The Use of Tetraspanin Based Peptides in the Prevention of *Staphylococcus aureus* Adherence to Human Skin

A Thesis Submitted for the Degree of Doctor of Philosophy

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Declaration

The work presented in this thesis is the work of the candidate, with the following exceptions:

- Cytometric bead arrays were performed by Sue Clark and Julie Swales from the core facility of flow cytometry at the University of Sheffield medical school.
- Injections of zebrafish were carried out by Dr Nelly Wagner and Mr Alex Williams, with assistance from the author.
- Recombinant EC2 domains were synthesized and purified by Dr John Palmer and Dr Marzieh Fanaei.
- FLG silencing was carried out by Miss Christabelle Goh at the IMB in Singapore.
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"Shun the non-believer, shunnnnn"

Charlie the Unicorn, by Jason Steele of Filmcow.com

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Abstract

Staphylococcus aureus is one of the primary causative agents of skin and wound infections. As bacterial adherence is essential for infection, blocking this step can reduce invasion of host tissues by pathogens. An anti-adhesion therapy, based on a host membrane protein family, the tetraspanins, has been developed that can inhibit the adhesion of *S. aureus* to human cells.

In Chapter 3, we show that peptides based on the EC2 domain of tetraspanins reduce the adherence of various strains of *Staphylococcus aureus* to host cells, and that the efficacy of these peptides correlates roughly with the expression levels of CD9 on each cell. We also show that one of these peptides, 800, reduces the quantity of viable adhered bacteria in a 3D model of a *Staphylococcus aureus* wound infection of human skin.

Chapter 4 measures the expression levels of various tetraspanins on cells found in the skin by microscopy and flow cytometry, and explores some of the potential ways that interfering with tetraspanins using this peptide therapy could interfere with normal host function, such as cytokine production, wound healing and cell metabolism. No major effects are seen with the peptides, other than a small negative effect of peptide 800 on migration, which was not observed in the skin model measuring epidermal migration.

Chapter 5 then looks at 2 ways that therapies could be improved for clinical use: combination therapy and drug delivery. Combining peptide 800 with flucloxacillin, an antibiotic only effective against MSSA, increased its efficacy in a cell line model, however, the opposite occurred in the 3D human skin model. Combining the peptide into nanoparticles using Nanocin™ increased the IC_{50} and $t_{1/2}$ of the peptide, however in the skin model anti-adhesive effects were lost.

Abbreviations

MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
DAPI	4,6-Diamidino-2-phenyl-indole Hydrochloride
AMP	Anti-microbial Peptide
AD	Atopic Dermatitis
Atl	Autolysin/Adhesin Protein
BSA	Bovine Serum Albumin
BHI	Brain Heart Infusion
Clf	Clumping Factor
cfu	Colony Forming Units
CA-MRSA	Community Aquired MRSA
CBA	Cytometric Bead Array
CBA	Cytometric Bead Array
DED	De-epidermised Acellular Dermis
DNA	Deoxyribonucleic Acid
DMSO	Dimethyl Sulfoxide
DMEM	Dulbecco's Modified Essential Medium
EMEM	Eagle's Modified Essential Medium
EDTA	Ethylenediaminitetraacetic Acid
EC2	Extracellular Loop 2
ECM	Extracellular Matrix
FnBP	Fibronectin Binding Protein
FnBP	Fibronectin Binding Protein
FCS	Foetal Calf Serum
GST	Glutathione S-transferase
GAG	Glycosaminoglycan
HBSS	Hank's Balanced Salt Solution
HI-FCS	Heat Inactivated Foetal Calf Serum
Hsp	Heat Shock Protein
HA-MRSA	Hospital Aquired MRSA
hBD	Human β-defensin
IMS	Industrial Methylated Spirits
IC50	Inhibitor Concentration
IP	Intellectual Property

IL	Interleukin
LPS	Lipopolysaccharide
LB	Lysogeny Broth
MCP	Macrophage Chemotactic Protein
MMP	Matrix Metalloprotease
MSSA	Methicillin Sensitive *Staphylococcus aureus*
MRSA	Methicillin Resistant *Staphylococcus aureus*
MW	Molecular Weight
MD	Monocyte Derived Macrophages
MOI	Multiplicity of Infection
NHK	Normal Human Keratinocyte
OD	Optical Density
PBP	Penicillin Binding Protein
PBS	Phosphate Buffered Saline
PHMB	Polyhexamethylene Biguanide
PI	Povidone-iodine
rcf	Relative Centrifugal Force
SiRNA	Silencing Ribonucleic acid
AgSD	Silver Sulfadiazine
SSTI	Skin and Soft Tissue Infection
SD	Standard Deviation
SEM	Standard Error of the Mean
TMR	Tetramethylrhodamine
TEM	Tetraspanin-enriched Microdomain
TEskin	Tissue Engineered Skin
WT	Wild Type

Chapter 1: Introduction

The skin is an important component of the innate immune system, and is the first barrier to infection. It protects the body from invasion by pathogens by acting as a semi-permeable barrier, allowing the exit of water and ions but not the entry of larger molecules such as proteins, lipids, or microorganisms. The skin surface is naturally colonised by a range of bacterial flora, including both commensal organisms and potential pathogens, which in standard conditions are not harmful.

Bacterial skin infections occur when the natural barrier is compromised, for instance in wounds such as diabetic ulcers, pressure sores and burns and in patients with psoriasis or other skin conditions. Infections are characterised by an uncontrolled and excessive growth of pathogenic bacteria in or on the skin, and are a widespread problem in healthcare. Infections can be chronic, in which the infection persists and stops the wound from healing; or acute, in which the wound heals rapidly and infection can be cleared easily by the immune system or medication. The most common bacterium isolated from skin and soft tissue infections (SSTIs) is *Staphylococcus aureus,* a gram-positive pathogen commonly found in the nasal epithelium and on skin as a commensal organism. Between April 2014 and March 2015 there were 9,827 reported cases of methicillin sensitive *Staphylococcus aureus* (MSSA) infection and 801 reported cases of methicillin resistant *Staphylococcus aureus* (MRSA) in the UK alone, amounting to a large financial burden to healthcare. *S. aureus* infections are especially prevalent individuals >85 years of age, due to an increased healing time and reduced immune efficiency in these patients (Wicke et al., 2009), and fast and efficient treatment with anti-microbial agents is an important step towards reducing the severity of these infections.

Wound infections are frequently treated with antibiotics which aim to directly kill any bacteria present, however, due to the rise in resistance to these drugs encouraged partially by incorrectly administered systemic treatment, alternate drugs are being increasingly frequently used (Alanis, 2005). Modern antimicrobial components of wound dressings include silver sulfadiazine, manuka honey and polyhexamethylene biguanide (PHMB), and antiseptics such as sodium chloride and chlorhexidine (Howell-Jones et al., 2005). These therapies have the capacity to reduce bacterial burden, however they are not as fast and effective as

antibiotic therapy and some, such as silver, can be expensive as a long-term treatment of chronic infections.

One new target of anti-microbial research is host adhesion, aimed at preventing the initial attachment stage of bacterial colonisation and which should therefore reduce the likelihood of infection. Although in its infancy, this field shows potential in the prevention of bacterial infections, with a more limited chance of resistance developing due to the lack of direct selective pressure on the pathogens.

Here we investigate one potential anti-adherence therapy and its possible application for the treatment of wound infections.

1.1 Innate Immunity as a Barrier

The skin is a key component of the innate immune system and is a complex, multi-component organ capable of mounting a full immune response. It can secrete anti-microbial peptides, modulate surface pH and secrete immune recruitment factors such as cytokines and chemokines in response to wounding and the presence of invading bacteria (Ong et al., 2002).

1.1.1 Skin Structure

Mammalian skin is a multi-layered structure, and consists of an epidermis, a dermis, and a subcutaneous layer which together can form an impenetrable barrier to micro-organisms. The skin consists of many cell types and distinct structures as shown in Figure 1.1 (Haake et al., 2001, MacNeil, 2007). The epidermis is the upper-most layer of the skin and is formed primarily of keratinocytes, alongside pigment forming melanocytes, Langerhans cells and nerve receptors. The epidermis is composed of layers (Fig 1.1B). Keratinocytes start as undifferentiated proliferated cells at the epidermal-dermal junction, gradually differentiating and stratifying as they move up through the layers towards the surface of the skin. Each epidermal layer has its own characteristics. At the base of the epidermis is the Stratum Basale, which consists of actively dividing cells and pigment-producing cells. Above this is the Stratum Spinosum, which is a thick layer of partially differentiated cells with oval nuclei and a spiny appearance caused by keratin build-up. The Stratum Granulosum above this is the layer in which keratinocytes begin to lose their nuclei and flatten to form a hard envelope. Finally, the keratinocytes die and form the Stratum Corneum, the uppermost layer of the epidermis

