, calculated as described in section 2.2.4, Results are mean ± standard error of the mean (SEM), N= 4. Data were analysed using One-way ANOVA with Sidak's multiple comparisons test (vs. isotype control), ***** $P \leq 0.05$, ****** $P \leq 0.01$, ******* $P \leq 0.001$ and ******** $P \leq 0.000$, ns is non-significant.

5.2.1.2 The effect of anti-tetraspanin antibodies on GFP-labelled *P. aeruginosa* adhesion to HaCaT keratinocyte cells

The immunofluorescence microscopy of HaCaT cells infected with GFP-labelled *P. aeruginosa* was used for counting. After 60 minutes of infection, the percentage of cells associated with bacteria was significantly reduced after pre-treatment with anti-CD151 (70 %), anti-CD9 (65 %), anti-CD63 (64 %) and anti-CD81 (48 %), when compared to the isotype control (Figure 5.2 A). Moreover, a significant reduction was observed in terms of the number of bacteria associated with keratinocyte cells pre-treated with anti-CD9 (83 %), anti-CD151 (36 %), anti-CD63 (35 %) and anti-CD81 (6 %), antibodies compared to the isotype control cells as shown in Figure 5.2 B. Furthermore, there was no significant reduction in the number of bacteria per infected cells, compared to the isotype control (Figure 5.2 C).

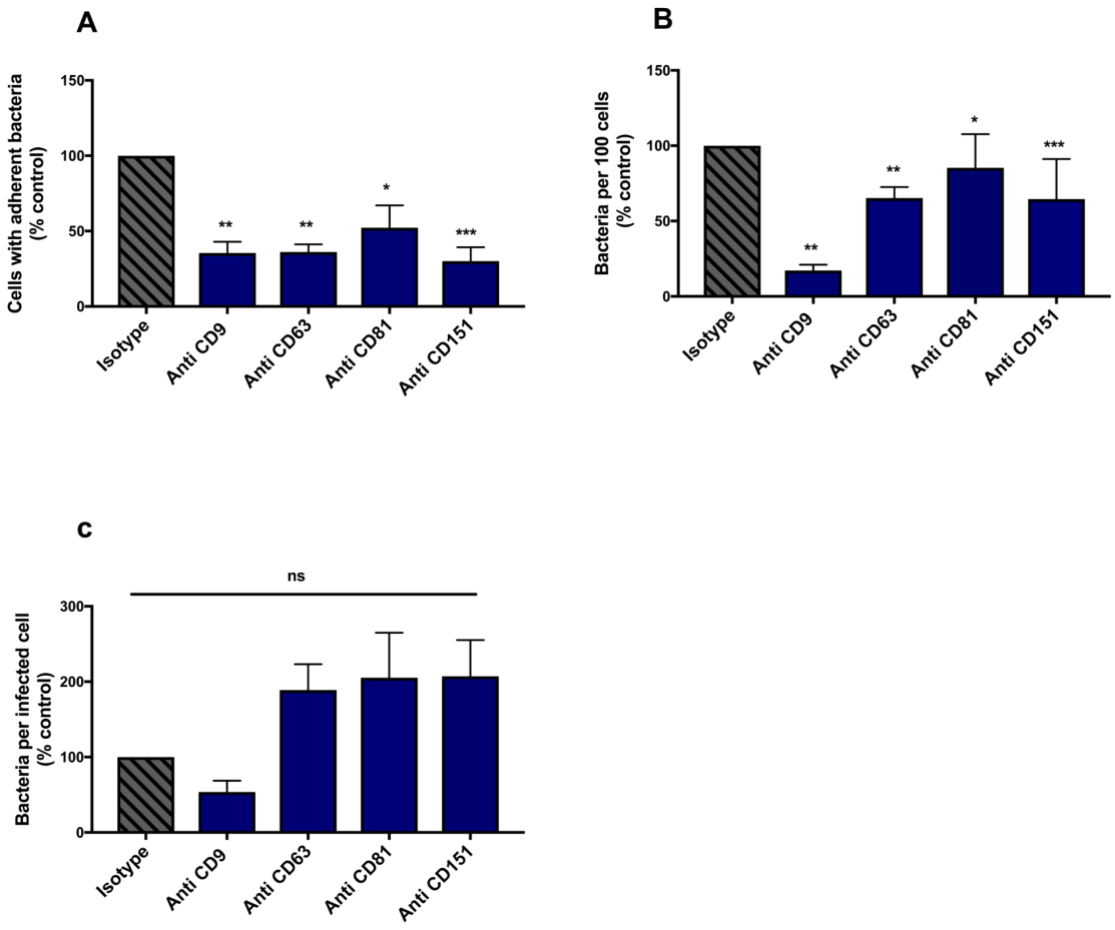

Figure 5.2 The effect of anti-tetraspanin antibodies on GFP-labelled *P. aeruginosa* adherence to HaCaT cells.

HaCaT cells were pre-treated with anti-tetraspanin antibodies (10 μg/ml) or the isotype control antibody for 30 minutes. GFP-labelled *P. aeruginosa* was incubated with adherent cells at MOI of 10 for 60 minutes, the cells were fixed, stained, then visualised using fluorescence microscopy and the number of infected cells was counted. (**A**) The percentage of cells with adherent bacteria. (**B**) The change in the number of bacteria per 100 cells. (**C**) The change in number of bacteria per infected cells, calculated as described in section 2.2.4, Results are mean ± standard error of the mean (SEM), N= 4. Data were analysed using One-way ANOVA with Sidak's multiple comparisons test (vs. isotype control), ***P* ≤ 0.05, ****P* ≤ 0.01 and *****P* ≤ 0.001, ns is non-significant.

5.2.1.3 The effect of anti-tetraspanin antibodies on GFP-labelled *P. aeruginosa* adhesion to HEC1B endometrial cells

The immunofluorescence microscopy of HaCaT cells infected with GFP-labelled *P. aeruginosa* was used for counting**.** After 60 minutes of infection, the percentage of cells associated with bacteria was significantly reduced after pre-treatment with anti-CD151(50 %), anti-CD9 (48 %), anti-CD63 (43 %) and anti-CD81 (30 %), when compared to the isotype control (Figure 5.3 A). Likewise, a significant reduction was observed in terms of the number of the bacteria associated with HEC1B cells pre-treated with anti-CD9 (63 %), anti-CD81 (54 %), anti-CD151 (43 %) and anti-CD63 (35 %) antibodies compared to the isotype control cells as shown in Figure 5.3 B. In addition, no significant reduction was seen in the number of bacteria per infected cells, compared to the isotype control (Figure 5.3 C).

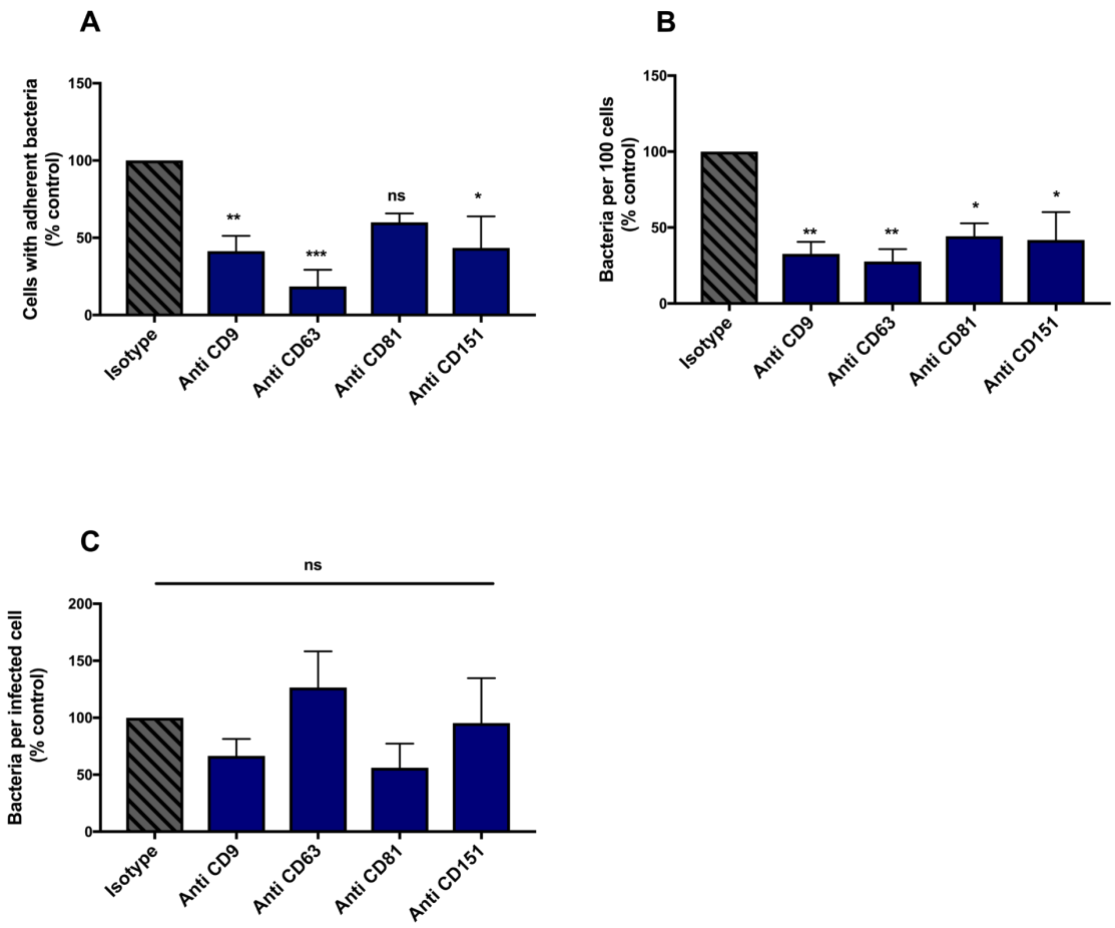

Figure 5.3 The effect of anti-tetraspanin antibodies on GFP-labelled *P. aeruginosa* adherence to HEC1B cells.

HEC1B cells were pre-treated with anti-tetraspanin antibodies (10 µg/ml) or the isotype control antibody for 30 minutes. GFP-labelled *P. aeruginosa* was incubated with adherent cells at MOI of 10 for 60 minutes, the cells were fixed, stained, then visualised using fluorescence microscopy and the number of infected cells was counted. (**A**) The percentage of cells with adherent bacteria. (**B**) The change in the number of bacteria per 100 cells. (**C**) The change in number of bacteria per infected cells, calculated as described in section 2.2.4, Results are mean ± standard error of the mean (SEM), N= 4. Data were analysed using One-way ANOVA with Sidak's multiple comparisons test (vs. isotype control), ***P* ≤ 0.05, ****P* ≤ 0.01 and *****P* ≤ 0.00, ns is non-significant.

5.2.2 The effect of EC2: GST proteins on GFP-labelled *P. aeruginosa* adhesion to host cells

Since anti-tetraspanin antibodies had an inhibitory effect on GFP-labelled *P. aeruginosa* infection of the three cell lines investigated, it appeared appropriate here to test the effect of the corresponding soluble EC2 proteins. These recombinant proteins were expressed as GST-fusion proteins in *E. coli* and had been prepared by our lab group (Fanaei *et al*., 2011).

In order to study the effect of EC2: GST proteins on GFP-labelled *P. aeruginosa*. adhesion to host cells by immunofluorescent microscopy, the cells were incubated with EC2: GST proteins at 500 nM or the glutathione S-transferase (GST) (the protein tag) control prior to the introduction of the GFP-labelled *P. aeruginosa*.

5.2.2.1 The effect of EC2:GST proteins on GFP-labelled *P. aeruginosa* adhesion to A549 epithelial cells

The effect of EC2: GST proteins on A549 epithelial cells is presented in Figure 5.4**.** After 60 minutes of infection, the percentage of cells associated with bacteria was significantly reduced after pre-treatment with EC2-CD9 (68 %), EC2-CD63 (51 %), EC2-CD81 (43 %) and EC2-CD151(34 %), when compared to the GST control (Figure 5.4 A). However, no significant reduction was observed in terms of the number of bacteria associated with epithelial cells compared to the GST control cells as shown in Figure 5.4 B. In addition, no significant reduction was observed in the number of bacteria per infected cells, compared to the GST control (Figure 5.4 C).

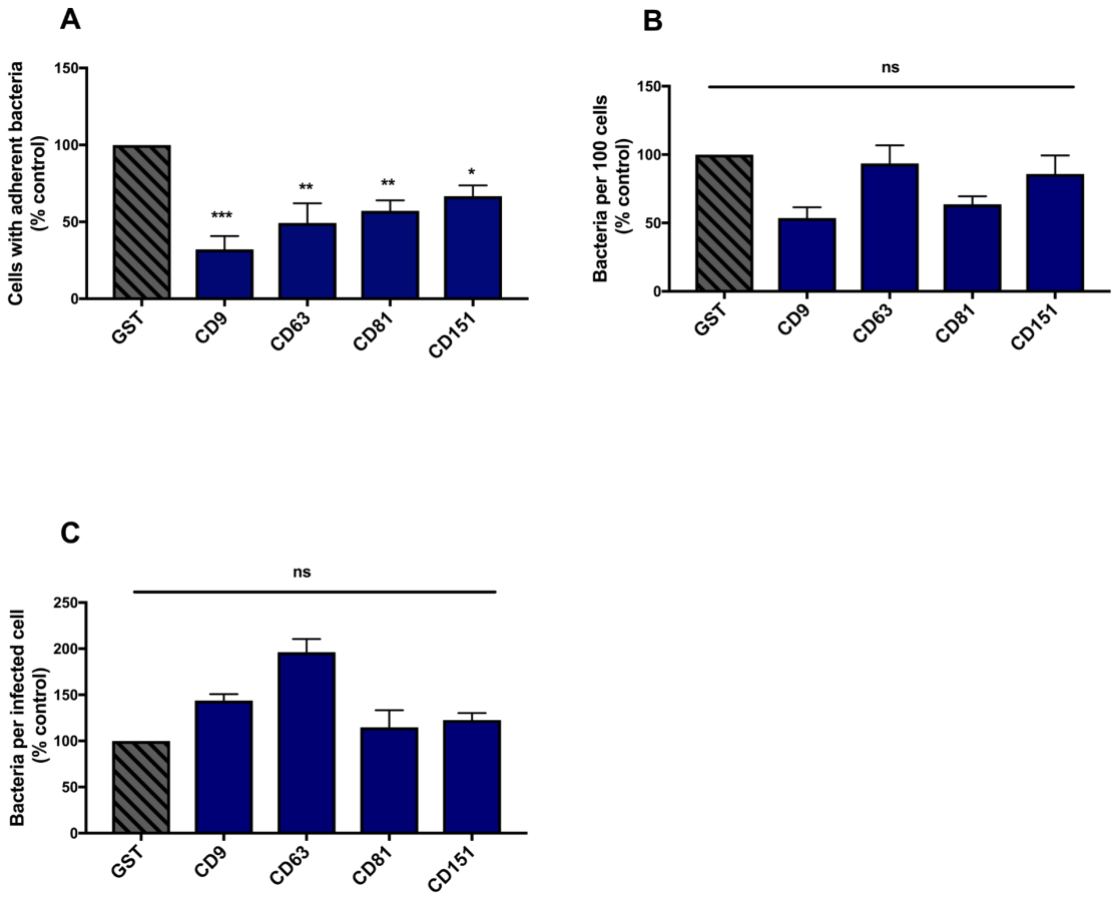

Figure 5.4 The effect of EC2: GST proteins on GFP-labelled *P. aeruginosa* adherence to A549 cells.