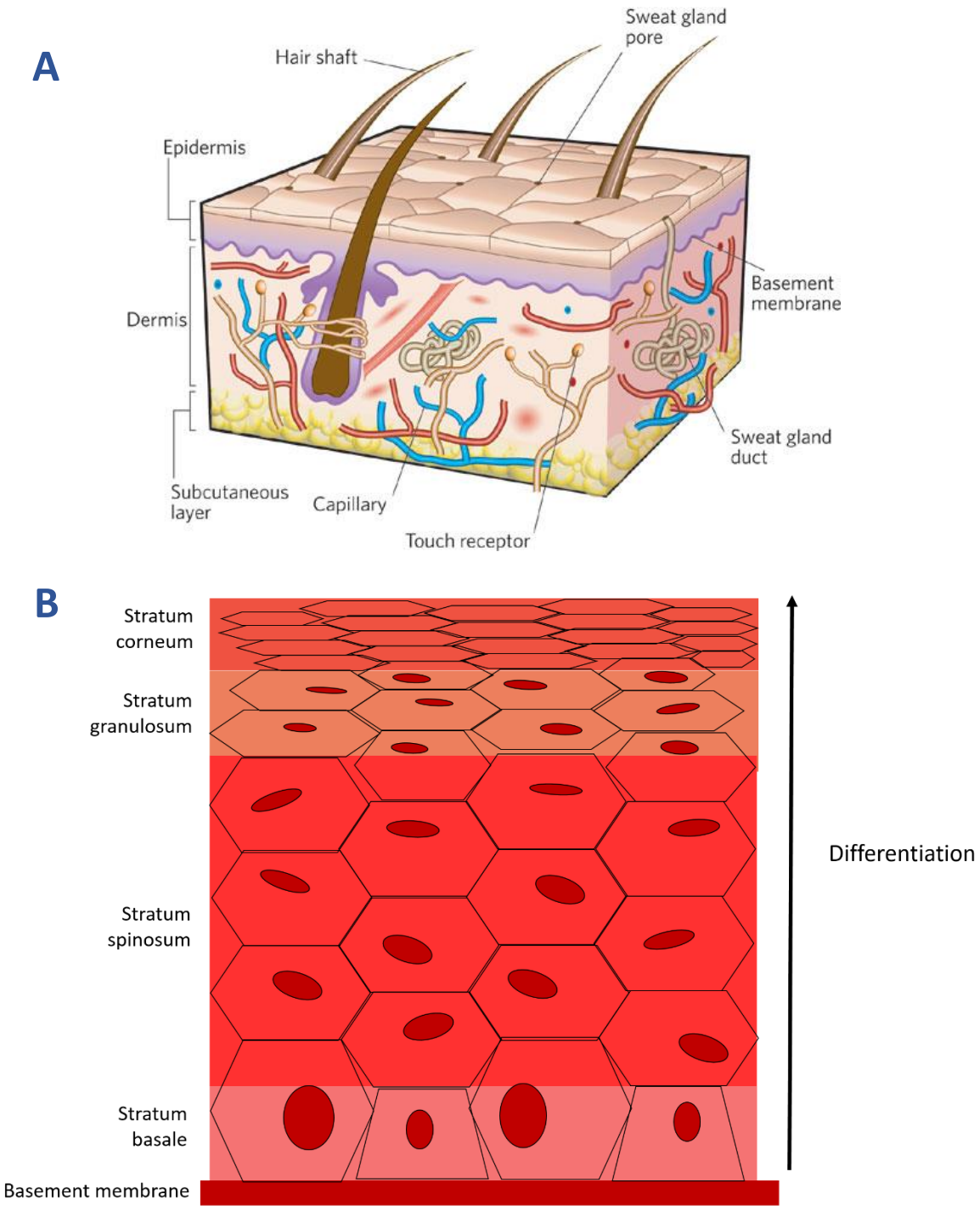


Figure 1.1: Structure of Human Skin: (A) Human skin is composed of a keratinocyte dense epidermis, connected by a basement membrane to a dermis containing immune cells, fibroblasts and structures such as sweat glands and hair follicles. Reproduced with permission from MacNeil et al (MacNeil, 2007). (B) Immediately above the basement membrane, are actively replicating cells. As newer cells are made, older cells are pushed up through the layers of skin as they differentiate and age. As they reach the upper surface of the skin cells fully differentiate, lose their nuclei and die, forming the desiccated stratum corneum which acts as the first barrier to infection.

(Haake et al., 2001). This layer is composed entirely of dead cells lacking nuclei with hard protein envelopes and forms an effective barrier against pathogens. Due to a lipid build-up and the close cell junctions, this layer is waterproof and impermeable to pathogens (Ong et al., 2002, Winsor and Burch, 1944, Madison, 2003). The dehydrated nature of the stratum corneum also discourages growth of some moisture-requiring microbial species on its surface, and a rapid turnover of dead keratinocytes reduces the time available for the establishment of infection by surface pathogens (Roth and James, 1988).

Below the epidermis, separated by the epidermal:dermal junction, is the dermis. The dermis is mostly connective tissue and acts as a protective layer for internal structures. It can be divided into 2 layers, the papillary region and the reticular dermis. The papillary region is located adjacent to the epidermis and is primarily formed of fibroblasts secreting extracellular matrix (ECM) proteins, including collagen, elastin and various glycoproteins (Bosman and Stamenkovic, 2003). This layer also contains capillaries and sensory structures for heat and pressure. The reticular dermis is a deeper layer formed of collagen, elastin and reticular fibres. This layer contains hair follicles, sweat glands, nail beds and cutaneous receptors. The main cell types in the dermis are fibroblasts (secreting ECM), adipocytes (secreting lipid) and macrophages (primarily for immune defence) (Menon, 2002).

The surface of the stratum corneum also has an acidic pH, first observed by Heuss et al in 1892 and quantified by Schade and Marchionini in 1928 (Schade and Marchionini, 1928), which conveys an extra line of defence against invading pathogens. Low pH is also required for lipid organisation and metabolism in the stratum corneum, and has functions in surface permeability (Schmid-Wendtner and Korting, 2006). A change in skin pH is observed in conditions involving a compromised skin barrier such as atopic dermatitis and psoriasis (Schneider et al., 2005).

1.1.2 Skin Defence Molecules

Keratinocytes, the primary cell type found in the epidermis, have been shown to secrete molecules that chemically protect the skin from microbial colonisation. These include lysozyme, secretory leukoprotease inhibitor or antileukoprotease, RNase 7, elafin, and dermcidin (Glaser et al., 2005) as well as small 20-46 amino acid peptides known as anti-microbial peptides which can cause the lysis of a range of resident microorganism. The

expression of members of these protein groups has been shown to increase in chronic wounds as a defence against continuing infection (Kesting et al., 2010).

Most anti-microbial peptides (AMPs) have a net positive charge and contain many basic residues (Zanetti et al., 2000). They bind to negatively charged components on the bacterial cell membrane, including lipopolysaccharides, polysaccharides, phospholipids and teichoic acids. Human β-defensins and cathelicidins (2 types of AMP) can also induce the production of cytokines such as interleukin 8 (IL-8), which further activate the immune system (Schneider et al., 2005).

Attempts at modifying of these host defence peptides to create an artificial barrier to infection are currently being conducted (Kesting et al., 2010). One example of this is the development of short hydrophobic peptides based on the IG-19 region of human LL-37, synthesised by Nan et al which show prokaryotic selection and LPS neutralising activity when applied to bacterial cultures (Nan et al., 2012).

1.1.3 Immune Response

Within the skin and underlying tissues there is a large range of active defence mechanisms that can act against invading pathogens. The first immune skin response upon wounding is the secretion of AMPs, matrix metalloproteases (MMPs) and cytokines such as IL-1β, IL-6, IL-18 and TNF by keratinocytes. These cytokines are largely pro-inflammatory, inducing an increase in blood flow to the wounded area and an influx of immune cells.

The first immune cells to respond to wounding and infection are Langerhans cells: dendritic cells which have been shown to have a range of functions in human skin. These innate cells can phagocytose pathogens and present antigen, and contribute further to the immune response by further secreting cytokines and chemokines, for example by secreting IL-15 for the recruitment of CD8+ T Cells (Klechevsky et al., 2008). Langerhans cells have also been shown to upregulate the production and activity of T helper cells, thus suggesting a role for them in tissue homeostasis (Seneschal et al., 2012). Cytokines and chemokines secreted by dendritic cells, keratinocytes and fibroblasts generate a chemotactic protein gradient to attract further immune cells such as neutrophils, T cells, monocytes and macrophages (Pasparakis et al., 2014).

Mast cells in the skin are one of the fastest responders to signals of a new threat. These cells possess specialised cytoplasmic compartments which contain pre-made pro-inflammatory cytokines and chemokines, that can be released rapidly upon the detection of antigens. This leads to the recruitment of natural killer cells to the site of infection, which clear invading pathogens via a range of killing mechanisms (St John et al., 2011).

Macrophages and monocytes also play a clear role in the initial response to skin wounding and infection. Circulating monocytes and resident macrophages both patrol the skin for antigens, and transport any antigens to lymph nodes for further immune activation (Jakubzick et al., 2013). Macrophages can be split into 3 categories based on their activation and function: pro-inflammatory M1, regulatory M2, and wound-healing macrophages which are activated at various stages of the wounding process (Mosser and Edwards, 2008).

Finally, initiation of a T cell response and then B cell defence leads to a longer lasting immunity. 2 times more T cells reside in the skin than are found in general circulation, and they have been shown to be sufficient to generate an immune response even in the absence of alternate lymphocytes (Clark et al., 2006). Figure 1.2, from a review by Nestle et al, shows the more complex picture of interactions within the immune response to wounding and infection (Nestle et al., 2009).

1.1.4 Wound Healing

Another way in which the skin protects wounds from infection is the process of wound healing. This is a complex process consisting of 3 main phases and involving many cell types secreting a large range of cytokines, chemokines and other signaling molecules (Werner and Grose, 2003). The first stage of wound healing, inflammation, is an immediate response to trauma: platelets clot to prevent bleeding, blood vessels deliver immune cells, nutrients and enzymes to the site of the trauma, and inflammation occurs. The second stage, proliferation, is when new granulation tissue is generated and new ECM proteins are synthesized and deposited in the wound by fibroblasts. Angiogenesis also occurs during the proliferation

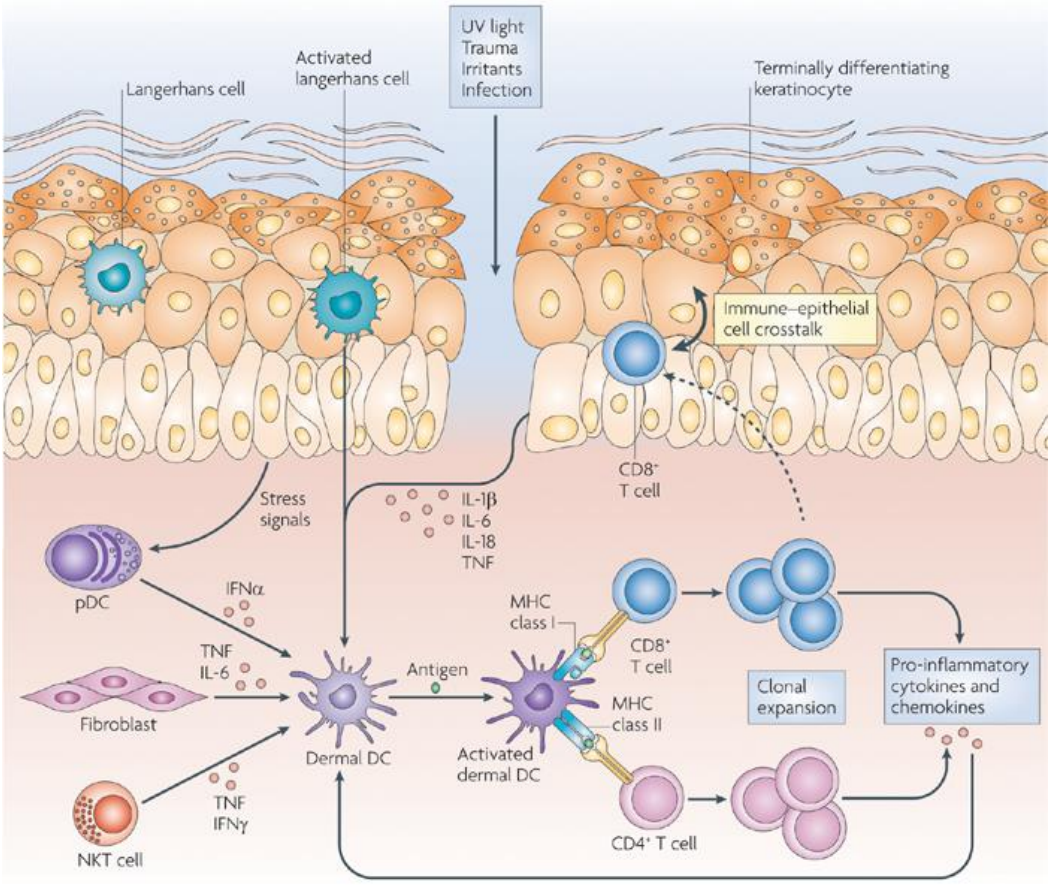


Figure 1.2: The Skin's Immune Response to Wounding and Infection: A trauma to the skin such as a wound, burn or infection instigates an immune response involving a plethora of cells and signalling molecules. Langerhans cells and keratinocytes are responsible for the first activation of the immune system, and secrete a range of cytokines such as IL-1β, IL-6 and IL-18. Fibroblasts in the dermis also secrete TNF and IL-6 and contribute to the inflammatory response. Dendritic cell, NK cells and T cells are all then recruited to the site of damage and inflammation is instigated. Reproduced with permission from Nestle et al (Nestle et al., 2009).

stage, creating new blood vessels within the wounded area. The third and final stage of wound healing is the matrix remodeling stage. In this stage, keratinocytes proliferate and migrate laterally to form a new epidermis upon the freshly placed collagen and ECM. The deeper dermal tissue is also gradually remodeled over a period of years to replace non-functional cells and weak connective tissue, reducing scarring (Barrientos et al., 2008).