A549 cells were pre-treated with EC2: GST proteins (500 nM) or the GST control for 30 minutes. GFP-labelled *P. aeruginosa* were incubated with adherent cells at MOI of 10 for 60 minutes, the cells were fixed, stained, then visualised using fluorescence microscopy and the number of infected cells was counted. (**A**) The percentage of cells with adherent bacteria. (**B**) The change in the number of bacteria per 100 cells. (**C**) The change in number of bacteria per infected cells, calculated as described in section 2.2.4, Results are mean ± standard error of the mean (SEM), N= 4. Data were analysed using One-way ANOVA with Sidak's multiple comparisons test (vs. GST control), *****$P \leq 0.05$, ******$P \leq 0.01$ and *******$P \leq 0.001$, ns is non-significant.

5.2.2.2 The effect of EC2:GST proteins on GFP-labelled *P. aeruginosa* adhesion to HaCaT keratinocyte cells

The effect of EC2: GST proteins on pre-treated HaCaT cells is shown in Figure 5.5**.** After 60 minutes of infection, the percentage of cells associated with bacteria was significantly reduced after pre-treatment with EC2-CD81 (80 %), EC2-CD9 (75 %), EC2-CD63 (70 %) and EC2-CD151 (64 %), when compared to the GST control (Figure 5.5 A). Moreover, a significant reduction was observed in terms of the number of bacteria associated with HaCaT cells pre-treated with EC2-CD9 (69 %), EC2-CD81 (69 %), EC2-CD63 (56 %) and EC2-CD151 (55 %), compared to the GST control cells as shown in Figure 5.5 B. Additionally, no significant reduction was seen in the number of bacteria per infected cells, compared to the GST control (Figure 5.5 C).

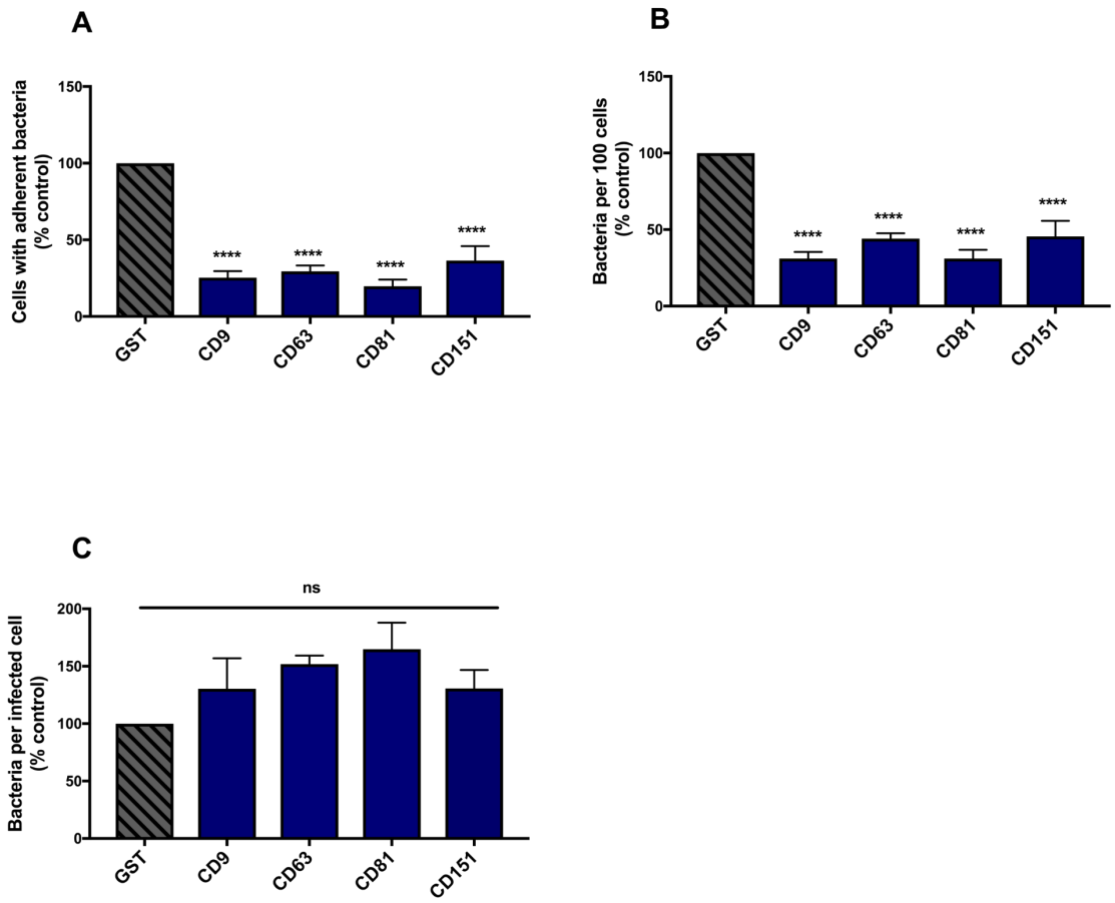

Figure 5.5 The effect of EC2: GST proteins on GFP-labelled *P. aeruginosa* adherence to HaCaT cells.

HaCaT cells were pre-treated with EC2: GST proteins (500 nM) or the GST control for 30 minutes. GFP-labelled *P. aeruginosa* were incubated with adherent cells at MOI of 10 for 60 minutes, the cells were fixed, stained, then visualised using fluorescence microscopy and the number of infected cells was counted. (**A**) The percentage of cells with adherent bacteria. (**B**) The change in the number of bacteria per 100 cells. (**C**) The change in number of bacteria per infected cells, calculated as described in section 2.2.4, Results are mean ± standard error of the mean (SEM), N= 4. Data were analysed using One-way ANOVA with Sidak's multiple comparisons test (vs. GST control), ***P* ≤ 0.05, ****P* ≤ 0.01 and *****P* ≤ 0.001, ******P* ≤ 0.0001, ns is non-significant.

5.2.2.3 The effect of EC2:GST proteins on GFP-labelled *P. aeruginosa* adhesion to HEC1B endometrial cells

The effect of EC2: GST proteins on pre-treated HEC1B cells is displayed in Figure 5.6**.** After 60 minutes of infection, the percentage of cells associated with bacteria was significantly reduced after pre-treatment with EC2-CD9 (82 %), EC2-CD81 (70 %), and EC2-CD63 (66 %), when compared to the GST control (Figure 5.6 A). However, a significant reduction was observed in terms of the number of bacteria associated with HEC1B cells pre-treated with EC2-CD9 (51 %) and EC2-CD63 (51 %), compared to the GST control cells as presented in Figure 5.6 B. Furthermore, no significant reduction was observed in the number of bacteria per infected cells, compared to the GST control (Figure 5.6 C).

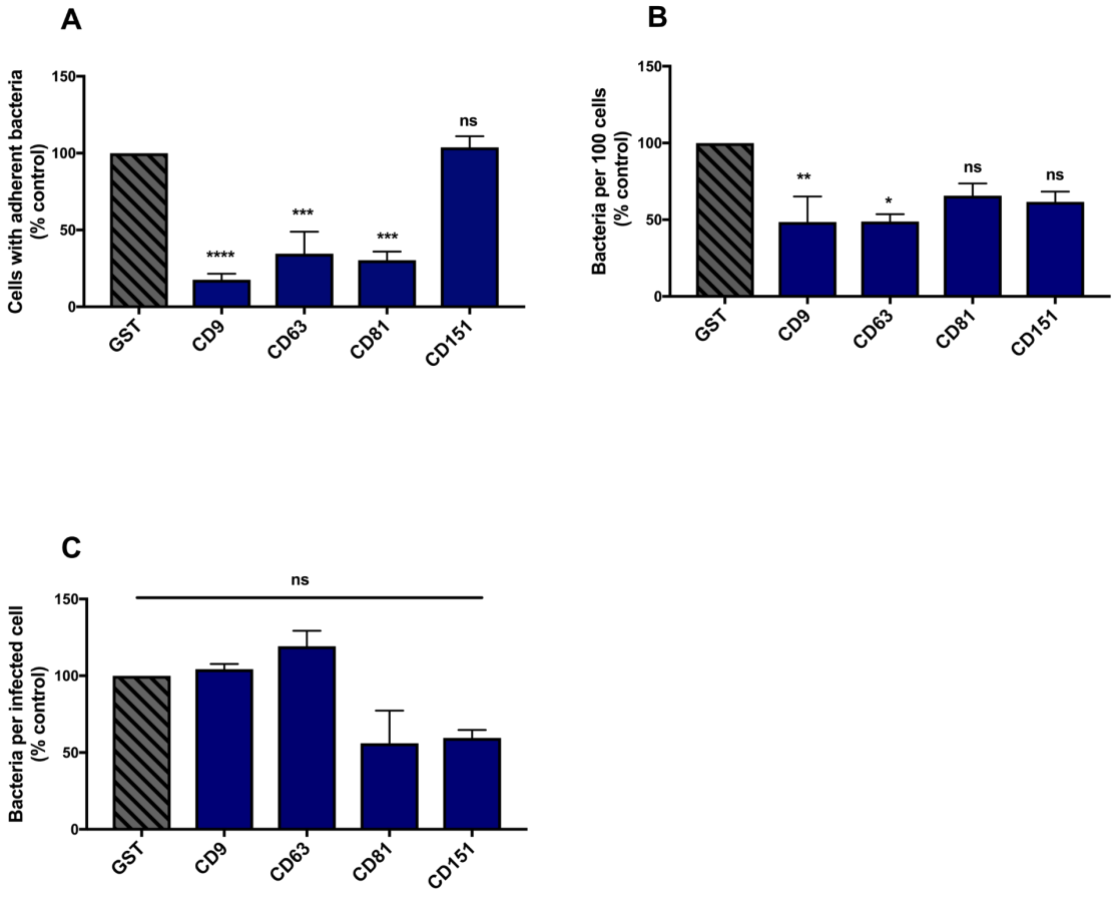

Figure 5.6 The effect of EC2: GST proteins on GFP-labelled *P. aeruginosa* adherence to HEC1B cells.

HEC1B cells were pre-treated with EC2: GST proteins (500 nM) or the GST control for 30 minutes. GFP-labelled *P. aeruginosa* were incubated with adherent cells at MOI of 10 for 60 minutes, the cells were fixed, stained, then visualised using fluorescence microscopy and the number of infected cells was counted. (**A**) The percentage of cells with adherent bacteria. (**B**) The change in the number of bacteria per 100 cells. (**C**) The change in number of bacteria per infected cells, calculated as described in section 2.2.4, Results are mean ± standard error of the mean (SEM), N= 4. Data were analysed using One-way ANOVA with Sidak's multiple comparisons test (vs. GST control), ***P* ≤ 0.05, ****P* ≤ 0.01 and *****P* ≤ 0.001, ns is non-significant.

5.2.3 The role of LPS contamination in EC2: GST protein induced adherence of GFP-labelled *P. aeruginosa* to host cells

A possible reason for the reduction in GFP-labelled *P. aeruginosa* adherence could be due to the downstream effects of the residual lipopolysaccharide (LPS), which is contained in the recombinant protein as a contaminant from the protein expression and purification process (chapter 2). Fanaei (2013) measured the residual levels of LPS as shown in Table 5.1. The experiment revealed that there is no significant effect of LPS on the adhesion of GFP-labelled *P. aeruginosa* to HEC1B cells and that the effect of the reduction in adhesion observed in the cells pre-treated with recombinant proteins is not due to LPS contamination (Figure 5.7).

Table 5.1: The LPS content of recombinant proteins (ng/ml) per 500 nM protein solution.

Recombinant Protein	LPS content (ng /ml in 500nM protein solution)
GST (Rosetta Gami)	3.25
CD9 EC2: GST (Rosetta Gami)	12.40

Data obtained by a previous student in our lab group (Fanaei, 2013) using the Limulus Ameobocyte Lysate chromogenic assay.

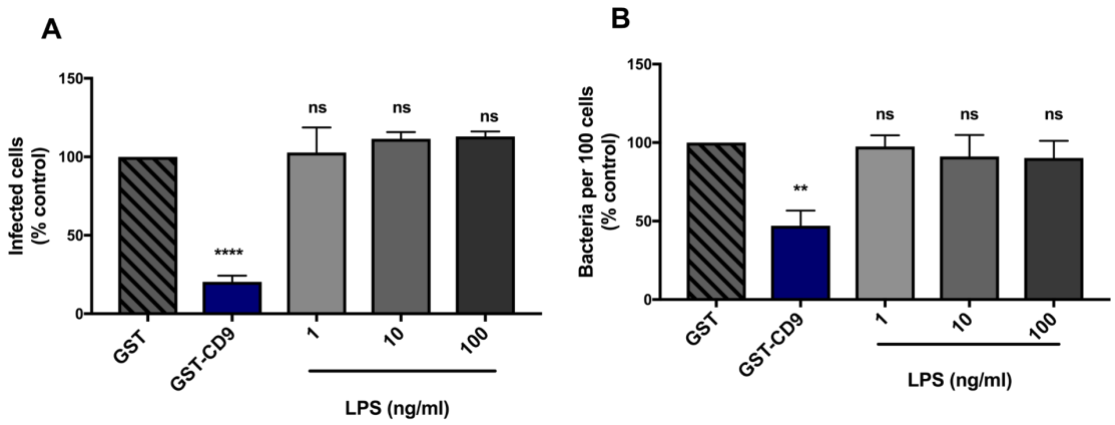

Figure 5.7 The effect of LPS on the adhesion of GFP-labelled *P. aeruginosa* to HEC1B cells.

HEC1B cells were pre-treated with a range of concentrations of LPS for 30 minutes. CD9 EC2: GST protein was used as a positive control. GFP-labelled *P. aeruginosa* were incubated with adherent cells for 60 minutes at an MOI of 10, the cells were then fixed, stained, then visualised by fluorescent microscopy. (**A**) The percentage of infected cells. (**B**) The percentage of number of bacteria per 100 cells. Data were normalised to the GST control cells. Results are mean ± standard error of the mean (SEM), N= 4. Data were analysed using One-way ANOVA with Sidak's multiple comparisons test (vs. GST control), $^{**}P \leq 0.01$ and $^{****}P \leq 0.0001$, ns is non-significant.

5.2.4 Anti-tetraspanin antibodies protect A549 and HEC1B cells from *P. aeruginosa* mediated toxicity

Anti-tetraspanin antibodies were examined for their ability to protect A549 and HEC1B cells against *P. aeruginosa*-induced cytotoxicity. The level of LDH activity post-infection of human cells was determined. Briefly, A549 and HEC1B cells were seeded in a 96-well plate (10,000 cells/well in 100 µl complete media) and allowed to attach at 37°C, 5 % CO_2 for 16 hours to achieve a 70-80 % confluent monolayer. The confluent cells were incubated with 10 µg/ml of anti-CD9 and anti-CD81 anti-tetraspanin antibodies for 30 minutes before the infection with *P. aeruginosa* at MOI of 10 for 16 hours, as described in section 2.2.8.

Following treatment with anti-CD9 and CD81 antibodies, the level of LDH released from A549 cells decreased to 40 % and 52 % respectively, these levels were significantly lower compared to the untreated infected cells (121 %) as shown in Figure 5.8 A. Similarly, treatment of HECB1 cells with the same antibodies decreased the level of LDH released to 9.3 % and 31 % respectively, compared to untreated infected cells (116 %) as shown in Figure 5.8 B.

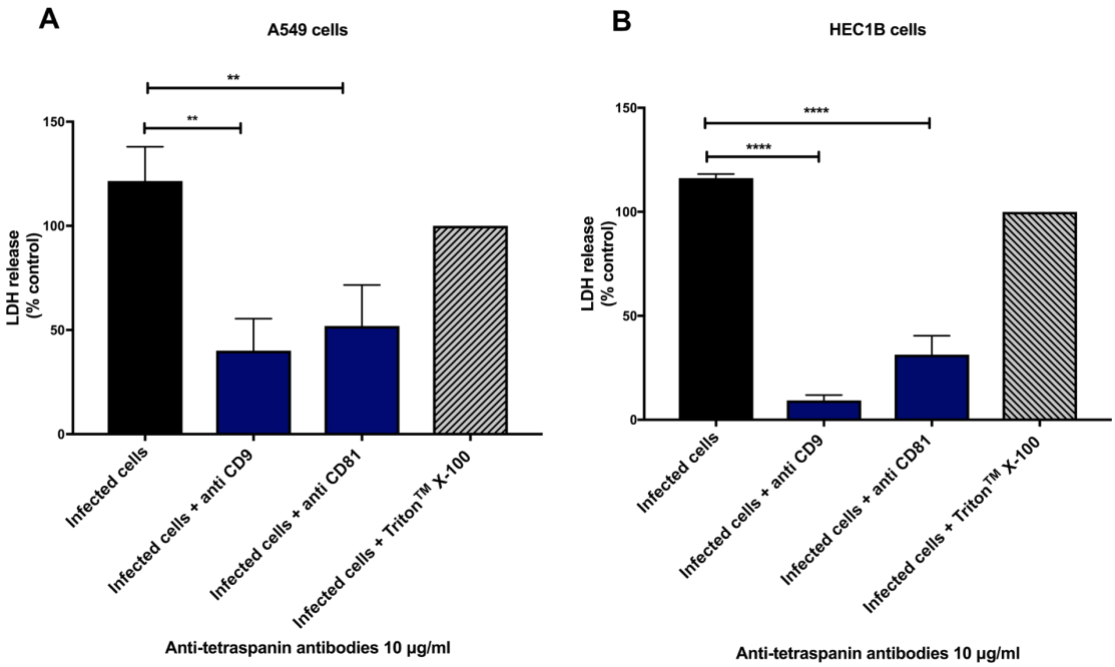

Figure 5.8 The effect of anti-tetraspanin antibodies treatment on A549 and HEC1B cell lines.

Monolayers of A549 (**A**) and HEC1B cells (**B**) were exposed to anti-CD9 and anti-CD81 antibodies at 10 µg/ml at 37°C, 5 % CO_2 for 30 minutes. Cytotoxicity was assessed by measuring the level of LDH released and expressed as a % of total LDH obtained from the control cell lysate (treated with Triton X-100). Results are mean ± standard error of the mean (SEM), N= 4. Data were analysed using One-way ANOVA with Sidak's multiple comparisons test (vs. infected cells), $^{**}P \leq 0.01$ and $^{****}P \leq 0.0001$.

5.3 Discussion

Here we have shown that pre-treatment of three different cell lines (A549, HaCaT and HEC1B) with certain reagents (anti-tetraspanin antibodies and EC2: GST recombinant proteins) significantly reduces GFP-labelled *P. aeruginosa* adhesion. In addition, this effect is not due to LPS contamination in the protein. Moreover, anti-CD9 and anti-CD81 protect human cells against *P. aeruginosa* -induced cytotoxicity.

This study found that the recombinant tetraspanin EC2: GST proteins affected the adherence of *P. aeruginosa* to a higher degree than the antibody treatment, in all the cell lines used. This is in agreement with a previous study which found that the EC2 fusion proteins were able to reduce the adherence of *N. meningitidis* to a higher degree than the interference of tetraspanins with antibodies (Green *et al.*, 2011b). In addition, anti-tetraspanin antibodies and EC2: GST proteins have been shown to be effective at decreasing the adhesion of several Gram negative and Gram positive bacteria to epithelial cells (Green *et al.*, 2011b). It seems that tetraspanins are involved in the adhesion of numerous bacteria to the host, including environmental strains such as *E. coli* and pathogenic such as *Staphylococcus and Pseudomonas*. This similar effect in different types of bacteria suggests that the tetraspanins have a general role in bacterial adhesion, rather than acting as specific receptors. There is an interaction with partner proteins on the cell membrane such as integrins, through TEM, and, TEMs act as adhesion platforms used by the bacteria to associate with the host cell. Our group has proposed that using tetraspanin proteins may disrupt TEM order in the cell membrane (Jennifer Wood, MSc dissertation, 2017) which results in clustering or changing of bacterial receptor on the host cell. However, the actual mechanism of action of these proteins has not been explained.

TEMs potentially contain receptors that the bacteria can use to bind to a host cell, by clustering these receptors that act as 'adhesion platforms' the bacteria will no longer recognise their receptor which will subsequently result in reducing the attachment to the cell membrane. This may explain the reduction in bacterial adhesion seen in this study.

Additionally, the role of LPS contamination in EC2 protein induced adherence of *Pseudomonas* to HEC1B cells was investigated. LPS is one of the major components of the cell wall of Gram-negative bacteria, it is a toxin that can affect many cellular processes in a human cell (Bausinger *et al*., 2002; Wakelin *et al*., 2006). It has been shown that a high dose of LPS may cause multiple organs failure and septic shock (Alexander and Rietschel, 2001). Therefore, it is important to investigate the possible effects of this Gram-negative endotoxin on bacterial interactions with host cells. HEC1B cells have been reported to express Toll-like receptor 4 **(**TLR4) (Aboussahoud *et al*., 2010). TLR4 may bind LPS and stimulate many immune cellular responses, such as cytokine and chemokine production (Medzhitov and Janeway, 2002). The existence of these molecules is able to severely change the interaction between the pathogen and the host cell. LPS also contributes to Gram-negative bacterial colonisation through hydrogen bonding on the surface of human cells (Sheng *et al*., 2008). As LPS contamination was unavoidable during the production and purification of the EC2: GST recombinant proteins (used in this study) (Petsch and Anspach, 2000), it was important to assess that the reduction in adhesion seen in the cells pre-treated with recombinant proteins is not due to LPS contamination. The data presented in Figure 5.7 shows that there was no obvious effect of LPS on the interaction between HEC1B cells and *P. aeruginosa* at concentrations significantly higher than the levels found as contamination in the EC2: GST recombinant proteins. Taken together, this provides evidence that the contamination is not causative to the phenomenon being examined.

In summary, the ability of recombinant human tetraspanin EC2: GST proteins and anti tetraspanin antibodies to inhibit the adhesion of GFP-labelled *P. aeruginosa* to three cell lines has been demonstrated. There is evidence that this effect is not due to LPS contamination, therefore it can be deduced that LPS existing in the recombinant protein samples did not contribute to the reduction in the numbers of *P. aeruginosa* adhering to host cells. Moreover, it is hypothesised that by intercalating into the tetraspanin web, the TEMs may disrupt the 'adhesion platforms' which may reduce the bacterial binding to the host cell receptor. Interestingly, when the efficacy of the tetraspanin treatments against GFP-labelled *P. aeruginosa* adherence is correlated with tetraspanin expression on the cell surface, as quantified by flow cytometry in chapter 3 we can see a direct correlation between the two parameters. In this study CD9 and CD81 were found to be expressed at high levels in A549, HaCaT and HEC1B cells, and they seem to have a direct role in GFP-labelled *P. aeruginosa* adherence, because the pre-treatment with anti-CD9 and anti-CD81 antibodies as well as EC2 proteins of CD9 and CD81 significantly reduced GFP-labelled *P. aeruginosa* adhesion. In addition, CD151 was found to have high expression level in A549 and HaCaT cells, and the pre-treatment with anti-CD151 and EC2 protein of CD151 reduced GFP-labelled *P. aeruginosa* adhesion. Furthermore, CD151 was expressed at very low level in HEC1B cells, and the pre-treatment with anti-CD151 and EC2 protein of CD151 had no effect in bacterial adhesion to this cell line.

Finally, anti-CD9 and anti-CD81 protect A549 and HEC1B cells from *P. aeruginosa*-induced cytotoxicity and that because they have an effect to in reducing GFP-labelled *P. aeruginosa* adhesion . The next chapter will investigate the role of peptide fragments derived from CD9 EC2 protein in reducing the adhesion and internalisation of GFP-labelled *P. aeruginosa* into host cells.

CHAPTER 6: The effect of CD9 EC2-derived peptides on GFP-labelled *P. aeruginosa* adherence to human cells

6.1 Introduction

In the work described in chapter 5, we investigated the role of tetraspanins in GFP-labelled *P. aeruginosa* attachment to epithelial cells. There was a significant reduction in adhesion when the host cells were incubated with the recombinant CD9 EC2 protein or anti-CD9 antibody. Similarly, previous studies have demonstrated the ability of CD9 EC2 proteins to reduce the adherence of various strains of both Gram-positive and Gram-negative bacteria to human epithelial cell lines (Hassuna *et al*., 2009; Green *et al*., 2011a; Ventress *et al*., 2016). Pre-treatment of human keratinocytes like HaCaT cells with low nanomolar concentrations of some CD9 EC2-derived peptides followed by infection with different *S. aureus* strains has been reported to significantly reduce bacterial adhesion (Ventress, Partridge *et al*., 2016). Therefore, in this chapter, an attempt was made to investigate the efficacy in reducing bacterial adhesion of eight peptides derived from the primary sequence of the tetraspanin CD9 EC2 domain (Figure 6.1). Furthermore, the possible cytotoxicity of the peptides on A549 cells and GFP-labelled *P. aeruginosa* was investigated. Finally, the effect of peptide 810 on the secretion of pro-inflammatory cytokines IL-6 and IL-8 was assessed.

6.1.1 Tetraspanin CD9 peptide fragments

CD9 EC2-derived peptides were developed based on the amino acid sequence of the EC2 region of CD9 (Tetraspanin 29) as shown in Figure 6.1 and Figure 6.2**.** Also, for each peptide a scrambled peptide control was designed with a randomised sequence of the amino acids (sequences in section 2.9.1.2).

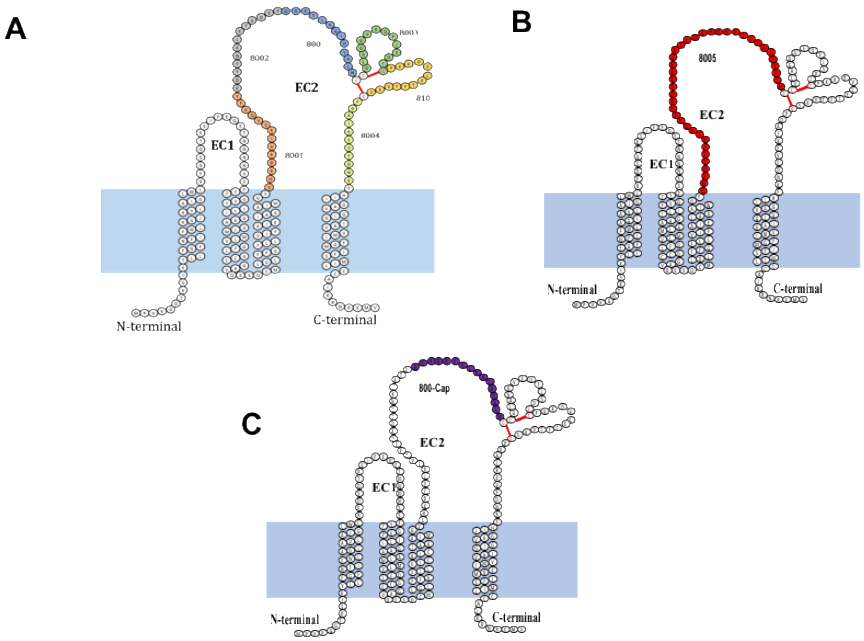

Figure 6.1 CD9 EC2-derived peptides corresponding to different regions of CD9EC2.

The amino acid sequence of (**A**) peptides 800, 810, 8001, 8002, 8003 and 8004. (**B**) peptide 8005 and (**C**) peptide 800-Cap, diagram generated by Dr Cozens.