Chronic wounds are wounds that are deficient in some aspects of the healing process, making wound closure a much slower process. One interesting hypothesis suggests that chronic non-healing wounds are caused by inefficient clearing of opportunistic pathogenic bacteria from the wound (Bjarnsholt et al., 2008).

1.1.5 Microbiome

The skin is a habitat for various commensal organisms, including many potentially pathogenic species of bacteria (Grice et al., 2009). The skin encompasses many different niches; from very dry, cold areas such as forearms to warm, moist areas such as underarms; and a different range of bacteria thrive in each area. The exact composition of the microbiome varies between individuals and can have a large effect on potential personalised treatments (ElRakaiby et al., 2014). In 2012 a role for skin microbiota in immune function was outlined by Naik et al. This research suggested that commensal organisms in mice can aid in immune function at the skin surface by increasing Interleukin-1 signalling and thus promoting the activity of T effector cells (Naik et al., 2012). This has not yet been confirmed in other organisms however it suggests that much is still not known about the interplay between commensal organisms and the skin, and treatments that effect the microbiota non-specifically could have dangerous effects on natural skin immunity.

1.2 Skin Pathogens

Some members of the microbiota of skin are opportunistic pathogens, and after a break in the skin barrier can penetrate into the tissue and cause infection. Common causes of skin and soft tissue infections are *Staphylococcus aureus, Pseudomonas aeruginosa, Staphylococcus epidermidis* and *Streptococcus pyogenes,* and often it is a combination of more than one of these pathogens that cause clinical conditions. In the wound setting, bacteria can act to cause

an infection individually, in their planktonic form or by the formation of a biofilm, which grants protection to the pathogens and causes harder to treat infections (Figure 1.3).

1.2.1 Staphylococcus aureus

Staphylococcus aureus is a leading cause of skin and wound infections in the developed world. It is a Gram positive coccus and commonly colonises the skin, respiratory tract and nasal epithelium. Diseases for which it is the causative agent including cellulitis, folliculitis, furuncles and impetigo (Tognetti et al., 2012). According to a recent NHS survey, there were 9,827 total cases of Methicillin Sensitive *Staphylococcus aureus* (MSSA) bacteraemias reported by the NHS in England between 1 April 2014 and 31 March 2015, and SSTIs accounted for roughly one-fifth of these MSSA infections. In that year, 92.5% of all *S. aureus* infections were sensitive to antibiotics, however 7.5% were resistant to one or more treatments (Gerver et al., 2015, Acute Trust et al., 2015). Like most bacterial skin pathogens, *S. aureus* gains access to target tissues via a break in the epidermis and once established in the skin, *S. aureus* causes noticeable symptoms associated with skin and soft tissue infections, including dryness, itchiness, and boils (NHS, 2011). It has a large range of adhesins and can adhere to fibronectin moieties on target epithelial cells via fnBP (Arrecubieta et al., 2008), and this adherence is enough to induce the internalisation of the bacterium by the host cell (Sinha et al., 2000). *Staphylococcus aureus* can also penetrate deeper into tissues and enter the blood stream, from where it can pass into different areas of the body. These systemic infections can often produce high mortality rates by multiple organ failure (Blot et al., 2002).

1.2.2 MRSA

Methicillin resistant *Staphylococcus aureus* (MRSA) is a staphylococcal infection characterised by a resistance to β-lactam antibiotics such as methicillin, oxacillin and cephroxadin. In 98% of environmental isolates, MRSA was found to also be resistant to other classes of antibiotics

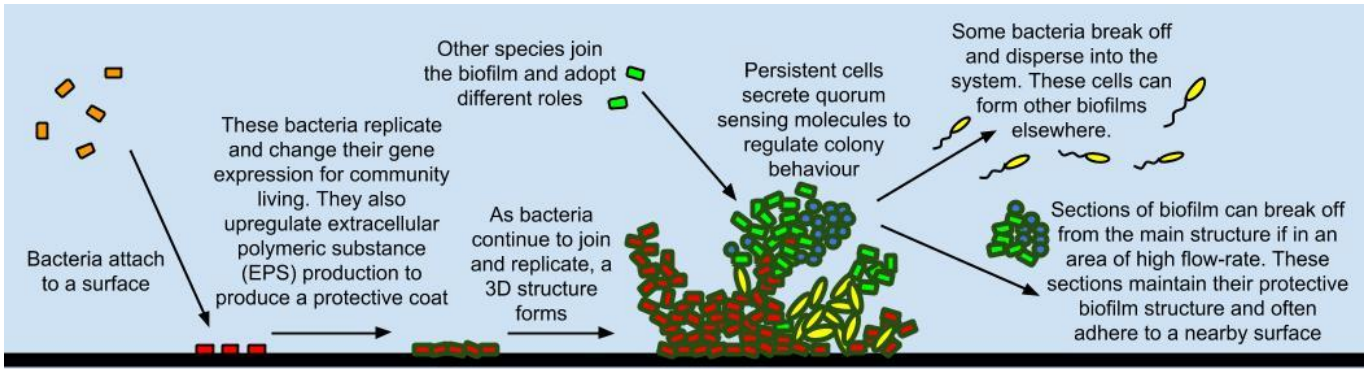


Figure 1.3: Biofilm Formation: Once a small number of bacteria are adhered to a surface, such as a wound or implanted medical device, they can replicate to form a biofilm. To do this, the bacteria first form a protective coating around themselves and change their gene expression drastically. Once established in this way, they can proliferate further and form a 3D colony. At this stage, other bacteria can adhere to and proliferate within the biofilm, and will fulfil different roles to live in symbiosis within the biofilm. Once established, a full biofilm is very difficult to treat, and constant bacterial shedding make secondary infections more likely.

(Roberts et al., 2013). MRSA can be categorised into hospital acquired (HA-MRSA) and community acquired (CA-MRSA) depending on the source of infection. It employs multiple mechanisms to convey resistance to antibiotics as outlined in section 1.4.2, such as mecA, a broad spectrum penicillin binding protein (PBP) which prevents the effects of penicillin and other β-lactam antibiotics within the cell by cleaving them into harmless products (Tong et al., 2012, Chambers, 1997).

Between April 2014 and March 2015 there were 801 reported cases of MRSA in the UK alone. The highest rates of infection are among those ≥85 years in age and in general a higher proportion of men exhibit the infections than women. These statistics are not limited to skin and soft tissue infections and include Staphylococcal medical device infections and pneumonia infections. The mortality rate associated with MRSA infections is almost three times higher than that of MSSA (Blot et al., 2002). This is because it is harder to diagnose, and doesn't respond to normal antibiotic treatment, allowing the infection more time to become firmly established. Resistant infections such as MRSA are causing significant problems for worldwide healthcare. Although 92.5% of infections identified in hospitals are still susceptible to non-lactam based antibiotics, resistance is still developing, and typical antibiotic treatment is becoming progressively less effective (O'Meara et al., 2000). Additionally, in recent decades, *Staphylococcus aureus* has developed resistance to non-lactam antibiotics such as vancomycin (Sieradzki et al., 1999) and ciprofloxacin (Blumberg et al., 1991), and therefore the need for new alternative treatments in this area is therefore becoming more pressing (Tong et al., 2012).

1.2.3 Other Common Bacterial Pathogens

1.2.3.1 Pseudomonas aeruginosa

Pseudomonas aeruginosa is an opportunistic infection which causes sepsis, inflammation, haemorrhaging and necrosis. After *S. aureus, P. aeruginosa* is the next most prevalent infectious agent in leg ulcers (Hansson et al., 1995), and is also commonly found in burn victims. It is also one of the principle bacteria found in wound biofilms (Dowd et al., 2008). This infection causes repetitive problems with hospital patients as it flourishes on medical equipment such as catheters and heart valves. *P. aeruginosa* adheres to target cells via a

series of adhesins including the lectins: LecA and LecB (Sato and Okinaga, 1987, Chemani et al., 2009).

1.2.3.2 Staphylococcus epidermidis

Staphylococcus epidermidis are Gram positive cocci which normally exists as a non-pathogenic commensal organism of the skin. In immunocompromised patients however, such as those with chronic wounds, it can cause serious infections. As with *P. aeruginosa* it is able to form biofilms on polymeric surfaces which leads to a wide range of medical device related infections, including infections of catheters and heart valves. Its adherence factors include capsular polysaccharide adhesins that form part of the slime component of biofilms (Knobloch et al., 2002, Ofek et al., 2003).

1.2.3.3 Streptococcus pyogenes

Streptococcus pyogenes is a less prevalent causative agent of wound and soft tissue infection. This species possesses a number of surface proteins that allow it to adhere to target epithelial cells, for example, Protein M on the streptococcal surface binds to GAGs (glycosaminoglycans) of the epithelium (Pancholi and Fischetti, 1989). Lipoteichoic acid (LTA) can also bind to fibronectin in the ECM (Beachey, 1981), and from there the bacteria can also bind albumin at the cell membrane for a tighter association (Egesten et al., 2011). *S. pyogenes* has been isolated from patients with SSTIs, otitis media, bronchitis, sinusitis, meningitis, and pneumonia (Nobbs et al., 2009).

1.3 Entry Pathways for Infection

As noted previously, colonisation often occurs after stress or trauma to the skin leading to a compromised barrier and adherence has been shown to be a key factor in this colonisation (Beachey, 1981). This damage is often in the form of a burn, a wound or a bite, or through a condition causing a weakened barrier such as atopic dermatitis or psoriasis. When the tissue is damaged, bacteria can easily adhere to exposed matrix factors and cellular receptors which would normally be concealed.

The ability of bacteria to adhere to cells via specific receptor interactions allows them to target favourable environments in a host and thrive, and after adherence bacteria can flourish extracellularly as a bio-film or induce internalisation by various methods. Once internalized,

some species of bacteria can pass from cell to cell and potentially into the blood stream where an infection can be transported to other sites around the body, resulting in systemic infections (bacteraemia, sepsis) (Cozens and Read, 2012).

The mechanism of adherence employed by bacteria is species-specific, however common mechanisms exist across species, as outlined below. Pathogens will often employ multiple mechanisms of adherence in order to establish tight docking onto a cell.