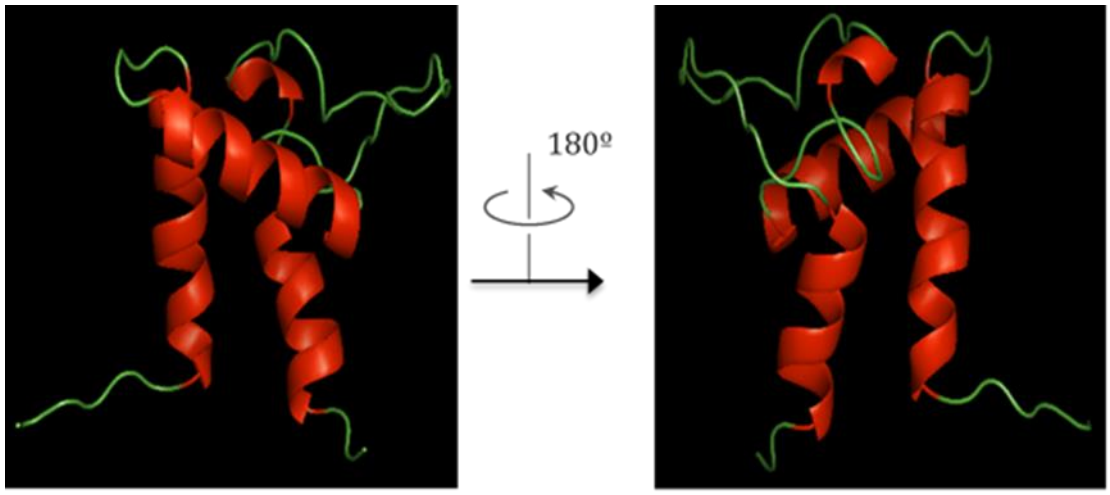

Figure 6.2 Tetraspanin CD9 structure.

The EC2 extracellular domain of the CD9 sequence shows the locations of the eight peptides, helices are shown in red and loops in green. The second image ('180') is rotated by 180 degrees (predicted CD9 EC2 structure using PyMOl by Dr.Issa).

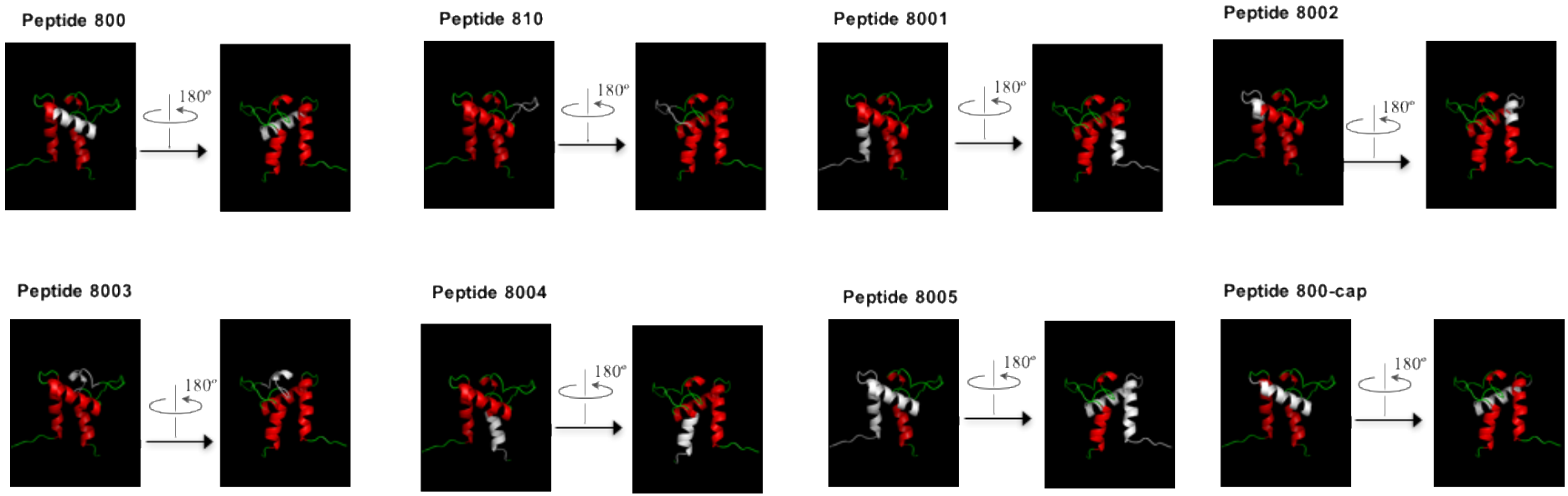

Figure 6.3 The peptide fragments from CD9 EC2.

The location of each peptide on the CD9 EC2 is shown in white. Other helices are shown in red and loops in green. The second image ('180') is rotated by 180 degrees, diagram generated by Dr Issa.

The aims of this chapter:

- To determine the effects of peptides on GFP-labelled *P. aeruginosa* growth and viability using the BacTiter-GloTM Assay.
- To investigate the effect of CD9 EC2-derived peptides (800, 810, 8001, 8002, 8003, 8004, 8005 and 800-Cap) on GFP-labelled *P. aeruginosa* adhesion to three human cell lines (A549 cells, HaCaT cells and HEC1B cells).
- To determine the dose-response of the most effective peptides.
- To investigate the effect of peptides 800, 810 and 8003 on GFP-labelled *P. aeruginosa* internalisation using a gentamicin protection assay.
- To assess the A549 cells viability using MTT assay.
- To determine the production of cytokines in cells treated with peptide 810 using CBA.

6.2 Results

6.2.1 The effects of CD9 EC2-derived peptides on GFP-labelled *P. aeruginosa* growth and viability

ATP is a direct measure of the metabolic activity in the cell and can be measured by quantifying the light constructed in a reaction with the naturally-occurring firefly enzyme Luciferase using a Luminometer. The quantity of light generated is directly proportional to the quantity of biological energy present in the cells. Therefore, to investigate the possible toxic effects of CD9 EC2-derived peptides on GFP-labelled *P. aeruginosa*, an ATP assay was performed to measure the metabolic activity (section 2.2.10.1). A linear correlation between rATP concentration and luminescence was obtained ($r^{\Lambda}2=0.9856$) $y=9E+X^{0.7401}$, where y represents the luminescence in RLU and x represents ATP

concentration in μM. This was used to determine the ATP concentration based on luminescence.

The rate of proliferation of GFP-labelled *P. aeruginosa* was monitored (section 2.2.2.2) in order to identify the characteristic phases of growth (Figure 6.4). The lag phase, the first stage where the bacterial cells are adjusting to the new media, was almost absent under the investigated experimental conditions. This is maybe because LB medium was used to prepare both the inoculum culture and sub-population culture. The log (exponential) phase was identified between 30 minutes and 6 hours, where GFP-labelled *P. aeruginosa* achieved the maximum rate of growth. GFP-labelled *P. aeruginosa* reached the mid-exponential phase within 2 to 3 hours of incubation, an OD_{600} of ~0.5 which was determined by calculating the average optical density between the start and endpoint of the log phase. Switch from the late-exponential (OD_{600} of 1.0) to the early-stationary phase (OD_{600} of 1.2) was displayed between 6 and 8 hours, respectively. The rate of proliferation was thereafter unchanged, with GFP-labelled *P. aeruginosa* cultures reaching the stationary phase of growth by 18 hours.

GFP-labelled *P. aeruginosa* cultures were treated with different peptides and scrambled control peptides (200 nM) during the log phase of growth and ATP production was monitored for 4 hours. The ATP production in GFP-labelled *P. aeruginosa* after treatment with peptides was measured in comparison to the control (untreated bacteria). Within 4 hours, the ATP concentration appeared to increase in the GFP-labelled *P. aeruginosa* (untreated) from 111 nM to 209 nM and in the GFP-labelled *P. aeruginosa* treated with all CD9 EC2-derived peptides, and the scrambled control peptides followed a similar increasing pattern which indicated that these peptides and the corresponding scrambled

control peptides were not toxic to the viability of GFP-labelled *P. aeruginosa* (Figure 6.5).

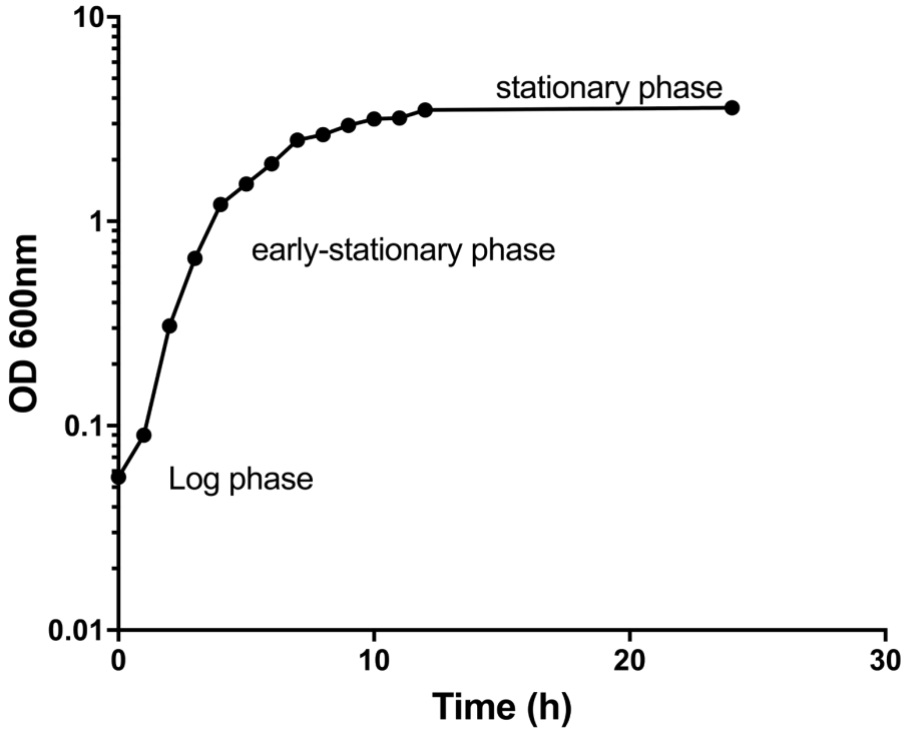

Figure 6.4 Growth curve of GFP-labelled *P. aeruginosa* over time in LB medium. Overnight cultures of GFP-labelled *P. aeruginosa* were diluted to an OD_{600} of 0.05 and grown in LB at 37°C for 24 hours, with agitation at 120 rpm. OD was measured at 600 nm, N= 3.

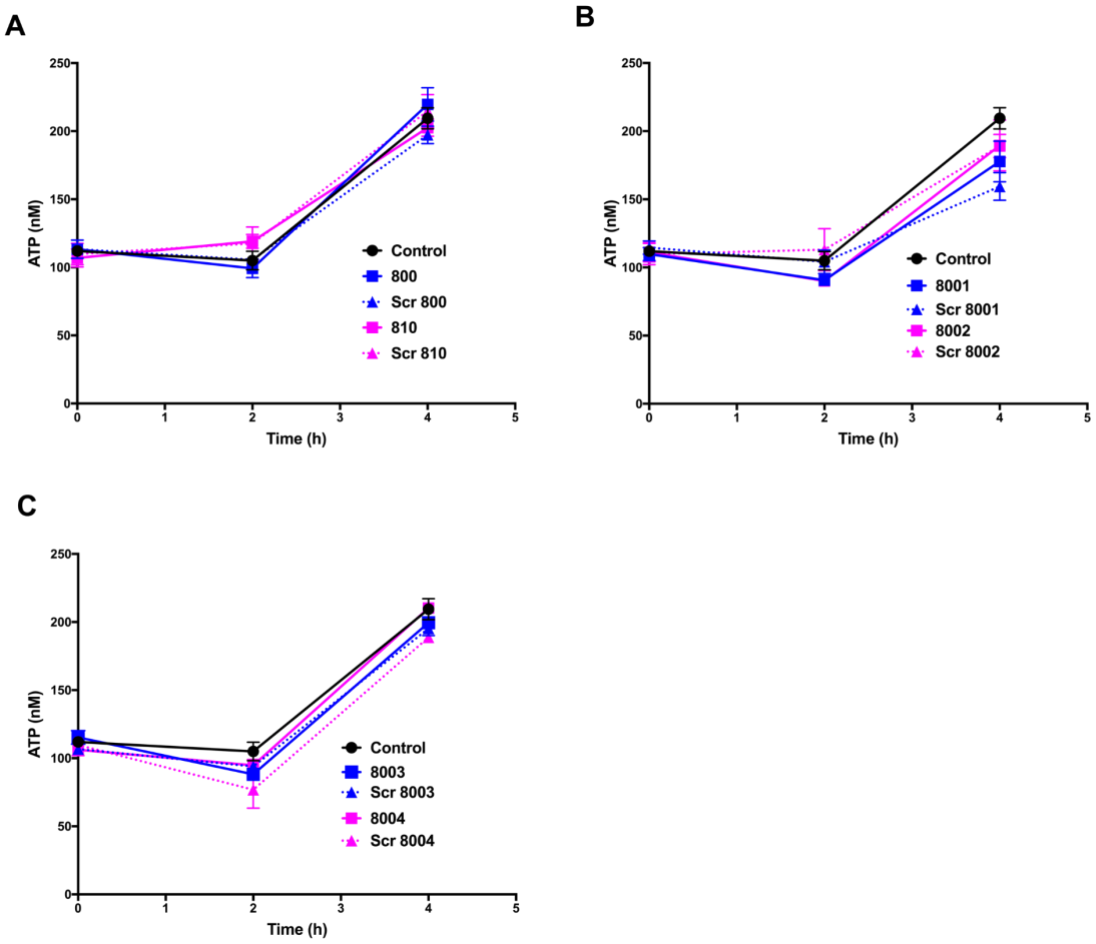

Figure 6.5 Quantification of ATP production in the GFP-labelled *P. aeruginosa* post-exposure to CD9 EC2-derived peptides.

Cultures of GFP-labelled *P. aeruginosa* were grown in LB in the presence of different CD9 EC2-derived peptides and scrambled control peptides (200 nM) for 4 hours at 37°C, with agitation at 120 rpm. ATP production was quantified using the BacTiter-GloTM Assay (Promega, UK). Readings were taken at 0, 2 and 4 hours after addition of the CD9 EC2-dervied peptides or scrambled control peptides. The data were analysed using Two-way ANOVA (vs. untreated *P. aeruginosa*), N= 4. Result are mean standard error of the mean (SEM).

6.2.2 The effect of CD9 EC2-derived peptides on GFP-labelled *P. aeruginosa* adherence to human cells

6.2.2.1 The effect of CD9 EC2-derived peptides on GFP-labelled *P. aeruginosa* adherence to A549 epithelial cells

To determine the effects of CD9 EC2-derived peptides on bacterial adherence, A549 cells were pre-treated with CD9 EC2-derived peptides and their corresponding scrambled control peptides at 200 nM (Figure 6.6 A) or 20 nM (Figure 6.6 B) followed by infection for one hour with GFP-labelled *P. aeruginosa* (section 2.2.4). All CD9 EC2-derived peptides used at 200 nM and 20 nM significantly decreased the binding of GFP-labelled *P. aeruginosa* to A549 cells when compared with the untreated cells. At 200 nM, the scrambled control peptides produced a significant reduction in GFP-labelled *P. aeruginosa* adhesion to A549 cells when compared with the untreated cells, however the scrambled control peptides used at 20 nM had no effect when compared with the untreated cells. Therefore, the CD9 EC2-derived peptides and their corresponding scrambled control peptides were used at a concentration of 20 nM in the further investigations.

6.2.2.2 The effect of CD9-derived peptides on GFP-labelled *P. aeruginosa* adherence to HaCaT keratinocyte cells

HaCaT cells pre-treated with all CD9 EC2-derived peptides at 200 nM (Figure 6.7 A) or 20 nM (Figure 6.7 B) significantly reduced the binding of GFP-labelled *P. aeruginosa* to HaCaT cells when compared with the untreated cells. Additionally, the scrambled control peptides had no significant effect on the binding of GFP-labelled *P. aeruginosa* to HaCaT cells at 200 nM and 20 nM when compared with the untreated cells.

6.2.2.3 The effect of CD9-derived peptides on GFP-labelled *P. aeruginosa* adherence to HEC1B endometrial cells

All CD9 EC2-derived peptides and their corresponding scrambled control peptides used at 200 nM significantly reduced the GFP-labelled *P. aeruginosa* adhesion to HEC1B cells when compared with the untreated cells (Figure 6.8 A). However, the lower concentration of the peptides showed specific effects; HEC1B cells pre-treated with peptides 800, 810, 8001, 8003, 8005 and 800-Cap at 20 nM significantly reduced the binding of GFP-labelled *P. aeruginosa* when compared to the untreated cells. While the pre-treatment with peptides 8002 and 8004 at 20 nM had no effect on the binding profile of GFP-labelled *P. aeruginosa* to HEC1B cells when compared with the untreated cells. Additionally, no effect was observed for all the scrambled control peptides used at 20 nM, when compared with the untreated cells *(*Figure 6.8 B).

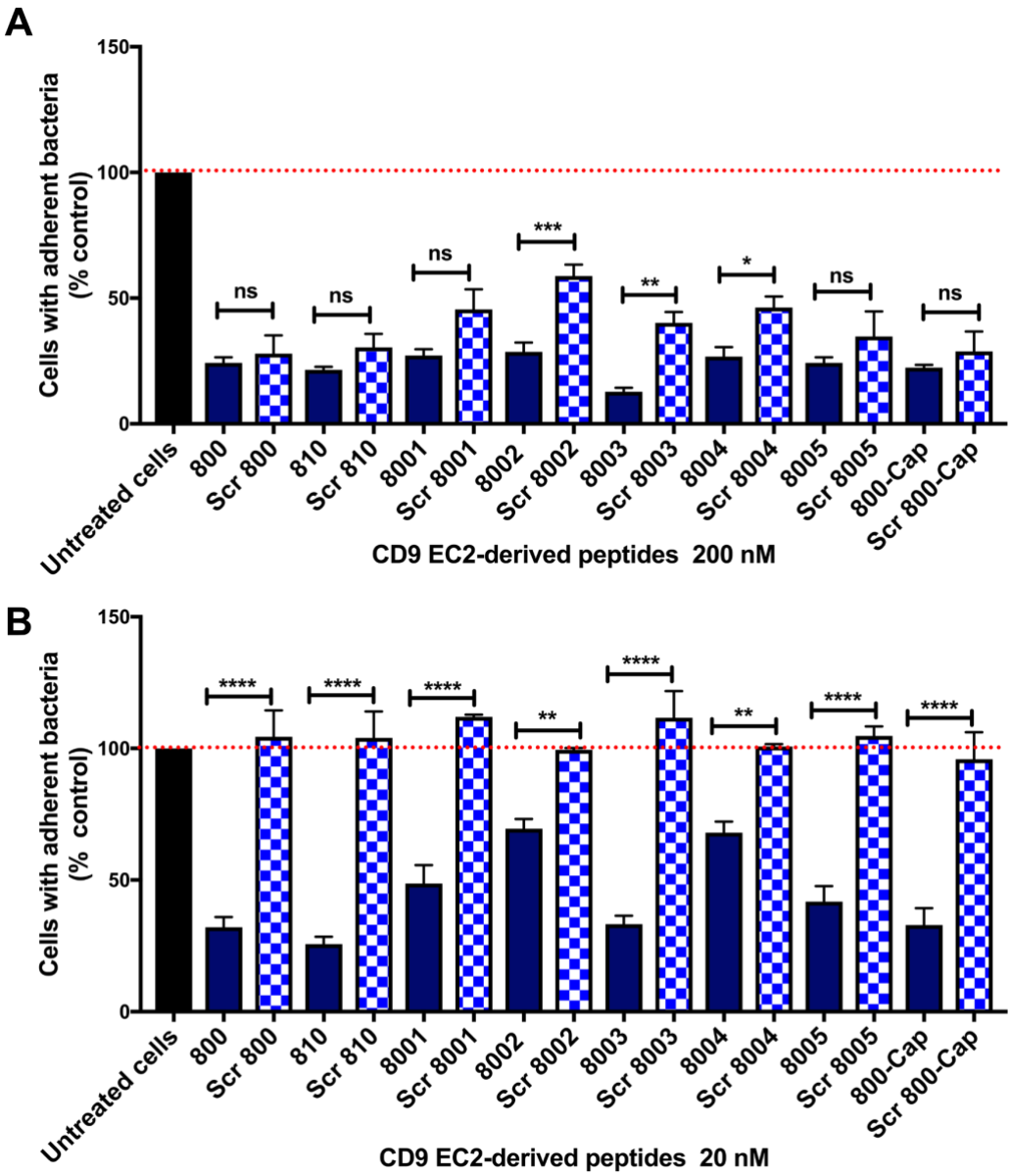

Figure 6.6 Effect of CD9 EC2-derived peptides on GFP-labelled *P. aeruginosa* adherence to A549 cells.

A549 cells were treated with CD9 EC2-derived peptides and their corresponding scrambled control peptides at (**A**) 200 nM or (**B)** 20 nM for 30 minutes. The adherent cells were washed and then infected with GFP-labelled *P. aeruginosa* for 60 minutes at an MOI of 10. A549 cells were fixed, stained, then visualised using fluorescent microscopy. The percentage of infected cells was normalised to the untreated control infected cells (100 cells). Results are mean ± standard error of the mean (SEM), N= 4. Data were analysed using One-way ANOVA with Sidak post-test (vs.the scrambled control peptides), ****$P \leq 0.0001$, ***$P \leq 0.001$.

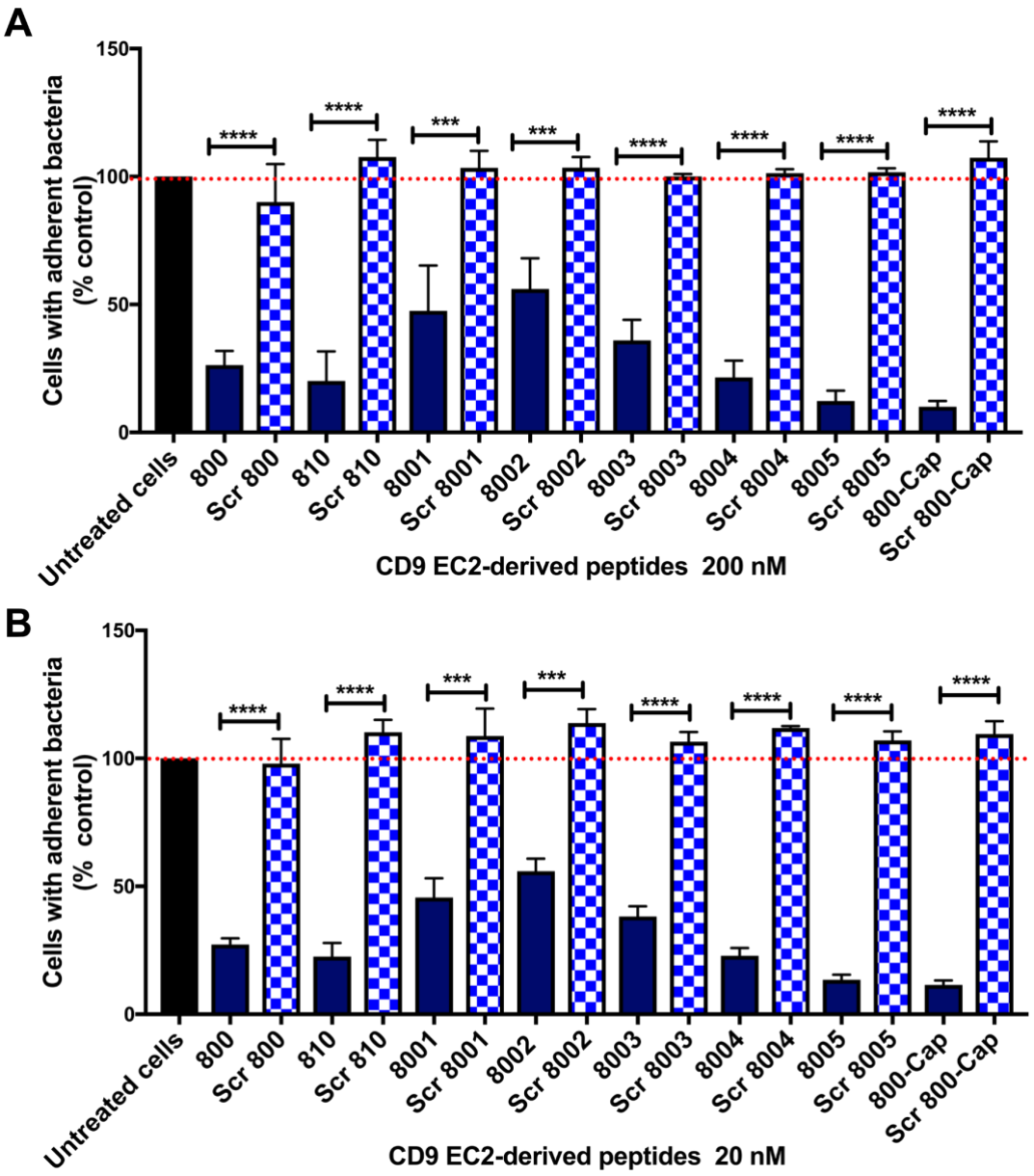

Figure 6.7 Effect of CD9 EC2-derived peptides on GFP-labelled *P. aeruginosa* adherence to HaCaT cells.

HaCaT cells were treated with CD9 EC2-derived peptides and their corresponding scrambled control peptides at (**A**) 200 nM or (**B**) 20 nM for 30 minutes. The adherent cells were washed and then infected with GFP-labelled *P. aeruginosa* for 60 minutes at an MOI of 10. HaCaT cells were fixed, stained, then visualised using fluorescent microscopy. The percentage of infected cells was normalised to the untreated control infected cells (100 cells). Results are mean ± standard error of the mean (SEM), N= 4. Data were analysed using One-way ANOVA with Sidak post-test (vs.the scrambled control peptides), ****$P \leq 0.0001$, ***$P \leq 0.001$.

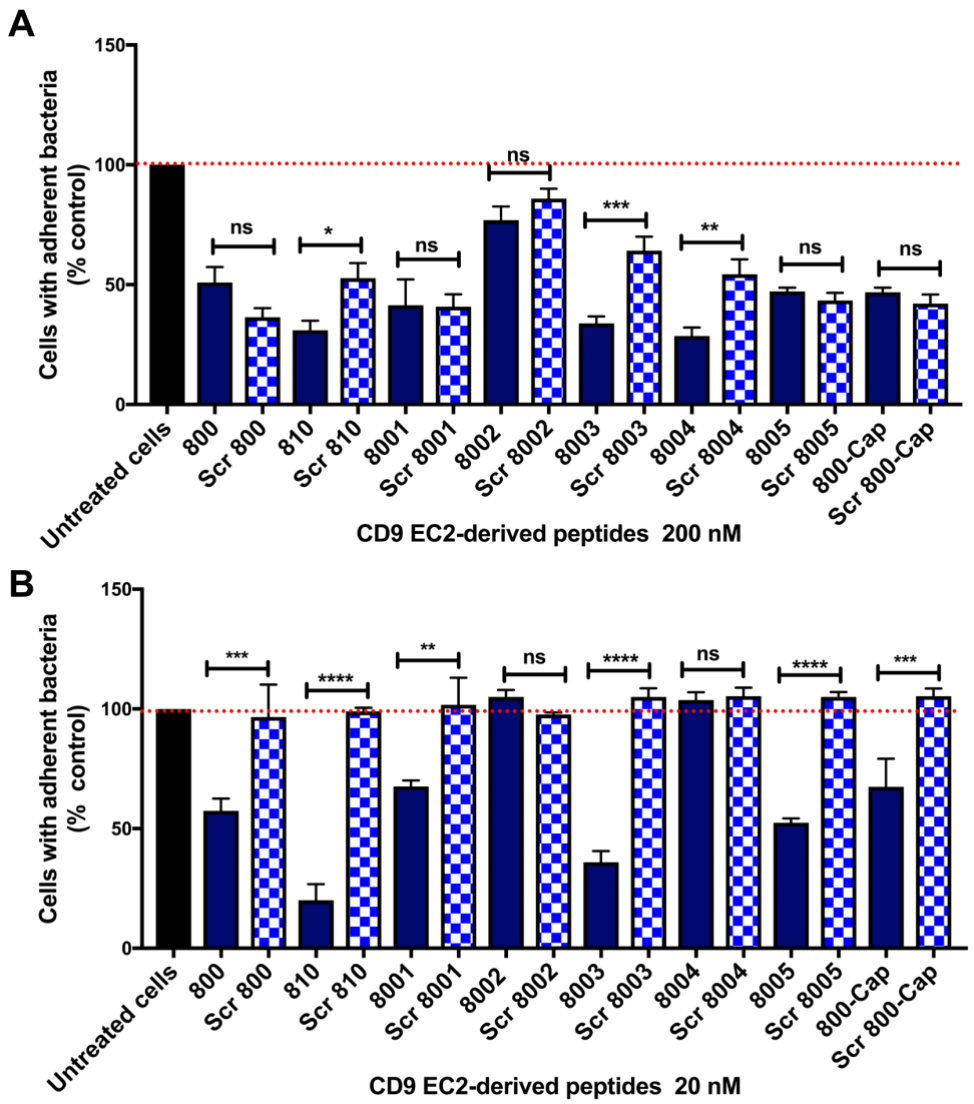

Figure 6.8 Effect of CD9 EC2-derived peptides on GFP-labelled *P. aeruginosa* adherence to HEC1B cells.