1.3.1 Hydrophobic Attachment

The initial mode of attachment used by most bacteria is a weak hydrophobic connection. Bacteria and cells both have an overall negative charge at physiological pH, which causes repulsion, one of the first barriers to adherence. To overcome this, some organisms will present positively charged or hydrophobic molecules on their surface, thus reducing the repulsive force. This allows for a weak association to form between bacteria and cell, which facilitates the formation of a stronger bond necessary to compete with other resident commensal organisms and to avoid host clearance (Cozens and Read, 2012, Absolom, 1988). It is worth noting that catheters, heart valves and other medical devices are frequently manufactured from hydrophobic materials, making it relatively simple for bacteria to adhere to them (Krasowska and Sigler, 2014).

1.3.2 Adhesins

A stronger connection between pathogen and host can be formed by attachment to host cell surface structures or to extracellular matrix. Direct interactions between bacterial adhesion molecules (adhesins) and host cell factors such as integrins, cadherins and immunoglobulin-superfamily cell adhesion molecules is the primary method of attachment. Some bacteria however do not bind directly to the host cell, rather they use adhesins to attach to extracellular matrix (ECM) proteins such as collagen or fibronectin.

Bacterial adhesins can be polysaccharide or protein based, and are generally species specific. Examples of adhesins include the protein subunit FimH of the pili, which facilitates the adhesion of uropathogenic *Escherichia coli* to the tetraspanin protein Uroplakin 1a on the cells of the urinary tract (Martinez et al., 2000, Zhou et al., 2001), and Opc (Opacity protein C)

which contributes to the adherence of *Neisseria meningitidis* to proteoglycan receptors on epithelial cells (de Vries et al., 1998).

1.3.2.1 Staphylococcus aureus Adhesins

Staphylococcus aureus has a diverse range of adhesins (Figure 1.4). This includes MSCRAMMs (microbial surface components recognising adhesive matrix molecules) such as fibronectin binding proteins (FnBPs) A and B which attach to fibronectin from the extracellular matrix and through a β-zipper mechanism, allowing connections to the cell through β1 integrins (Schwarz-Linek et al., 2003). FnBPs also bind to fibrinogen and to Heat shock protein 60 (hsp60) (Dziewanowska et al., 2000). A surface clumping factor, ClfA, of *S. aureus* has also been shown to bind to fibronectin and fibrinogen in the stratum corneum (Cho et al., 2001, McDevitt et al., 1994), and the autolysin/adhesin protein Atl interacts with Hsc70 expressed on the cell membrane (Hirschhausen et al., 2010). Additional adhesins include but are not limited to: IsdB, Extracellular Adherence Protein (Eap), Sle1, and extracellular matrix protein-binding protein (Emp) (Foster and Hook, 1998). Many of these attachment mechanisms are also exploited to induce internalization of the *S. aureus*, further demonstrating the link between adherence and infection.

1.4: Treatment of Wound Infections

Various studies have been undertaken in an attempt to determine the financial burden of current wound management in hospitals (Posnett et al., 2009). Figures are inconclusive due to the likelihood of multiple complications in skin infections, however average figures have been published which suggest treating general wound and infections costs in the area of 2-4% of the national healthcare budget and that treating antibiotic resistant infections (such as MRSA) costs the United Kingdom in excess of £1 billion each year. Finding ways in which to treat these wounds more effectively, and prevent the onset of chronic infections, is therefore an important goal financially, as well as for the improvement of mortality rates and healthcare (Gottrup et al., 2009, Posnett et al., 2009).

1.4.1 Drugs and Antibiotics

Systemic drug delivery is commonly used for the treatment of wound infections, in which antibiotics are taken orally or intravenously. Vancomycin has been used extensively as a

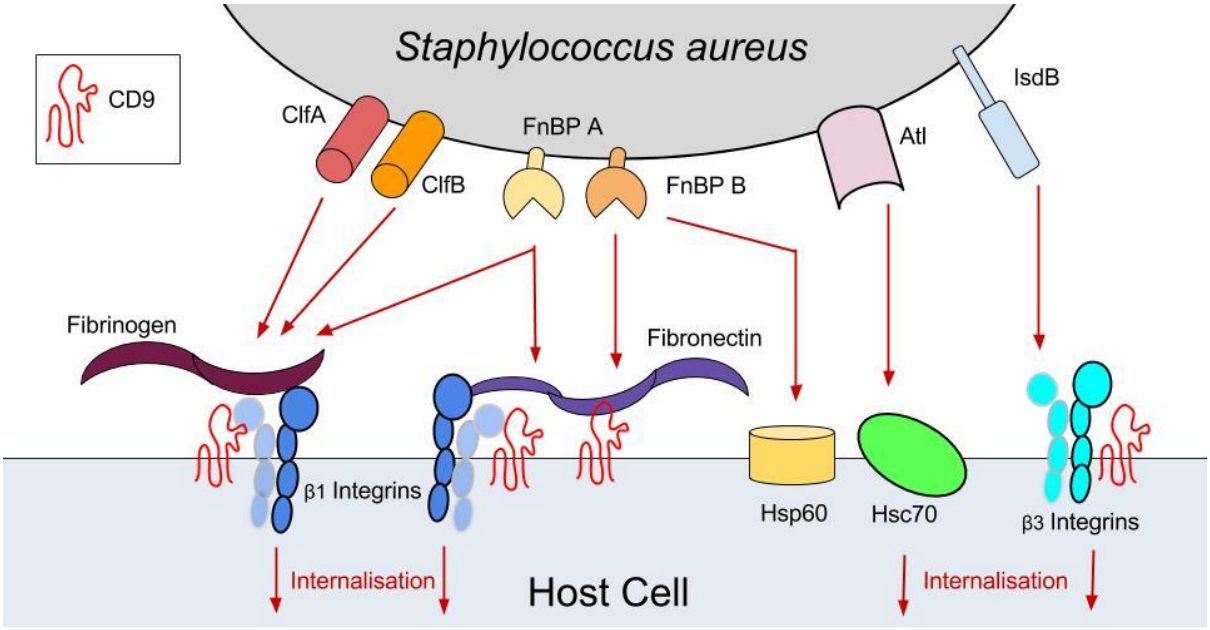


Figure 1.4: Adherence of *Staphylococcus aureus*: *Staphylococcus aureus* has a wide range of adhesins for binding to host cells including ClfA/B, FnBP A/B, Atl and IsdB amongst others. These help the bacteria bind to the cell surface, to β3 integrins, Hsp60 and Hsc70, or to the ECM, to fibronectin or fibrinogen. The tetraspanin CD9 is known to be a binding partner to many of the host receptors.

systemic treatment against MRSA infection; however, as with many other antibiotics, strains of MRSA have been identified which are also resistant to vancomycin, with up to 74% of soft tissue infection isolates having this resistance. Recent guidelines by the Infectious Disease Society of America recommend clindamycin, trimethoprim-sulfamethoxazole, tetracycline (minocycline or doxycycline) or linezolid for the treatment of suspected community acquired MRSA infected wounds (Stevens et al., 2014). Antibiotics generally inhibit essential processes found in bacterial metabolism, such as cell wall synthesis and type II fatty acid synthesis. Systemic delivery of these drugs however can also have wider unwanted systemic effects, and does not always lead to optimal concentrations in the wound, leading to failure in treating the infection (Zilberman and Elsner, 2008).

1.4.2 Antimicrobial Resistance

Recent decades have seen the emergence of antibiotic resistance as an ever-changing challenge for global healthcare. Several 'super bugs' have arisen which threaten the efficacy of antibiotic treatment and have led to the need for alternate treatments. This resistance has emerged due in part to the misuse of antibiotics (Alanis, 2005). Finishing a course of antibiotic treatment early or using levels that are too low creates selective pressure on the bacterial population. This will often be sufficient to kill sensitive strains, however strains with a mutation that conveys resistance or an enhanced potential for survival will be less effected. In this situation, competing strains have been removed and a population bottleneck occurs, allowing the resistant strain to flourish (Davies and Davies, 2010, Levy and Marshall, 2004). Resistance mechanisms can also be transferred between bacteria and potentially species by lateral gene transfer by conjugation (Arai, 1967).

Additionally, antibiotic resistance is known to arise in livestock and in water bodies such as lakes, rivers and waste water effluents due to selective pressure exerted by drug contamination in the water and the increased occurrence of genetic exchange (Lupo et al., 2012). Multiple mechanisms of resistance exist in the population and are too numerous to list here. Some common examples include β-lactamases which act by breaking down β-lactam antibiotics, and multidrug efflux system which can pump multiple types of antibiotics out of the bacterial cytoplasm (Levy and Marshall, 2004, Davies and Davies, 2010). Additionally,

some pathogenic bacteria can form biofilms which provide protection from antimicrobials (Stewart and Costerton, 2001).

MRSA exhibits a large number of virulence genes which convey antibiotic resistance. The first appearance of MRSA was in the 1940s, with a strain which produced penicillinase which cleaves and inactivates the essential β-lactam ring of penicillin (Kirby, 1944). The second resistant strain identified contained a low affinity penicillin binding protein (PBP 2a) coded by the *mecA* gene conveying resistance to a broad range of methicillin-like antibiotics (Chambers and DeLeo, 2009, Kirby, 1944). Another mechanism of resistance in MRSA is known as the Erm mechanism, conferring resistance to erythromycins in which the Erm methyl transferase enzyme methylates part of the 23S RNA subunit of the ribosome, allowing protein synthesis but preventing the binding of erythromycins (Westh et al., 1995).

Due to the constant increase in the occurrence of resistance, other categories of drugs are required to potentially target other aspects of bacterial virulence, such as adherence, toxin production or biofilm formation (Kollipara et al., 2014).

1.4.3 Combined treatments

Combination therapy as a method to combat bacterial resistance is the treatment of infections with 2 or more compounds with different modes of action, for example a fluoroquinolone with a macrolide, or a β-lactam with an aminoglycoside or tetracycline. This has shown some success in the treatment of resistant infections, for example the combination of levofloxacin and rifampicin, or daptomycin and rifampicin, in the treatment of MSSA infection (El Haj et al., 2015), however more detailed clinical studies regarding the success and consequences of these combinatory treatments need to be undertaken, and more detailed information of the speed of drug delivery and wound concentration need to be sought (Davies and Davies, 2010)

1.4.4 Wound Dressings

The main requirement of a therapeutic antimicrobial agent is that it prevents infection of human cells without negatively affecting cell or tissue function. Therefore, most commercially available wound dressings have antibacterial and antifungal activity. Most modern wound dressings are now designed as a bilayer, with the layer adjacent to the wound designed to

absorb fluid in the wound, and the upper layer designed to stop bacterial penetration into the wound. Newer versions of these also secrete anti-microbials into the wound (Zilberman and Elsner, 2008) and can aid in wound healing (Mori et al., 2014, Han et al., 2012).

Modern antimicrobial components of wound dressings include antibiotics, silver sulfadiazine, Manuka honey and polyhexamethylene biguanide (PHMB), and antiseptics such as sodium chloride and chlorhexidine (Howell-Jones et al., 2005). These therapies are capable of reducing bacterial burden, however some such as silver can be expensive as a long-term treatment of chronic infections, and in the case of silver sulfadiazine can also be toxic to host cells (AshaRani et al., 2009). Resistance to these treatments has also been observed, such as a resistance to sulfadiazine silver identified in *Pseudomonas aeruginosa* (Modak and Fox, 1981).