HEC1B cells were treated with CD9 EC2-derived peptides and their corresponding scrambled control peptides at (**A**) 200 nM or (**B**) 20 nM for 30 minutes. The adherent cells were washed and then infected with GFP-labelled *P. aeruginosa* for 60 minutes at an MOI of 10. HEC1B cells were fixed, stained, then visualised using fluorescent microscopy. The percentage of infected cells was normalised to the untreated control infected cells (100 cells). Results are mean ± standard error of the mean (SEM), N= 4. Data were analysed using One-way ANOVA with Sidak post-test (vs. the scrambled control peptides), ****$P \leq 0.0001$, ***$P \leq 0.001$ and ns is non-significant.

6.2.3 The optimum dose for CD9 EC2-derived peptides 810, 8003 and 800-Cap on GFP-labelled *P. aeruginosa* adherence to A549 cells

As observed from the previous results (section 6.3.2.1), the most effective peptides to reduce GFP-labelled *P. aeruginosa* adherence to A549 cells were the 810, 8003 and 800-Cap peptides. Thus, the effect of these CD9 EC2-derived peptides in GFP-labelled *P. aeruginosa* adherence to A549 cells was investigated by producing dose response curves to determine the concentration that inhibits 50 % of bacterial adhesion (IC_{50}). In this experiment, A549 cells were pre-treated with different concentrations of the peptides ranging from 0.3 nM to 20 nM for 30 minutes after which they were infected with GFP-labelled *P. aeruginosa* for 60 minutes at MOI of 10. The cells were fixed and counted manually under a fluorescent microscope and analysed using a three parameters nonlinear regression. These peptides were capable of significantly reducing the percentage of infected cells (Figure 6.9). The results show that peptide 810 had an IC_{50} of 2.4 nM (Figure 6.8 A), whereas peptide 8003 had an IC_{50} of 3.4 nM (Figure 6.8 B) and peptide 800-Cap had an IC_{50} of 3.2 nM (Figure 6.8 C). Thus, peptide 810 presented the most effective inhibition of GFP-labelled *P. aeruginosa* adhesion to A549 cells.

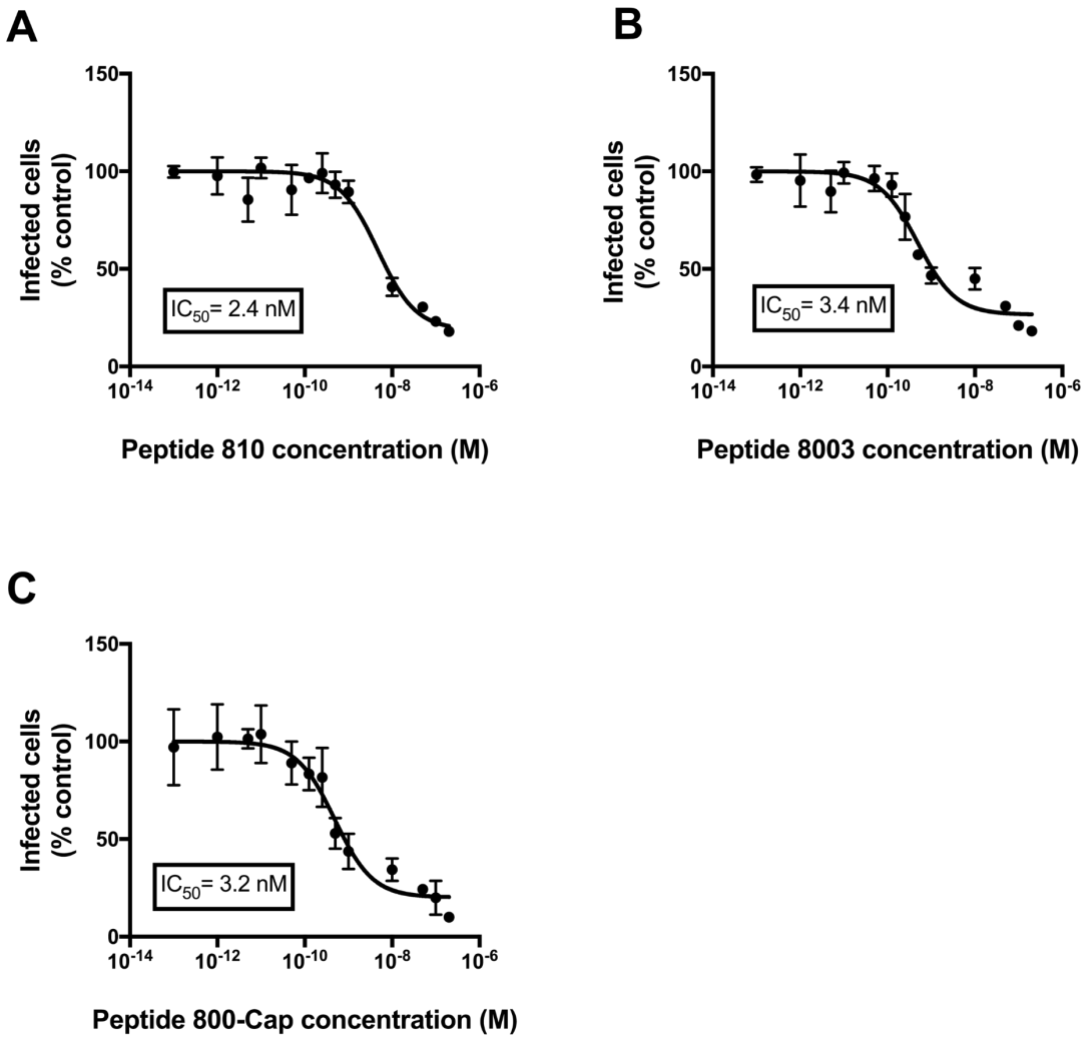

Figure 6.9 Dose-response curves of CD9 EC2-derived peptides 810, 8003 and 800-Cap in A549 cells infected with GFP-labelled *P. aeruginosa.*

A549 cells were pre-treated with CD9 EC2-derived peptides over a range of concentrations, from 0.3 nM to 20 nM for 30 minutes. The adherent cells were washed and subsequently infected with GFP-labelled *P. aeruginosa* for 60 minutes at an MOI of 10. A549 cells were fixed and DAPI stained, allowing for analysis of adherence using fluorescent microscopy (section 2.2.4). Samples were calculated as a percentage against a control of untreated cells, which was normalised 100 %. X scale plotted as power of 10. Line represents log (inhibitor) vs. response (three parameters). Result are mean ± standard error of the mean (SEM). The R square for peptide 810 is 0.891, for peptide 8003 is 0.918 and for peptide 800-Cap is 0.906, N= 4.

6.2.4 The effect of CD9 EC2-derived peptides on *P. aeruginosa* internalisation into A549 epithelial cells

CD9 EC2-derived peptides were tested on A549 cells for their ability to reduce internalisation of GFP-labelled *P. aeruginosa*. Alveolar epithelial (A549) cells were chosen because they are an established model for the study of *Pseudomonas* internalisation (Hawdon *et al*., 2010).

6.2.4.1 Determination of the optimal (MOI)

In order to investigate the effects of CD9 EC2-derived peptides on GFP-labelled *P. aeruginosa* internalisation into A549 cells, a range of MOIs (1, 10 and 50) were tested to determine the optimal MOI, using an antibiotic exclusion assay (section 2.2.9.1). The results showed that the percentage of internalised bacteria for MOI of 1 was 5 %, MOI of 10 was 8 % and MOI of 50 was 7 %. MOI of 10 was chosen as there was no statistically significant difference vs MOI of 50 and MOI of 1 gave a lower percentage of internalised bacteria (Figure 6.10 A)**.**

6.2.4.2 The effect of CD9 EC2-derived peptides on GFP-labelled *P. aeruginosa* internalisation into A549 cells

As mentioned in section 6.2.2.1 peptides 800, 8003 and 810 reduced the adherence of GFP-labelled *P. aeruginosa* to A549 cell by 40 %, 30 % and 23 %, respectively, at MOI of 10. The ability of these peptides to reduce the internalisation of GFP-labelled *P. aeruginosa* into A549 cells was assessed using an antibiotic exclusion assay. The results showed that there was no significant difference in bacterial internalisation post-treatment with the peptides and also with the corresponding scrambled control peptides, when compared to the untreated cells. Surprisingly, A549 cells pre-treated with the scrambled

control peptide 800 reduced the percentage of internalised GFP-labelled *P. aeruginosa* by only 2 %, when compared to the untreated cells, while peptide 800 reduced by only 3 %. Additionally, peptides 810 and 8003 together with the corresponding control peptides exhibited a similar reduction in the percentage of internalised bacteria, when compared to the untreated cells. However, for all the tested peptides and their corresponding control peptides the difference was not statistically significant when compared to the untreated cells (Figure 6.10 B).

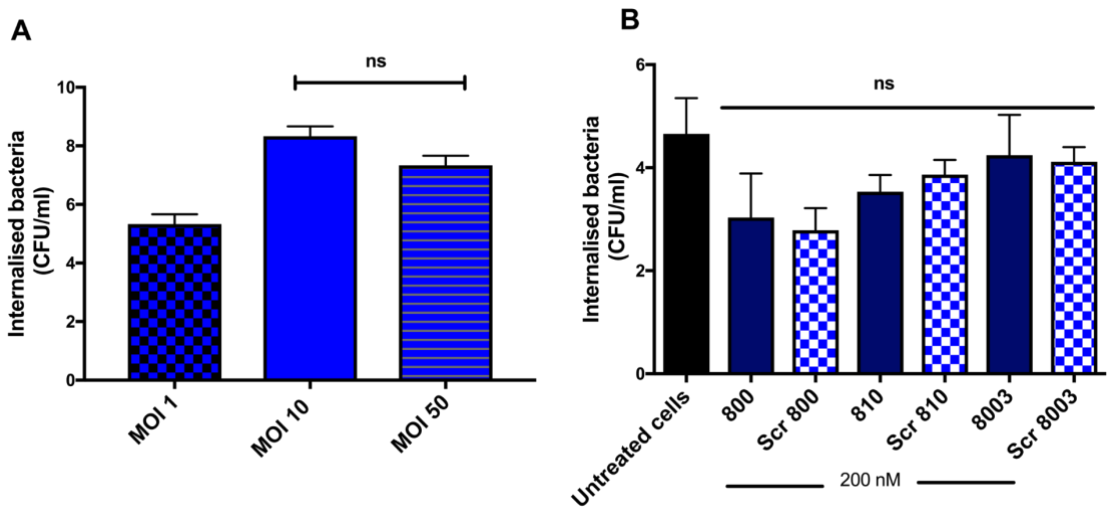

Figure 6.10 Internalisation of GFP-labelled *P. aeruginosa* into A549 cells.

(**A**) A549 cells were seeded in 6 well plates at 37°C, 5 % CO_2 for 48 hours. Cells were washed before being infected with GFP-labelled *P. aeruginosa* at a range of MOIs (1, 10 and 50) at 37°C for 60 minutes, this was followed by washing, and then 1 ml of gentamicin was added at 37°C for 60 minutes to kill extracellular bacteria. A549 cells were washed before adding 1 ml of lysis buffer for 20 minutes, after incubation A549 cells were washed and a cell scraper was used to detached the cells. Aliquots of 100µl were then spread onto LB plates. Agar plates were incubated at 37°C for 24 hours before the bacterial colonies were enumerated and expressed as percentage of input bacteria, N= 4. (**B**) MOI of 10 was used to infect the cells after the peptides and scrambled control peptides treatment for 30 minutes. Result are mean ± standard error of the mean (SEM). Data were analysed using One-way ANOVA (vs. untreated cells), ns is non-significant, N=4.

6.2.5 The cytotoxic effect of CD9 EC2-derived peptides on A549 cells

The viability of A549 cells was determined in the context of the percentage of MTT metabolised by untreated cells to quantitatively assess the effects of CD9 EC2-derived peptides at 500 nM on A549 cells. Briefly, A549 cells were seeded in a 96-well plate (10,000 cells/well in 100 µl complete media) and allowed to attach at 37°C, 5 % CO_2 for 16 hours to achieve a 70-80 % confluent monolayer. A549 cells were then incubated with CD9 EC2-derived peptides for 30 minutes before the infection with *P. aeruginosa* with MOI of 10 for 16 hours, section 2.2.10.2.

The percentage of viable A549 cells after exposure to CD9 EC2-derived peptides was not significantly different compared to the control (untreated cells), but significantly higher compared to the cells treated with Triton X-100. The results indicate that the concentration of 500 nM did not cause a significant loss in mitochondrial function, as indicated by the high cell viability measurements. This concentration was therefore considered non-cytotoxic to A549 cells under these conditions (Figure 6.11).

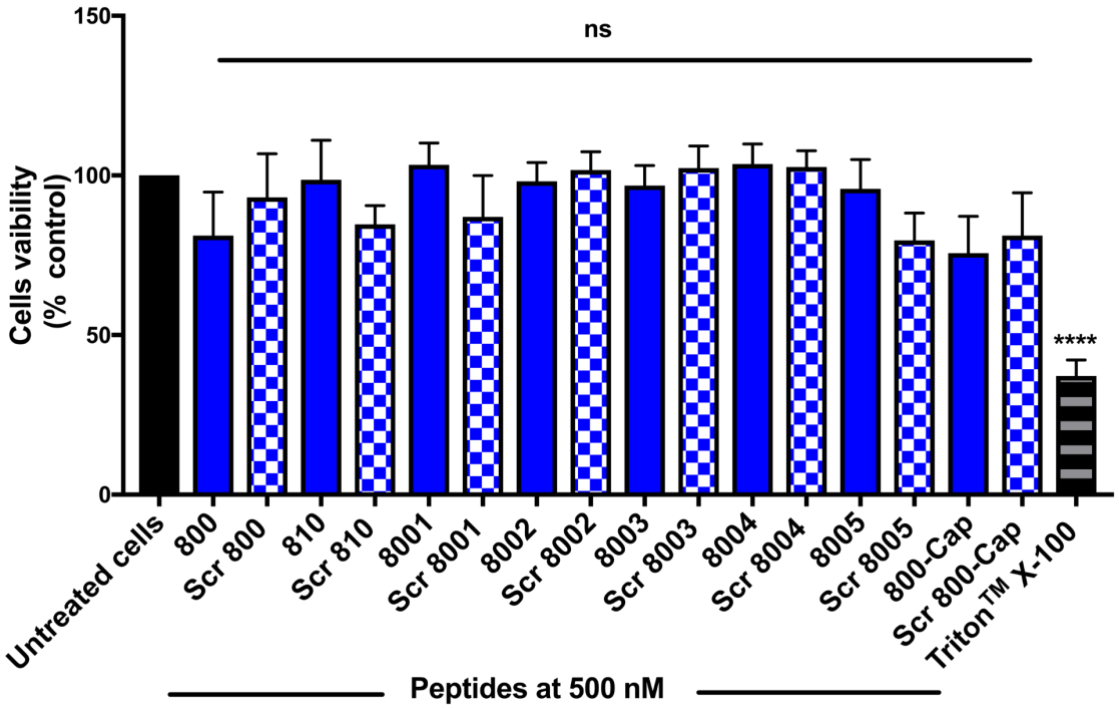

Figure 6.11 Viability of A549 cells following exposure to CD9 EC2-derived peptides. A549 cells were incubated with 500 nM of CD9 EC2-derived peptides for 30 minutes at 37°C, 5 % CO_2. Viability was assessed using the MTT assay and expressed relative to the untreated control cells (100 %). Results are mean ± standard error of the mean (SEM). Data were analysed using One-way ANOVA with Dunnett test (vs. untreated cells), ****$P \leq 0.0001$ and ns is non-significant, N= 4.

6.2.6 The effect of peptide 810 on cytokine release from A549 and HEC1B cells

Having established that peptide 810 effectively reduced bacterial adhesion to the A549 cells and HEC1B cell lines (chapter 6), it was decided to measure cytokine release to determine if the loss of adhesion also affected the immune response. The cytokines released by A549 and HEC1B cells pre-treated with peptide 810, and then infected with GFP-labelled *P. aeruginosa* were investigated (section 2.2.7). Initial experiments were undertaken to establish the optimal time for the highest cytokine release from peptide 810 treated and infected cells (Appendix 1).

Figure 6.12 A shows the level of IL-8 secreted by A549 cells pre-treated with peptide 810 and infected with GFP-labelled *P. aeruginosa* at MOI of 35 (this MOI was determined from previous flow cytometry optimisation expirements). The results show that there was a significant increase (by 32%) in the level of IL-8 released when compared with the untreated cells. Moreover, cells pre-treated with the scrambled control had no significant difference in the cytokine release, $P > 0.9$ vs untreated. Additionally, the level of IL-6 released was not significantly different when comparing the peptide 810-treated A549 cells and scrambled control vs untreated cells, Figure 6.12 B. However, the levels of the two cytokines IL-8 and IL-6 were not significantly different for HEC1B cell pre-treated with peptide 810 or scrambled control peptide when compared to the untreated cells (Figure 6.12 C and D).

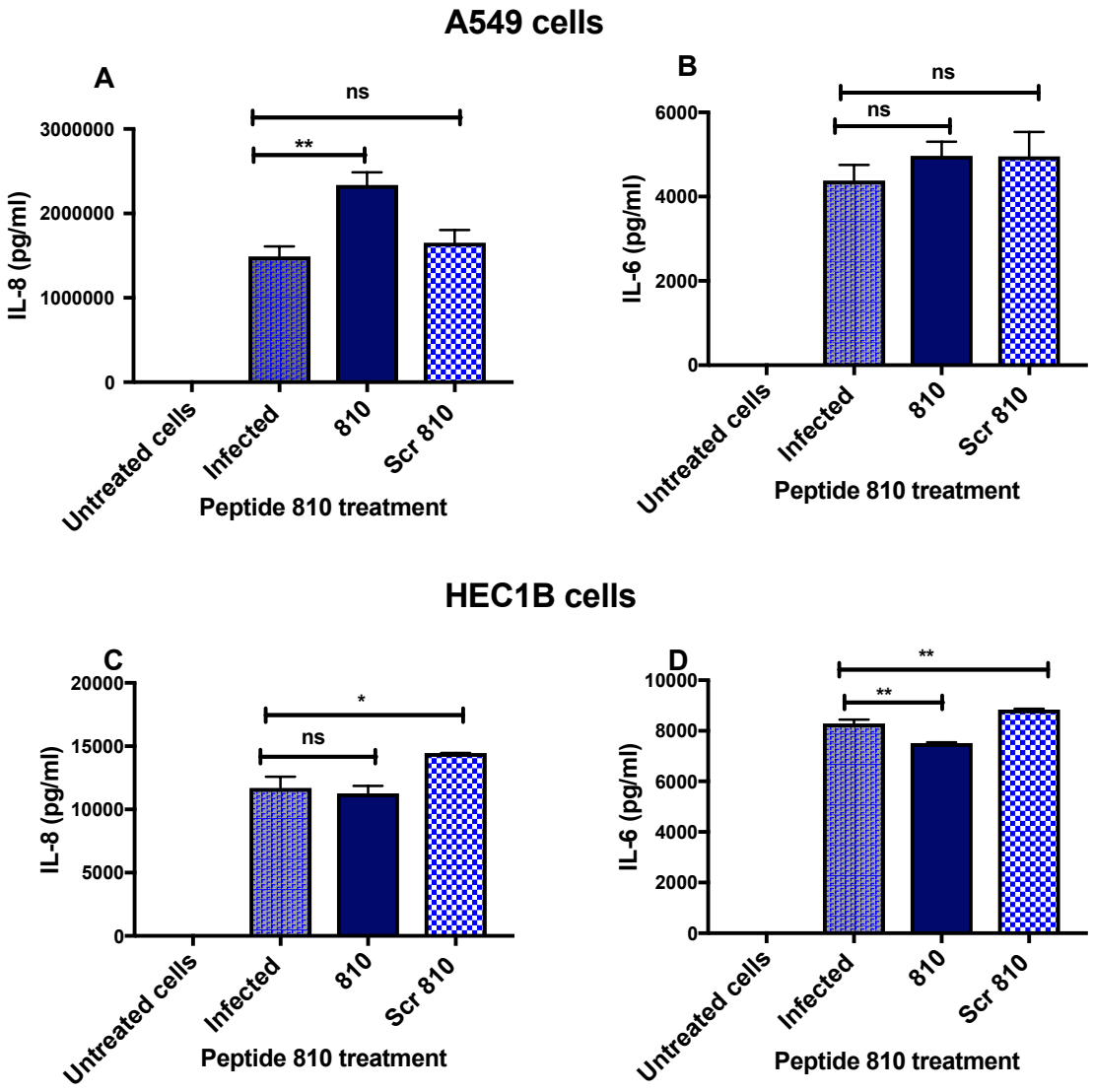

Figure 6.12 The effect of peptide 810 on IL-6 and IL-8 release from A549 and HEC1B cells following infection with GFP-labelled *P. aeruginosa.*

The supernatants from peptide treated cells that were then infected with GFP-labelled *P. aeruginosa* were collected after 18 hours of incubation at 37°C, 5 % CO_2 for analysis using CBA. Results are mean ± standard error of the mean (SEM), N= 3. Data were analysed using One-way ANOVA with Bonferroni's multiple comparisons test (vs. untreated cells), *$P \leq 0.05$ and ns is non-significant.

6.3 Discussion

From a therapeutic point-of-view, tetraspanin based anti-adherence peptides display low toxicity towards human cells and the pathogen, which makes them promising candidates for anti-bacterial therapy (Ventress *et al*., 2016). There is no evidence yet of the exact mechanism of action for tetraspanins peptides, but they are thought to work in a similarly way to the tetraspanin EC2 domains or antibodies (discussed in chapter 4), perhaps by interpolating into TEMs on the cell membrane. This could lead to disruption of the proteins through protein interactions and disordering of the TEMs. This would affect bacterial adhesin molecule interactions with TEM and thus reduce adhesion.

In this study, we demonstrate that pre-treatment of three cell lines with short (≤ 50 amino acid) peptides directly derived from the sequence of the EC2 domain of tetraspanin CD9 significantly reduced the adherence of GFP-labelled *P. aeruginosa*, but not internalisation. This study also found that in the three cell lines tested, the peptides were not toxic towards the bacteria. In addition, no significant effects were seen on human cell metabolism, indicating that this may be a promising drug in an anti-adhesive therapy against bacterial infection. Finally, a slight increase was observed in the release of cytokine IL-8 after A549 cells were pre-treated with peptide 810.

Previous work from our lab has shown that these peptides are capable of reducing Gram-negative and Gram-positive bacteria adhesion to host cells (Green *et al*., 2011a). Cozens (2015) demonstrated that six peptides from the sequence of EC2 domain of CD9 were able to reduce up to 50 % of meningococcal adhesion to human epithelial cells. Furthermore, Ventress *et al*., (2016) provided some evidence for the potential of using these peptides as inhibitors of *S. aureus* adherence to keratinocytes. Pre-treatment of human keratinocytes like HaCaT cells or primary human keratinocytes with 50 nM of

peptides 810, 8001 or 800, followed by infection with different strains of *S. aureus* including SH1000, S235 or JE2 MRSA, resulted in a significant reduction in bacterial adherence. Peptide 810 was most effective against HaCaT cells infected with the S235 strain, causing 60 % in bacterial adherence. Recent work by Issa (2017) (unpublished data) found that some peptides including 8005 and 800-Cap are very effective in reducing the adherence of *S. aureus* (SH1000) to HaCaT cells. Correspondingly, in this study, pre-treatment of the tested cell lines with CD9 EC2-derived peptides significantly reduced the adherence of GFP-labelled *P. aeruginosa* by more than 80 % to A549 and HaCaT cells, and 37 % to HEC1B cells. This is consistent with the expression level of CD9 in the different cell lines as discussed previously in chapter 4, suggesting that the peptides may have been affected by the amount of TEMs on the cell surface of different cell types when inhibiting bacterial adhesion.

Previous studies in our lab have shown that scrambled peptides at low concentration have no effect on the attachment of various pathogens to host cells. In agreement with this study were the scrambled control peptides had no effect at 20 nM, this indicates that the anti-adhesive effect is tetraspanin specific and not a non-specific peptide effect (Cozens, 2015; Ventress, 2016). In this study, the pre-treatment with scrambled control peptides at 200 nM has been shown to have an effect on *Pseudomonas* adhesion to host cells compared to the untreated cells (control) and this could be due to non-specific activity. Work in our lab has shown that some scrambled control peptides e.g. 800-Cap, are hydrophobic, with strong affinity to plastic. Moreover, structural analysis of other scrambled control peptides e.g 8005, using circular dichroism, has shown that the ∝-helical confirmation is maintained despite the change in sequence (Dr. Issa, Department of Infection and immunity and cardiovascular, University of Sheffield, personal communication). This suggests that the activity of peptides depends on both amino acid

sequence and helical structure.

The dose-response experiments to ascertain inhibitory concentration (IC_{50}) values have shown differences between peptides, host cells and the bacteria used. In this study, peptides showed efficiency at very low concentration (2.4 nM for peptide 810) towards the GFP-labelled *P. aeruginosa*, which was in agreement with previous studies which have shown that peptide 8001 and peptide 8003 exhibit low IC_{50} (4.06 nM and 3.72 nM respectively) towards *N. meningitidis* (Cozens *et al.*, 2016), and even 1 nM of peptide 800 has been reported efficinet towards *S. aureus* (Ventress *et al.*, 2016). This difference in IC_{50} values could be due to virulence factors amongst different bacterial types, suggesting that the adhesion molecules for the bacterial pathogen may play a role in this process. Many Gram-negative and Gram-positive bacteria such as *N. gonorrhoea* and *S. aureus* express two major adhesin proteins, the type IV pili (Tfp) and phase-variable opacity-associated (opa), which promote adherence to epithelial cells (Carbonnelle *et al.*, 2005). It has been found that a reduction in *Neisseria* adhesion to cells treated with tetraspanin CD9 peptides is related to tfp expression. In contrast, expression of the major adhesion protein (opa) in *Neisseria* (Virji *et al.*, 1996) had no significant effect on bacterial adhesion to cells treated with tetraspanin-based peptides (Cozens, 2015). This suggests the hypothesis that TEMs play a role as "adhesion platforms" that cluster cell membrane protein and aid some bacterial/host cell interactions.

As mentioned previously, peptides 800, 8003 and 810 were tested to identify their abilities to inhibit the adhesion and internalisation of GFP-labelled *P. aeruginosa* into A549 cells after gentamicin treatment, and no effect was observed. In the untreated control cells, only 5 bacteria /10000 cells were internalised. This finding is in agreement with previous studies that investigated multiple anti-adhesive peptides, and on different epithelial cell

line with only about 1-2 % bacteria invading epithelial cell line (Issa, personal communication 2016). However, the frequencies of invasion in epithelial cell lines are considerably different. Therefore, invasion characteristics cannot always be compared across cell lines or bacterial species.

Furthermore, there was no toxic effect on GFP-labelled *P. aeruginosa* growth and viability during incubation with CD9 EC2-derived peptides, demonstrating that the reduction in the number of adherent bacteria is not due to bacteriostatic or bactericidal effects. These peptides are therefore unlikely to produce resistant bacteria.