Wound dressings and topical treatments can also contain a drug delivery material which helps maintain the moist environment conducive to wound healing and also delivers the antibiotics and antiseptics mentioned above into the wound slowly, over a period of hours or days without degradation (Zilberman and Elsner, 2008). These platforms differ in composition based on the type of wound and infection and can take on many forms including foams, hydrofibers® (Aquacel) and hydrocolloids. One common type are based on dehydrated wafers; freeze dried dressings which rehydrate and swell upon application to the wound, becoming viscous gels. They can be synthesized from sodium alginate, xanthan gum, methylcellulose and other emulsifiers, and allow for the steady release of drugs solubilized within the wafer (Matthews et al., 2005) Collagen is also frequently used as a dressing. This has the disadvantage of being broken down rapidly by wound enzymes, however this can also work in its favour as it allows for steady release of drug from the collagen matrix (Radu et al., 2002). Collagen is also one of the main components of the ECM and therefore many biological dressings have been generated using this as a base, incorporating keratinocytes and fibroblasts to secrete molecules useful for would healing (Pruitt and Levine, 1984).

A new type of wound dressing has also been developed which can release a drug in response to the presence of the bacteria. One example of this is the gentamycin polyvinyl acetate hydrogel, in which the gentamycin is bound to the hydrogel by a peptide linker which can be cleaved by a proteinase secreted by *Pseudomonas aeruginosa* (Suzuki et al., 1998).

1.4.5 Anti-adherence Therapies

Anti-adhesion therapies are a relatively new area of research focus which are becoming increasingly popular with the rise in antibiotic resistance, as they exert a weaker selective pressure on bacteria and are therefore less likely to cause resistance to develop. They reduce adherence of bacteria, and thus reduce infection and can be host-targeted or pathogen-targeted (Cozens and Read, 2012).There are a variety of mechanisms by which they can work, including: changing host receptor expression, interfering with host receptor function, directly blocking host-bacteria interactions, coating the host with non-adhesive molecules, and blocking bacterial adhesins (Krachler and Orth, 2013).

There are currently a number of anti-adhesion therapies being developed to treat bacterial skin and soft tissue infections (Krachler and Orth, 2013, Cozens and Read, 2012). One example in development are the proanthocyanidins, extracted from cranberry juice, which are being developed for the treatment of urinary tract infections (Shmuely et al., 2012). These molecules have also been shown to inhibit the binding of *Helicobacter pylori* (Burger et al., 2000) and *Porphyromonas gingivalis* (Labrecque et al., 2006), making them a potentially wide spectrum treatment. Alternatively, biphenyl mannosides are known antagonists of FimH, a major binding factor of *E.coli*, first identified over 40 years ago (Hartmann et al., 2012). These saccharides have been used to successfully inhibit the adhesion of *E.coli* to host cells (Hartmann et al., 2012).

Peptide based adhesion inhibitors are much rarer, with issues frequently arising during early stages of their development in downstream toxicity, specifically alterations in host cell signalling and metabolism (Krachler and Orth, 2013), and degradation in the body. Peptides that mimic host receptors and those that competitively inhibit bacterial adhesins can easily activate signalling pathways downstream and cause unwanted negative side effects in the host. Despite this, there are a number of peptide based anti-adhesion molecules in various stages of development. For example, p1025, developed by Kelly et al, is a peptide designed to prevent dental cavities by inhibiting the adherence of *Streptococcus mutans.* It acts by preventing the binding of surface protein streptococcal antigen (SA) I/II to hydroxylapatite on the tooth surface (a naturally occurring mineral found in bones and teeth) and has shown success at preventing the re-colonisation of teeth by *S. mutans in vivo* (Munro et al., 1993, Kelly et al., 1999). Another series of peptides in development are MAM-7 based inhibitors,

which have been shown to inhibit the adherence of *Escherichia coli*, *Yersinia pseudotuberculosis*, *Vibrio cholerae*, and *Vibrio parahaemolyticu*s to host cells (Krachler and Orth, 2011, Krachler et al., 2011) and show promise as an anti-adhesion therapy.

1.5 Drug Discovery

The route to the discovery of a potential new therapeutic and the development necessary to prepare it for use in the clinic is a long one, full of potential pitfalls. Most molecules that show promise as anti-microbials often have unwanted side effects in the host. Problems also arise in how the drugs are distributed in the body and their retention in a system. The final pitfall encountered is often the expense. On average it costs £1.15 billion to get a drug to market in the UK (Industry, 2016). This funding is almost exclusively available to industrial companies and not in an academic setting, which affects which drugs are prioritised for development. Below is a basic outline of the main steps of getting a potential therapeutic from the initial discovery in the lab (by library screening, structural studies, computer based design or serendipity) and into the clinic.

1.5.1 Cell Lines

Once identified, therapeutics are then tested on cell line models to give an idea of toxicity, dosage and effectiveness. Frequently, immortalized cell lines are used for this as they are reproducible and simple to test, however it is known that through repeated culture they can pick up small mutations that can make them drastically different to the target cells. Primary cells isolated from donors give a more accurate model of human response, however these are harder to obtain and cells from different donors behave differently, leading to greater variance in the data (Pastor et al., 2010, De Saint Jean et al., 2004).

1.5.2 Tissue Models

An optional way of testing potential therapeutic compounds before, or as an alternate to, animal testing, is in a 3D tissue model. These are favourable over animal models as they indicate human responses, and if used as an alternative also eliminate the element of suffering possible in animal testing. Models exist in various stages of development, of organs, various epithelial surfaces, and skin (Mroue and Bissell, 2013, Oliveira et al., 2015).

One primary example of a developed tissue testing model, used in this study, is the human skin infection model developed by Shepherd et al. This model uses keratinocytes and fibroblasts derived from patient biopsies which are seeded onto a de-cellularised dermal scaffold, also derived from a biopsy, between 2 metal rings. The centre of this inner ring forms a model wound with a 10mm diameter. The construct is cultured for 7-14 days at an air-liquid interface, and then is ready to be infected (Figure 1.5) (Shepherd et al., 2009). An advantage of this model is that it is easily reproducible, allowing for multiple standardized repeats, and uses primary human cells, thus giving a more accurate cellular response. Another simpler model used in this study is a model of just the human epidermis, sourced by companies such as CELLnTEC, in which keratinocytes are seeded onto a porous membrane and grown to confluence before moving to air liquid interface and triggering 3D growth and differentiation (CELLnTEC, 2015). These models have been optimised by different research groups using different scaffolds, such as electro-spun collagen scaffolds, and by using various immortalised cell lines.

Some skin models are commercially available for this type of testing, including Alloderm® from LifeCell Inc, EZ Derm™ from Brennen Medical and Integra® from Integra LifeSciences Corp. The aim of developing tissue models is to eventually negate the need for animal testing, and to develop more complex models for the study of skin diseases such as psoriasis and atopic dermatitits.

1.5.3 Animal Models

The next stage of drug testing is generally animal testing. Testing at this stage gives good indications of how the drug reacts in a whole system with all immune components present and many potential drugs fail at this point. The specific organism chosen for testing varies depending on availability and relevance of model.

1.5.3.1 Zebrafish

Danio rerio, commonly known as zebrafish, are increasingly being used as a model to test the effects of small systemic drugs. Prajsnar et al have developed a model in which the fish are injected with *Staphylococcus aureus*, and the drug being studied is put in the media or injected. It is not an ideal system, as even certain antibiotics are above the size threshold to diffuse into the fish, and therefore must be injected separately to the *S. aureus*, which is

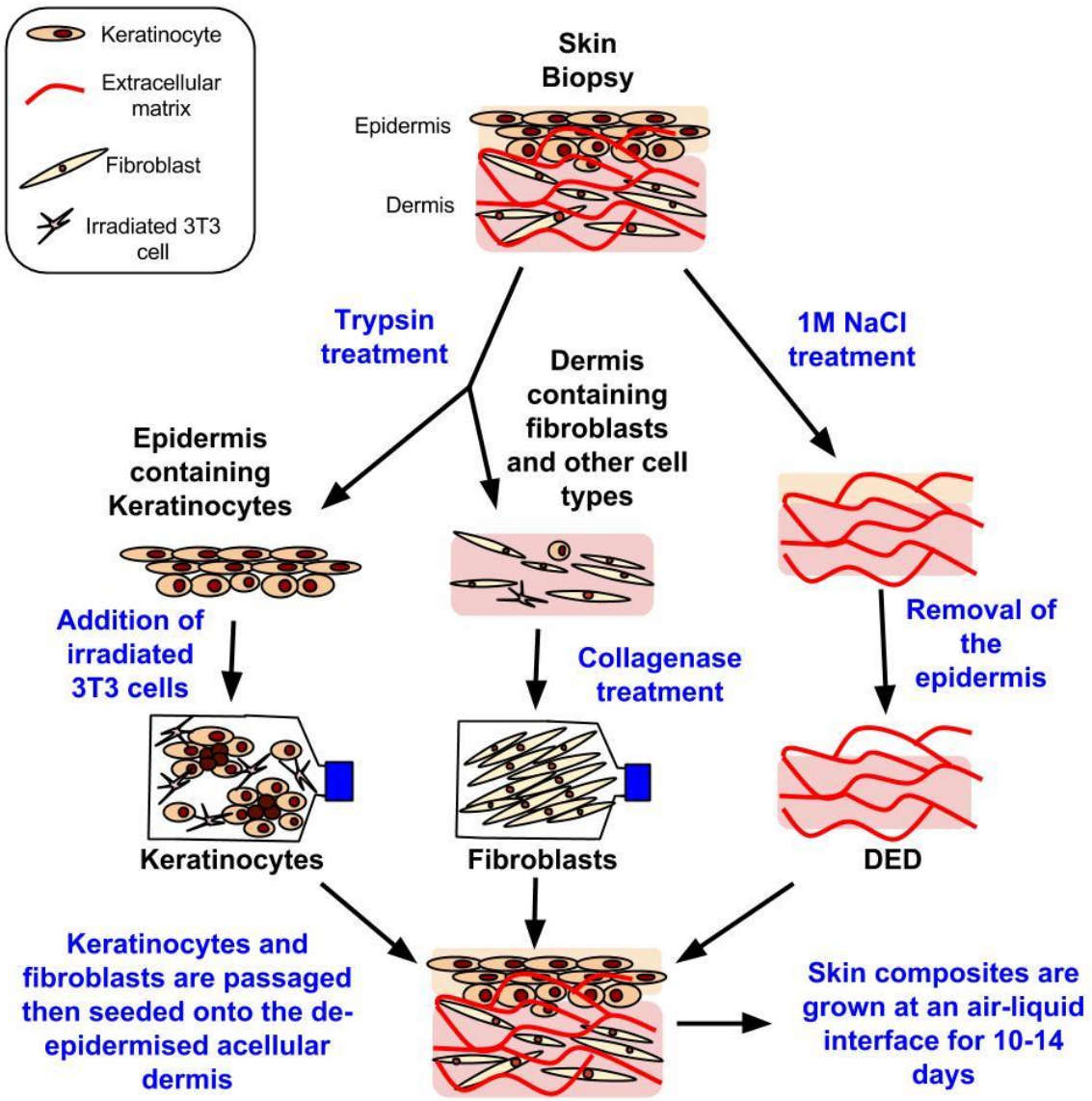


Figure 1.5: The Construction of a 3D Model of Human Skin with Reproducible Qualities: A skin biopsy is cut into fragments and treated in 2 different ways. To obtain a de-epidermised acellular dermis, the skin is treated with 1M sodium chloride, and the epidermis is manually removed and discarded. To obtain primary cells, the skin is treated with trypsin, which breaks down connection in the dermal:epidermal junction. The bottom side of the epidermis and the top side of the dermis is then scraped into media in order to isolate keratinocytes. These are cultured with irradiated 3T3 cells which secrete fibroblasts growth factors to help the keratinocytes to adhere to the flask and grow. The remaining dermis is then treated with collagenase, releasing fibroblasts which can be expanded in culture. After these cells have been scaled up in the laboratory, they are seeded back onto the decellularised dermis and allowed to grow at air-liquid interface for 10-14 days until they are ready to use.

traumatic for the fish and causes injury and some fatalities. It is however a relatively simple and rapid way to achieve results that might indicate any possible effects or any toxicity associated with the compound being tested (Prajsnar et al., 2008). Zebrafish also have a similar immune response to humans, and therefore provide good indicators of possible immune reactions (Nuesslein-Volhard et al., 2002, Prajsnar et al., 2008).