In summary, peptides based on the EC2 domain of the tetraspanin CD9 have been established as effective inhibitors of GFP-labelled *P. aeruginosa* adherence to human cells, but not internalisation. The concentration used was lower than that used for whole CD9 EC2 domains, with variations between the IC_{50} of the peptides. It has been shown that these peptides are not toxic to GFP-labelled *P. aeruginosa*. Moreover, there was no significant effect on cell viability after CD9 EC2-derived peptides treatment on A549 cells, indicating that they could be a valuable addition to present treatments for bacterial infection. Finally, the level of cytokines released were measured after treatments with peptide 810 and no significant effects were seen. Treatment with these peptides will therefore not elicit an immune response.

CHAPTER 7: General Discussion

7.1 Summary

In this thesis, we have investigated the action of tetraspanin antibodies, recombinant EC2 proteins and CD9 EC2-derived peptides on the adhesion of GFP-labelled *P. aeruginosa* to host cells. We hypothesised that tetraspanins may mediate adhesion platform formation on the plasma membrane by TEM and form an important mechanism of bacterial adhesion to the host cell.

P. aeruginosa is an opportunistic pathogen, which causes infections in immunocompromised individuals, in chronic leg ulcers, burn wounds, pneumonia and the lungs of cystic fibrosis patients (Fothergill *et al*., 2012; Morita *et al*., 2014; Savoia, 2014). *P. aeruginosa* is notorious for its antibiotic resistance and has been implicated in nosocomial infections (Breidenstein *et al*., 2011). Therefore, developing effective antimicrobial agents against multi-drug resistant bacteria is a growing concern in the medical community (Kang *et al*., 2012; Bjarnsholt *et al*., 2013). Most antimicrobials available for treating *P. aeruginosa* infections directly kill or inhibit the bacterial growth, subsequently causing the growth of resistant strains through genetic changes (Breidenstein *et al*., 2011; Morita *et al*., 2014). Most antimicrobial peptides have less specific mechanisms of action (e.g. membrane damage), and a variety of targets, which decreases the development of resistance by the pathogen (Jorge *et al*., 2012).

One effective method is to target bacterial adherence (Cozens and Read, 2012), as this method of treatment should develop less resistance because it does not kill the bacteria directly and therefore exerts less pressure on the bacteria.

The present work was in an agreement with the previous findings obtained by our group, who demonstrated that tetraspanin reagents (EC2 proteins and anti-tetraspanin

antibodies) may reduce the adherence of other Gram-negative and Gram-positive pathogens to host cells. Furthermore, CD9 EC2-derived peptides are also being investigated for their role in *Salmonella* adherence (Ali, 2016), *Staphylococcus* adherence (Ventress *et al*., 2016) and monocyte giant cell fusion (Elgawidi, 2016).

In **Chapter 3**, we provided evidence demonstrating that the selective tetraspanins are variably expressed on keratinocyte and epithelial cell lines. Consistent with previous studies (Ali, 2016; Ventress, 2016), we hypothesised that each cell line may produce a specific type of TEM depending on the variable expression level of the tetraspanins. These TEMs are able to form different function specific for each cell type.

In **Chapter 4**, we successfully generated a stable fluorescent-labelled *P. aeruginosa* and attempted to investigate the bacteria on a flow cytometric method that can detect the disruption of *P. aeruginosa* adhesion to host cell using tetraspanin reagents. Flow cytometry analysis is potentially a rapid and powerful method to investigate bacteria-host interaction, and many studies have reported the effectiveness of using this method, particularly when combined with fluorescence-labelled bacteria (Valdivia *et al*., 1996; Trouillet *et al*., 2011b).

Surprisingly this method was not sensitive enough to detect the effect of tetraspanin reagents treatment in GFP-labelled *P. aeruginosa* adhesion to host cell. This was in agreement with the previous investigation looking at the role of tetraspanin EC2 proteins in *Salmonella* adherence to J774 cells, where flow cytometry could not detect any significant effects but the fluorescent microscopy obtained significant results (Ali, 2016). Many factors could interfere with the results from flow cytometry including using a specific flow cytometry machine to detect bacterial interaction, as the analysis of bacteria may differ depending on the growth conditions used, or the sources from which the

bacteria were taken (Winson and Davey, 2000). Additionally, bacteria are much smaller than human cells, therefore, the analysis of pathogenic bacteria poses specific challenges for flow cytometry. Furthermore, if the flow cytometry machine is not specifically designed for microbial investigation, it is necessary to determine whether it is capable of such analysis and then optimise its performance (Harkins and Harrigan, 2004). Therefore, fluorescence microscopy was used in **Chapter 5 and 6** to investigate the role of tetraspanin treatments (anti-tetraspanin antibodies, EC2: GST proteins and CD9 EC2-derived peptides) in GFP-labelled *P. aeruginosa* adherence to host cell. Here, we obtained the first evidence that tetraspanin reagents could significantly reduce GFP-labelled *P. aeruginosa* adherence to human cell lines, but not the internalisation. This is consistent with previous studies on Gram-negative and Gram-positive bacteria (Green *et al*., 2011b).

Using a selection of anti-tetraspanin antibody treatments on epithelial and keratinocyte cells, we have shown that adherence of GFP-labelled *P. aeruginosa* can be significantly reduced. Some anti-tetraspanin antibodies had more of an effect than others, suggesting that some tetraspanins are more involved in *P. aeruginosa* association to human cells than others; for example, CD63 showed a great reduction in bacterial adherence to HEC1B cells while CD81 demonstrated no effect. However, in A549 and HaCaT cells all the anti-tetraspanin antibody treatments appeared to result in a similar level of reduction in bacterial adherence. But, the number of GFP-labelled *P. aeruginosa* per cell was found to be unaffected by some tetraspanin treatments, this suggests that there is a subpopulation of the cells, around 40 %, on which the tetraspanin reagents are ineffective. Furthermore, we found that anti-CD9 and anti-CD81 protect A549 and HEC1B cells from GFP-labelled *P. aeruginosa*-induced cytotoxicity.

Our studies have also tested the effect of recombinant tetraspanin EC2: GST proteins on

GFP-labelled *P. aeruginosa* adherence to epithelial cells and keratinocyte cells. Using similar concentrations of protein to those of the antibody treatment we have found that the GST: EC2 proteins are able to significantly reduce GFP-labelled *P. aeruginosa* adherence to a better degree than the interference of tetraspanins with antibodies, and this effect was not due to LPS contamination of the recombinant protein.

In **Chapter 6**, CD9 EC2-derived peptides were similarly tested for their ability to reduce GFP-labelled *P. aeruginosa* adhesion to human cells. The peptides significantly reduced, by 50%, the adherence of GFP-labelled *P. aeruginosa* to epithelial and keratinocyte cells when compared to the scrambled control peptides which were also used as controls for non-specific effects. Moreover, the effect displayed by peptides was a dose dependent response, and peptides were active at a concentration of 3 nM. Also, the scrambled control peptides displayed no effect on GFP-labelled *P. aeruginosa* adhesion to human cells at a concentration of 20 nM, this indicates that the effect seen was specific for the peptides. However, the scrambled control peptides were found to have unspecific effect at a concentration of 200 nM on A549 and HEC1B cell lines but not HaCaT cells.

These peptides may intercalate into TEMs and could work in synergy with the common antibiotics such as gentamicin and mupirocin to provide more antibacterial protection at subminimal inhibitory concentrations. *P. aeruginosa* first adhere to epithelial cells, and this adherence is mediated by Tfp. This adhesin is required for tetraspanins inhibition of bacterial adhesion (Cozens and Read, 2012). The Tfp can recognise CD46 (Kallstrom *et al.*, 1997b) which forms complexes with the tetraspanin CD9 (Takeda *et al.*, 2003). However, the exact mechanism of tetraspanin function in bacterial adhesion is still unclear and needs to be further investigated.

In addition, the effects of CD9 EC2-derived peptides on GFP-labelled *P. aeruginosa* growth and viability were tested and no effect was observed, this indicates that these peptides were non-toxic to the bacteria. We also investigated the ability of selective CD9 EC2-derived peptides to reduce GFP-labelled *P. aeruginosa* internalisation into A549 cells but no effect was seen, therefore the internalisation might not be a tetraspanin dependent process.

Tetraspanins are cell membrane molecules with varying important functions across the body. It was important to test the effects of CD9 EC2-derived peptides on cell metabolism, therefore MTT assay was used and no effect was seen in the pre-treated epithelial cells. Additionally, a slight increase in production of the cytokine IL-8 was observed after A549 cells were pre-treated with peptide 810.

To summarise, we have shown that tetraspanin treatments (anti-tetraspanin antibodies-EC2: GST proteins and CD9 EC2-derived peptides) are capable of reducing GFP-labelled *P. aeruginosa* adhesion to epithelial and keratinocytes cells. We hypothesise that tetraspanins treatment may result in integration with TEMs on the cell membrane and through this process the peptides become capable of disordering the microdomains. This effect has been observed in a broad spectrum of bacterial species to different cell lines (Table 7.1), indicating that tetraspanin treatments may be an important new class of antibacterial drug development.

7.2 Future work

- Use high resolution microscopy such as (STORM) to identify how TEMs work and are organised by using either labelled antibodies or GFP-tagged tetraspanins in transfected cell lines.
- Use different strains of *P. aeruginosa* (e.g. virulent and avirulent) to determine

whether the pathogenicity of these strains is related to the usage of the tetraspanin on human cells.

- Use other pathogenic bacteria that have similar entry mechanism for invading human epithelial cells to determine if tetraspanins are involved in mediating adhesion in extended range of bacteria.
- Use complex tissue culture systems that resemble lung epithelium or other tissues such as skin to investigate whether tetraspanins are likely to work in vitro.

Table 7.1 Tetraspanin treatments are effective on many bacterial species.

Bacterium	Cell line	Treatments	Reference
Neisseria meningitidis	DETROIT HEC1B MD Macrophages HDMECs	Antibodies(CD9,63,81,151) Combination of antibodies (CD9,63,81) EC2: GST proteins (CD9,63,151) CD9 EC2-derived peptides (800,810,8001,8002,8003,8004)	(Green *et al*., 2011a).
Escherichia coli	HEC1B	Combination of antibodies (CD9,63,81) EC2: GST proteins (CD9,63,151)	(Green *et al*., 2011a).
Salmonella enterica	HEC1B J774 MD Macrophages	Combination of antibodies (CD9,63,81) EC2: GST proteins (CD9,63,151)	(Hassuna, 2010; Ali, 2016).
Streptococcus pneumoniae	HEC1B	Combination of antibodies (CD9,63,81)	(Green *et al*., 2011b).
Staphylococcus aureus	HEC1B HaCaT	EC2: GST proteins (CD9,63,81,151) CD9 EC2-derived peptides (800,810,8001,8005,800-Cap)	(Ventress, 2016) unpublished
Pseudomonas aeruginosa	A549 HaCaT HEC1B	Antibodies(CD9,63,81,151) EC2: GST proteins (CD9,63,81,151) CD9 EC2-derived peptides (800,810,8001,8002,8003,8004, 8005,800-Cap)	Our study

- **DETROIT** is a human epithelial pharynx carcinoma cells line.
- **HEC1B** cells are human endometrial adenocarcinoma cell line.
- **MD macrophages** are monocyte derived macrophages from human peripheral blood.
- **HDMECs** are human dermal microvascular endothelial cells.
- **J774** cell line is a murine macrophage tumour cell line.
- **HaCaT** cells are spontaneously immortalised human keratinocyte cell line.

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Appendix 1

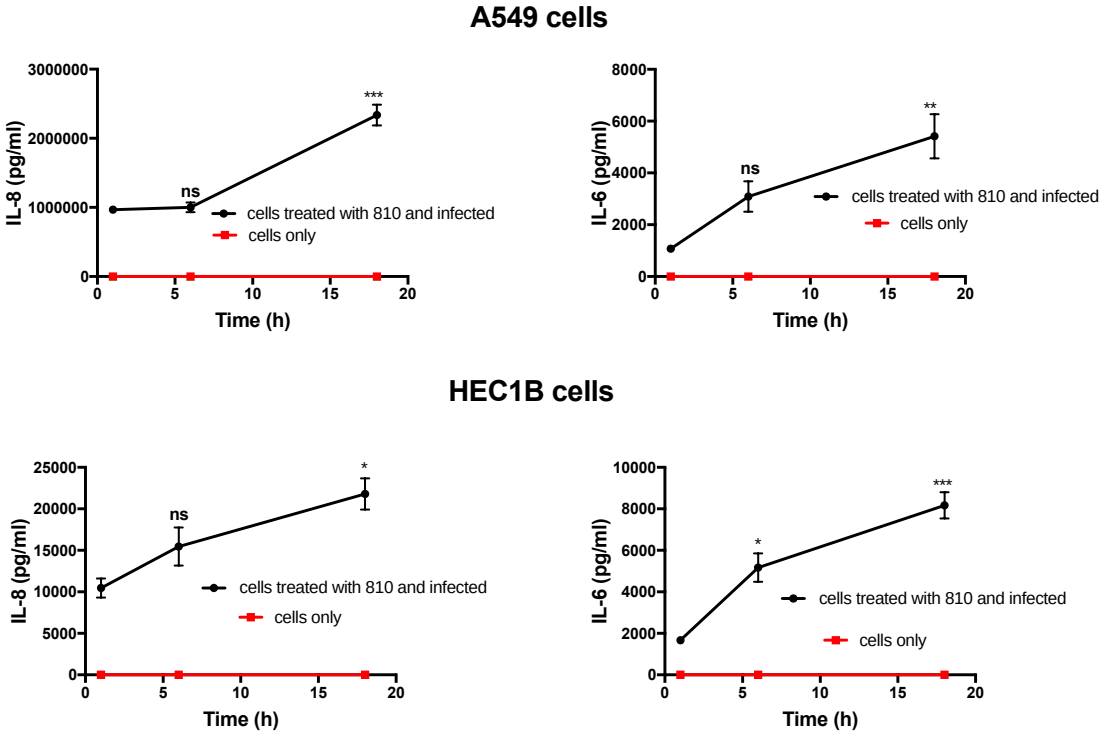

Figure A.1 The optimal time for cytokine release from peptide 810 treated and infected cells.

The supernatants from peptide treated cells that were then infected with GFP-labelled *P. aeruginosa* were collected after 1, 6 and 18 hours of incubation at 37°C, 5 % CO_2 for analysis using CBA. Results are mean ± standard error of the mean (SEM), N= 3. Data were analysed using One-way ANOVA with Sidak's multisple comparisons test (vs. 1 hour incubation), *$P \leq 0.1$, **$P \leq 0.01$, ***$P \leq 0.001$ and ns is non-significant.