1.5.3.2 Mice

A more traditional organism used for animal testing is mice. These have a relatively short lifespan and, as a mammal, are similar to humans in many ways such as body temperature, skin structure and immune functioning. Testing on mammals such as mice, guinea pigs and rabbits is generally kept to a minimum due to ethical concerns.

1.5.4 Clinical Trials

Clinical trials are the final stage in testing therapeutic compounds, and can be dangerous due to the possibility of the compounds being specifically toxic to humans. These trials show the exact clinical effects of the drugs in a range of human subjects, and are essential due to the inability to predict individual immune responses. They are normally conducted in 3 phases of increasing magnitude, and a pre-test may also be carried out using 'clinical trial simulation', however this cannot predict individual human response and is not therefore not enough on its own to prove drug safety (Holford et al., 2010). Clinical trials looking at wounds specifically are difficult to standardize, due to the variety in wound size, type and location, as well as individual age and health status, and for this reason very few comprehensive studies of wound dressings have been undertaken.

1.6 Tetraspanins

Tetraspanins are a superfamily of membrane proteins expressed in a range of eukaryotes including humans. There are 33 members of this family in mammals. Tetraspanins are known as the 'molecular organisers' of the cell membrane as their primary function is suggested to be the formation of lateral interactions with other tetraspanins, as well as with other protein, lipid and polysaccharide components of the cell membrane in order to form specialized microdomains known as tetraspanin enriched microdomains (TEM), or tetraspanin webs (Levy and Shoham, 2005). Their expression patterns differ between cell types (Figure 1.6, (Kim

et al., 2014, Wilhelm et al., 2014, Wang et al., 2015)), as some tetraspanins such as CD9, CD53 and CD81 are expressed across various different cell types, whereas other such as uroplakins 1a and 1b are specialised to one cell type. Previous research by Green et al has also suggested that tetraspanin expression varies based on the stage of the cell cycle (Green, 2010).

TEM were initially studied by biochemical approaches including isolation of detergent resistant membranes, co-immunoprecipitation, protein crosslinking and proteomics (Yanez-Mo et al., 2009, Le Naour et al., 2006, Charrin et al., 2001, Charrin et al., 2002). While these techniques have been instrumental in the original identification of TEMs, they do not provide insight in the spatiotemporal characteristics of TEM in the plasma membrane. A recent study by Zuidscherwoude et al used high resolution microscopy to determine these characteristics and challenge the original view of the tetraspanin web (Zuidscherwoude et al., 2015).

Tetraspanins have been implicated in a wide range of cell functions including signalling, migration, adherence and fusion. The exact function of individual tetraspanins is difficult to elucidate, as there is redundancy within the tetraspanin web system (Boucheix and Rubinstein, 2001).

1.6.1 CD9

CD9 is one member of the tetraspanin family found in humans and many other eukaryotes. Its structure is as noted above, and it has 4 cysteines in its EC2 domain, forming 2 sub-loops (Boucheix et al., 1991). It has been implicated in sperm-egg fusion (Higginbottom et al., 2003), multinucleated giant cell formation (Takeda et al., 2003) and cell migration (Penas et al., 2000, Jiang et al., 2013). CD9 function in migrating cells of a wound is outlined in Section 5.6, and its roles in immunity are outlined in section 5.4. Section 5.7 describes its roles in *Staphylococcus aureus* attachment to skin cells. Common binding partners of CD9 include Claudin-1, CD81, a6b1 and a1b1 integrins and PSG17 (Boucheix and Rubinstein, 2001).

1.6.2 Tetraspanin Structure

Tetraspanins have 4 transmembrane domains, often containing polar residues, with 1 intracellular and 2 extracellular loops and 2 intracellular termini (Figure 1.7). All tetraspanins contain a conserved CCG motif in the EC2 domain, characteristic of the tetraspanin family. At the time of writing, there have been no 3D crystal structures published for a complete

tetraspanin molecule, however the structure of CD81 EC2 domain has been characterised (shown in figure 1.7) and is said to resemble a mushroom (Kitadokoro et al., 2003). Further theoretical modelling has supported this theory, suggesting that the closely associated transmembrane coils hold the protein's extracellular loops close to one another to support the EC2 structure. Tetraspanins also have varying numbers of conserved cysteine residues in the EC2 capable of forming disulphide bridges and thus creating a sub-loop structure. This structure generally displays the lowest levels of sequence conservation within the molecule, and thus contains much of the functional specificity (Hassuna et al., 2009). Tetraspanins also have palmitoylation sites adjacent to the membrane which aid in tetraspanin-tetraspanin interactions (Charrin et al., 2002, Kitadokoro et al., 2001, Kitadokoro et al., 2003).

1.6.3 Tetraspanin Enriched Microdomains

Tetraspanins associate with each other in the membrane, driven partly by the membrane-proximal palmitoylation sites as well as membrane lipids such as cholesterol, to alter the localisation of cell surface components and to form TEM (Charrin et al., 2002). This dynamic structure is thought to be essential for many critical cell functions. TEM have been implicated in many cell functions, including cell adherence and fusion, membrane trafficking, endocytosis, leukocyte adherence and motility (Berditchevski and Odintsova, 2007, Hemler, 2005).

At the time of writing, 2 main models for the organisation of TEM have been proposed (Figure 1.8). The first widely accepted hypothesis is that within the tetraspanin microdomains there are multiple different tetraspanins that all have multiple specific partner proteins. In this model each TEM is made up of a large number of mixed interacting tetraspanins. Multiple proteomic studies support this theory, with 3 to 7 tetraspanins found in differently isolated TEM (Le Naour et al., 2006). Recently, however, the structure of TEM has been revisited with super-resolution dual colour stimulated emission depletion microscopy. This study looked at the localisation of multiple tetraspanins and their binding partners on a membrane, and it was observed that each TEM was only made up of 1-2 types of tetraspanin and their partner proteins, and that they were much smaller than originally thought, at less than 120nm in diameter (Zuidscherwoude et al., 2015).

	Protein Expression by Spectral Counting (ppm)			
Tissue	CD9	CD63	CD81	CD151
Skin	575	163	24	-
Heart	36.1	1.85	55.1	13.1
Kidney	327	43.1	267	46.7
Brain	1516	-	835	3.79
Platelet	1506	917	-	110
Liver	11.5	5.45	249	1.26
Pancreas	319	278	418	32.2
Colon	2674	535	709	233
Lung	258	68.5	116	45.7

Figure 1.6: The Expression of Tetraspanins in Different Tissues: PaxDB was used to assess the relative levels of 4 common tetraspanins, CD9, CD63, CD81 and CD151 in various human tissues. CD9 is highly expressed in the platelets, colon, and brain, similarly to CD81. CD81 however is not expressed in platelets. CD151 is also expressed highly in the colon and platelets but not in the brain whereas CD63 is not expressed in the brain but is highly expressed in platelets (although at lower levels than CD9). Data from PaxDB, based on studies by Kim et al and Wilhelm et al combined (Kim et al., 2014, Wilhelm et al., 2014,

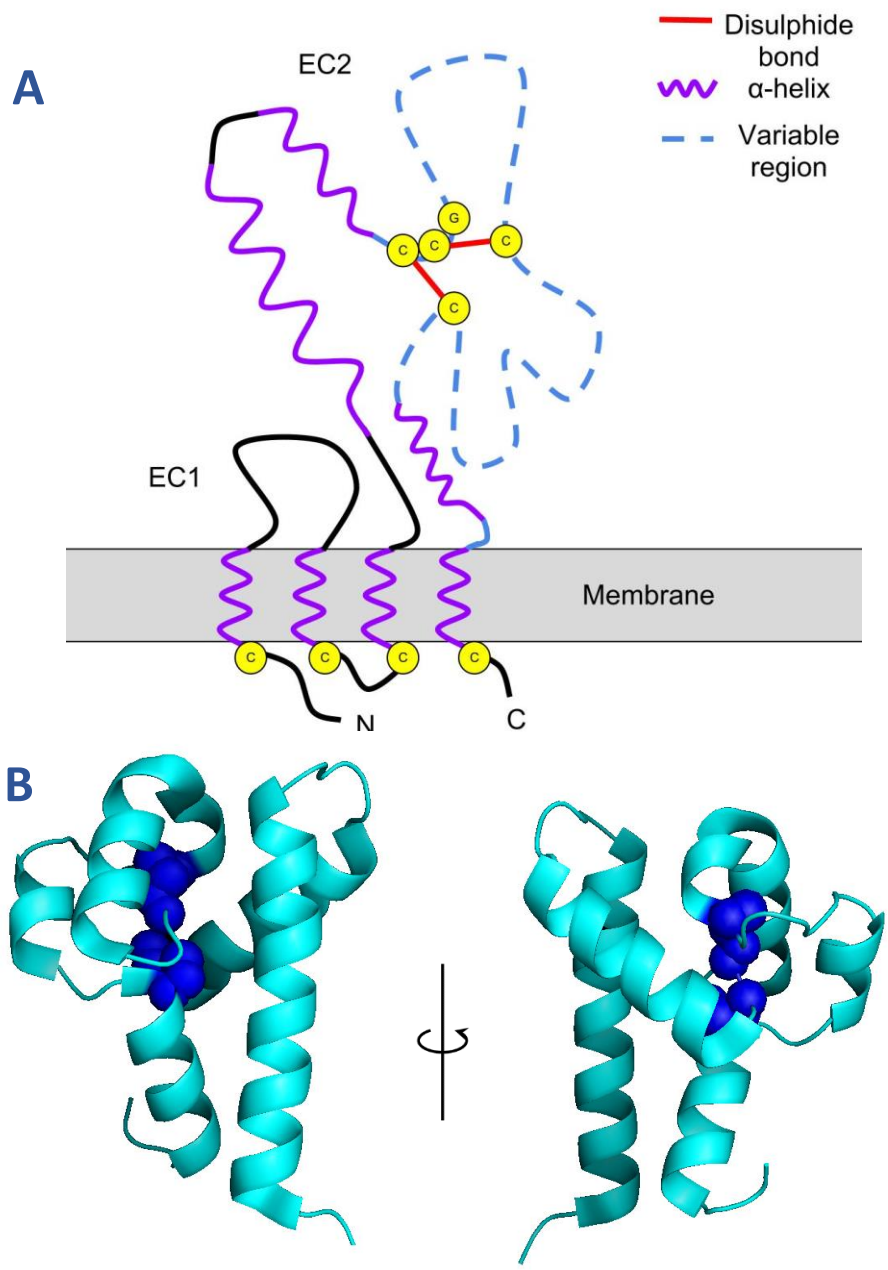


Figure 1.7: Structural Features of Tetraspanins: All tetraspanins have a conserved structure, consisting of 4 transmembrane domains, a small intracellular loop, and two extracellular loops, as well as two intracellular termini. They have a conserved CCG motif and multiple additional conserved cysteines in their EC2 domains. They also have cysteines proximal to the cytoplasmic membrane which can be palmitoylated to alter function. 3 of the transmembrane helices are also known to contain polar residues. A) Diagrammatic representation of a tetraspanin: The area represented by a blue dotted line is the most variable part of the tetraspanin family, with the greatest amount of sequence diversity. In different members of the family this loop also contains different numbers of cysteine residues, which alter its tertiary structure. B) The structures of CD81 EC2 domain as characterized by X-ray crystallography by Kitadokoro et al (Kitadokoro et al., 2003).

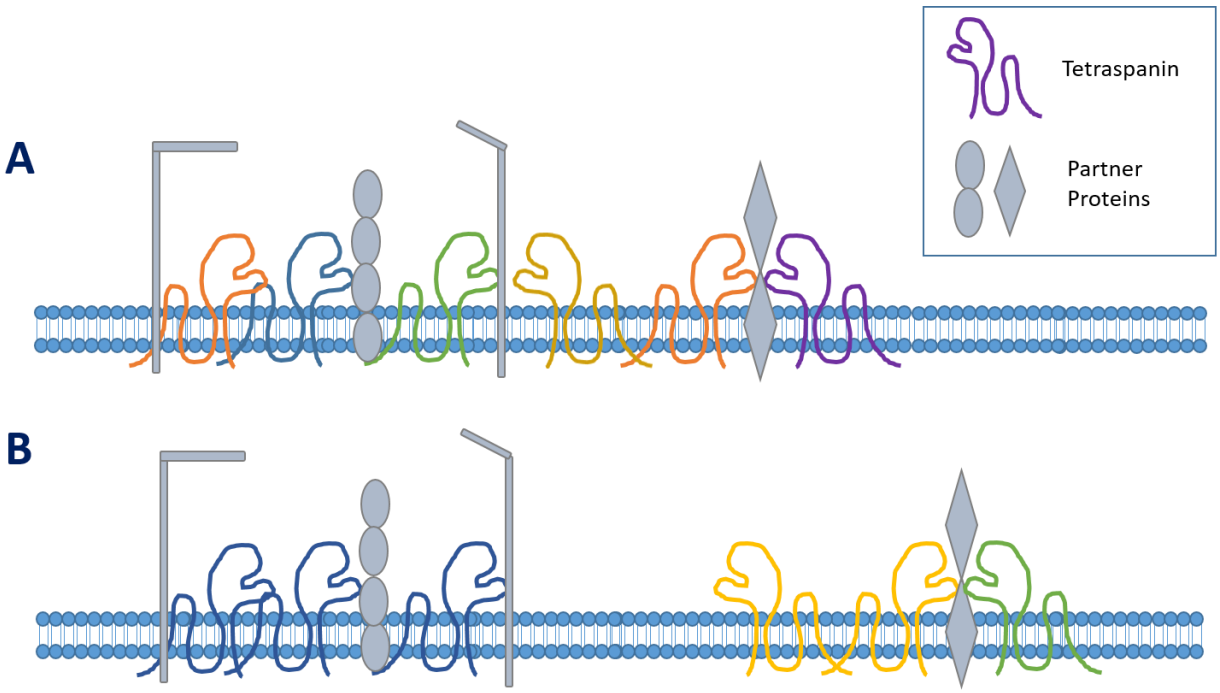


Figure 1.8: Two Conflicting Models of a Tetraspanin Enriched Microdomain (TEM): (A) The currently accepted model of TEM, in which multiple different tetraspanins with their partner proteins pull together to form a mixed domain. (B) The newly proposed model by Zuidscherwoude et al, in which each microdomain is made primarily of one type of tetraspanin and its partners (Zuidscherwoude et al., 2015).

TEMs were first characterised using three detergents of varying strengths combined with co-immunoprecipitation. This showed 3 levels of interactions between tetraspanins and their partners. Primary interactions, which endure the strongest detergent, are the direct strong interactions between tetraspanins and their partners, such as between CD151 and integrin α3β1 (Serru et al., 1999). Secondary interactions are suggested to be between tetraspanins, pulling together their partner proteins observed in primary interaction, and thus starting the formation of the tetraspanin web. Tertiary interactions are detected using a weak detergent such as Brij98 but not a stronger one. These partners are most weakly associated with the tetraspanin web and could contain proteins that are merely located proximal to TEMs (Hemler, 2005, Charrin et al., 2009).

Different regions of the tetraspanin have been seen to be involved in the lateral associations required to generate TEMs. For example, the variable region of the EC2 loop of CD151 interacts with the membrane-proximal stalk region of its partner protein α3β1. The transmembrane domains of CD81 and CD9 however are the domains essential for their interaction with EWI-2.

One of the most characterised series of tetraspanin interactions is between CD151 and the laminin binding integrins: α3β1, α6β1 and α6β4; a subject of interest due to the overexpression of CD151 in various cancers (Romanska and Berditchevski, 2011). These interactions occur during the initial stages of biosynthesis and involves the EC2 region of CD151. In CD151 knockdowns, usual integrin functions such as signalling and internalisation are reduced (Sheng et al., 2009). Another strongly characterised interaction is between CD19 and CD81, which complex with CD21 and increase B cell activation. When CD81 was knocked down in mice, CD19 failed to traffic to the cell surface and glycosylation of CD19 failed to occur, supporting a role for tetraspanin in trafficking partners to the membrane (Shoham et al., 2003).

1.5.4 Tetraspanin Knockouts

Knockouts of tetraspanins in model organisms has provided us with much information on the function of tetraspanins. The primary thing that we have learnt from these model is the level of redundancy within the tetraspanin web system. Knockouts of many different tetraspanins known to be involved in essential cell functions had no obvious effect on cell phenotype. It is

speculated that due to the conservation within the tetraspanin family, certain tetraspanins can fill in for the function of others when necessary. However, some tetraspanin knockdowns have striking phenotypes. For example, CD151-deficient mice had impaired angiogenesis, platelet function and immune function (Takeda et al., 2007, Lau et al., 2004, Wright et al., 2004). CD9 knockdown mice are phenotypically normal, however they have a defect affecting sperm-egg fusion, rendering females infertile, and increased monocyte fusion (Takeda et al., 2003, Miyado et al., 2000, Le Naour et al., 2000). These knockouts also only exhibit a small difference in platelet function, despite CD9 being very highly expressed on platelets (Mangin et al., 2009).

1.6.5 Tetraspanins in Immunity

At the onset of infection, pathogen associated molecular patterns (PAMPs) from invading organisms are detected by pattern recognition receptors (PRRs) on host immune cells which then trigger full activation of the immune system, including the secretion of cytokines and chemokines and an increase in immune cell migration to the site of infection. Tetraspanins have been implicated in many of these processes.

For example, monoclonal antibodies against several tetraspanins increased the migration of dendritic cells and natural killer cells in response to a chemotactic gradient (Kramer et al., 2009, Mantegazza et al., 2004). CD9, CD37, CD53, CD81 and CD82 have all been shown to interact with antigen presenting major histocompatibility complexes (MHC), a key surface protein in immune activation, and the localisation of MHC complexes could be essential for antigen presentation (Kropshofer et al., 2002, Vogt et al., 2002). Alternately, recent research has shown that CD37 and CD82 have opposing function in antigen presentation and migration of dendritic cells (Jones et al., 2016).

CD81 also plays a strong role in B cell and T cell responses. CD81 is essential for the trafficking of CD19 to the cell surface, as mentioned previously. Mutations in either CD19 or CD81 reduces the production of antibodies by B cells (Boucheix and Rubinstein, 2001). Additionally, CD81 is enriched at the immune synapse, the area of interaction between immune cells such as T lymphocytes and antigen presenting cells involved in cell signalling (Mittelbrunn et al., 2002).

CD9 also has a role in immunity. When CD9 is knocked down in macrophages, the TNF-α acute inflammatory response is greatly upregulated upon stimulation with LPS, in turn upregulating the inflammation in response to pathogens (Suzuki et al., 2009). Additionally CD9, CD14 and TLR4 are all seen to interact directly (Suzuki et al., 2009), and removing CD9 from this system leads to the re-localisation of TLR4:CD14 complexes to low density membrane fractions, possibly upregulating TLR4 signalling (Pfeiffer et al., 2001, Suzuki et al., 2009). Major histocompatibility complexes (MHCs) are also known to form supramolecular cell surface structures with a range of tetraspanins including CD9 and CD81, helping to stabilise immunological synapse formation (Vogt et al., 2002).

Dendritic cells are immune cells located in the skin and other peripheral tissues. They express high levels of PRRs and simulate T cells as well as producing extensive cytokines for immune cell recruitment (de Koning et al., 2012). Many of the immune processes in dendritic cells have been shown to be reliant on tetraspanins including CD9 which interacts with MHC class II, CD37 which promotes dendritic cell migration to sites of infection, and CD82 which is involved in antigen presentation. The organisation and function of PRRs has also recently shown to be influenced by tetraspanins, however the mechanism with which they do this has not yet been defined (Figdor and van Spriel, 2010). A more in depth review of the role of tetraspanins in immunity, can be found in Boucheix et al (Boucheix and Rubinstein, 2001).

1.6.6 Tetraspanins and Integrins

The most common binding partners of tetraspanins are integrins (Peddibhotla et al., 2013, Scherberich et al., 1998, Hong et al., 2012), cell surface receptors involved in adhesion and cross-membrane signaling. They can bind both ECM proteins such as fibronectin and collagen, and internal structural proteins such as actin via adaptor proteins, and are formed of 2 subunits, α and β. In mammals there are 18 individual α subunits which can be found in complex with one of 8 different β subunits (Laflamme et al., 1994).

1.6.7 Tetraspanins and Wound Healing

Tetraspanins and involved in the process of wound healing, via their roles in cell migration and interactions with structural proteins. CD9 specifically was seen to be down-regulated at the leading edge of the migrating epidermis and is localised to motility related structures (Penas et al., 2000). However, when CD9 was knocked out in mice, re-epithelisation of the

wound was delayed through an enhancement of JNK signalling and matrix metalloprotease MMP-9 expression (Jiang et al., 2013, Zhang et al., 2012). This process was found to be regulated in the HaCaT cell line through an integrin switch from αvβ5 to αvβ6, which is confirmed by the presence of α5β6 in human wounds (Haapasalmi et al., 1996). Additionally, it was found that antibodies to integrins upregulate the expression of collagenase in keratinocytes, also increasing epidermal migration (Larjava et al., 1993). After tissue damage, platelets bind to the exposed basement membrane via integrins (Ginsberg et al., 1988). Additionally, cytokines secreted by white blood cells stimulate the upregulation of integrins on fibroblasts and macrophages, which in turn promotes their migration into the wound (Toda et al., 1987, Albelda and Buck, 1990).

1.6.8 Pathogens and Tetraspanins

Studies have suggested that both viruses and bacteria may be exploiting tetraspanins as a gateway for infection (Green et al., 2011). For example, diphtheria toxin receptor, a key virulence factor receptor for *Cornyebacterium diphtheria* infection, has been shown to associate with CD9. Furthermore, over-expression of CD9 in target cells increases susceptibility to diphtheria binding (Nakamura et al., 2000). Uropathogenic *E.coli* has also been shown to exploit tetraspanins in order to adhere to and infect cells. TSPAN21, a member of the human tetraspanin family (also known as Uroplakin 1a) has been shown to associate with the lectin FimH, which is found at the tip of type 1 fimbrae of *E.coli* (Zhou et al., 2001). CD81 also has a direct role in hepatitis C virus infection and it has been demonstrated that antibodies to the EC2 domain of CD81 can inhibit viral binding to cells (Zhu et al., 2012, Helle and Cocquerel, 2008, Pileri et al., 1998).

CD9 is known to be involved with many of the host cell receptors exploited by *Staphylococcus aureus* to instigate an infection. For example, CD9 closely interacts with β1 integrins, as identified by co-immunoprecipitation experiments (Rubinstein et al., 1994) and these integrins are required for Staphylococcal adherence via FnBP (Wilkinson et al., 1995). CD9 is also known to associate with a wide range of other integrins as outlined in Berditchevski et al (Berditchevski, 2001), including α6β4 in keratinocytes (Jones et al., 1996) and αvβ3 (Peddibhotla et al., 2013) , which associates with both CD9 and Hsc70, another cellular receptor which *S. aureus* exploits for adhesion to host cells (Guerrero and Moreno, 2012).

Fibronectin, one of the major cell receptors for *Staphylococcus aureus*, has also been shown to bind directly to recombinant CD9 and immobilised platelet CD9, suggesting a direct interaction between CD9 and Fibronectin (Guerrero and Moreno, 2012). Additionally, in platelets, fibrinogen receptors become exposed when cells are treated with anti-CD9 antibodies (Hato et al., 1988).

These examples show how bacteria can exploit tetraspanin interactions in order to adhere to and subsequently infect host cells. Recent studies have suggested that tetraspanins could be utilized as a potential therapeutic target. Unlike traditional drugs, this therapy would target components of the host as opposed to the bacteria, and thus would decrease the likelihood of resistance developing (Hassuna et al., 2009).

1.6.9 Tetraspanin Based Therapies

Hassuna et al have shown that the application of antibodies to various tetraspanins, and recombinant EC2 domains, disrupts the adherence of bacteria such as *Neisseria meningitidis, Staphylococcus aureus* and *Salmonella enterica* to mammalian cells (Hassuna et al., 2009). This work has now been extended to include additional bacterial species and cell types (Table 1.1). As mentioned previously, tetraspanins connect together molecules on the membrane of host cells to create tetraspanin webs, and can create adhesion platforms for bacteria such as *E. coli*. It is likely that as is demonstrated with leukocyte adhesion platforms (Barreiro et al., 2005), tetraspanins are involved heavily in the organization of bacterial adhesion platforms and that treatments are disrupting TEM and therefore these adhesion platforms (Figure 1.9).

In a model of Neisserial infection of Hec-1-B endometrial cells carried out by Dr Daniel Cozens, CD9 EC2 domain showed high levels of success in inhibiting *Neisseria meningitidis* adherence, whereas CD81 EC2 domain had no effect. Chimeric proteins were therefore produced, where sections of CD9 were substituted with sections of CD81 to determine the essential sites for the anti-adhesive effect. The segments which showed the greatest change in binding were generated as short peptides to be developed as potential therapeutics (Figure 1.10). There are many proteases in the wound environment, and large molecules such as antibodies and EC2 domains would be broken down rapidly, leading to the decision to generate smaller peptides with greater permeability and ease of production. (O'Driscoll et al., 2013) These

peptides showed an equal success to EC2 domains in reducing the adherence of *Neisseria meningitis* and *Salmonella enterica* to host cells.

It was therefore hypothesised that the peptides could have an effect on the adherence of *Staphylococcus aureus in vivo*, and therefore could potentially have a therapeutic role in a wound environment. Despite the lack of evidence for a direct interaction, it has been shown that tetraspanins interact with the host cell adhesion targets of *Staphylococcus aureus.* For example, Hsc70 is seen to interact with CD38 and CD81 in human lymphoblastoid B cells (Zumaquero et al., 2010). *S. aureus* receptors could therefore be dispersed using a tetraspanin targeted therapy.

Table 1.1: Tetraspanin Treatments Are Effective on a Range of Cells and Bacteria

Treatment	Targets	Cell Type	Bacterial Species	Reduction	Source
Antibody	CD9,63,81	DETROIT	*Neisseria meningitidis*	50%	(Green et al., 2011)
	CD9,63,81,151	HEC-1-B	*Neisseria meningitidis*	25%	(Green et al., 2011)
	CD9,63,81	MD Macrophages	*Neisseria meningitidis*	20%	(Green, 2010)
	Combi (9,63,81)	MD Macrophages	*Neisseria meningitidis*	60%	(Green, 2010)
	Combi (9,63,81)	HDMECs	*Neisseria meningitidis*	60%	(Green, 2010)
	CD9,63,151	Hec-1-B	*Neisseria lactamica*	70%	(Green, 2010)
	Combi (9,63,81)	Hec-1-B	*Neisseria lactamica*	50%	(Green et al., 2011)
	Combi (9,63,81)	Hec-1-B	*Escherichia coli*	50%	(Green et al., 2011)
	Combi (9,63,81)	Hec-1-B	*Salmonella enterica*	30%	(Green et al., 2011)
	Combi (9,63,81)	Hec-1-B	*Streptococcus pneumoniae*	40%	(Green et al., 2011)
	CD9	J774	*Salmonella enterica*	45%	Unpublished
	CD81	J774	*Salmonella enterica*	25%	Unpublished
	CD63	MD Macrophages	*Salmonella enterica*	50%	(Hassuna, 2010)
	CD81	HaCaT	*Pseudomonas*	20%	Unpublished
Recombinant EC2 domain	CD9,63,151	DETROIT	*Neisseria meningitidis*	50%	(Green, 2010)
	CD9,63,151	Hec-1-B	*Neisseria lactamica*	40%	(Green, 2010)
	CD9,63,151	Hec-1-B	*Escherichia coli*	40%	(Green, 2010)
	CD9,63,151	Hec-1-B	*Salmonella enterica*	50%	(Green, 2010)
	CD9,63,151	MD Macrophages	*Salmonella enterica*	50%	(Hassuna, 2010)
	CD9,63,151	Hec-1-B	*Staphylococcus aureus*	40%	(Green, 2010)
	CD9,63,151	Hec-1-B	*Streptococcus pneumoniae*	50%	(Green, 2010)
	CD9,63,82,TSPAN3	Hec-1-B	*Neisseria meningitidis*	50%	Unpublished
	CD9,81	Hec-1-B	*Staphylococcus aureus*	50%	Unpublished
Peptides	All	Hec-1-B	*Neisseria meningitidis*	50%	Unpublished
	All	Macrophages	*Neisseria meningitidis*	50%	Unpublished

Detroit 562 is a human epithelial pharynx carcinoma cells line. Hec-1-B cells are a 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 are a murine macrophage tumour line, and HaCaT cells are a spontaneously immortalised human keratinocyte cell line. Work was carried out by Dr Luke Green, Dr Daniel Cozens, Dr Fawwaz Ali and Ms Jehan Alrahimi.

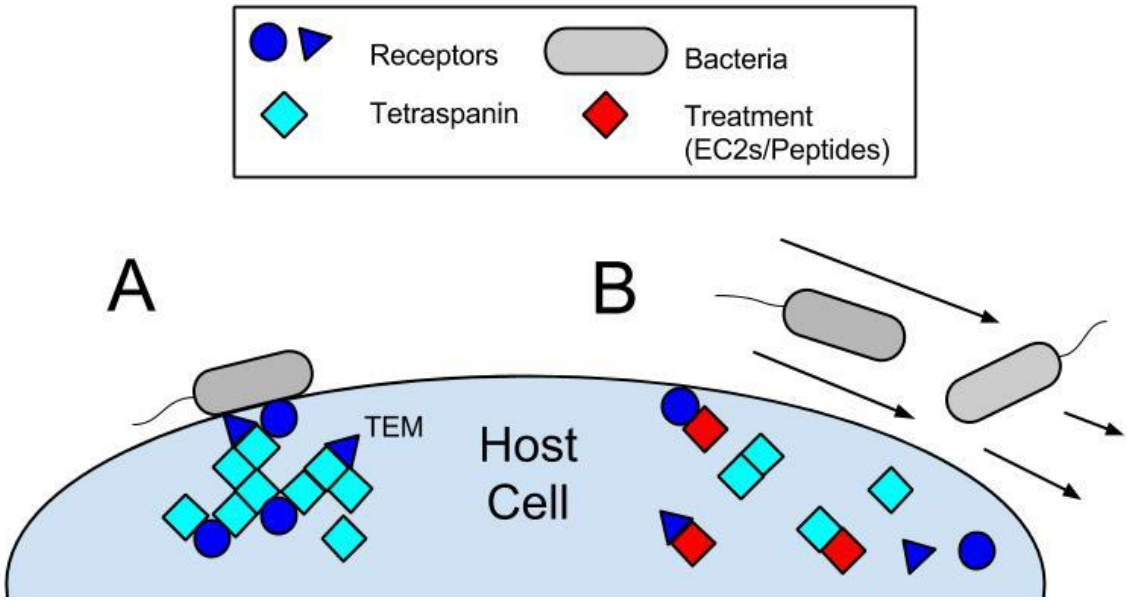


Figure 1.9: Disruption of TEM: The treatment of cells with recombinantly expressed EC2 domains, antibodies, or tetraspanin based peptides is thought to disrupt TEMs. This can cause the redistribution of host cell receptors and therefore prevent the adherence of bacteria the cell. A) Normal organization of adhesion platforms by tetraspanins. B) After therapy, receptors are redistributed and bacteria can no longer adhere and are cleared by sheer pressure.

Peptide Sequences:

810: GPKKDVLETFTVKS
810SCR: TSKEKLVGPDTKVF

8001: SHKDEVIKEVQEFY
8001SCR: EEVKKFESQHDIYV

800: EPQRETLKAIHYALN
800SCR: AYPHLERNLQEIAKT

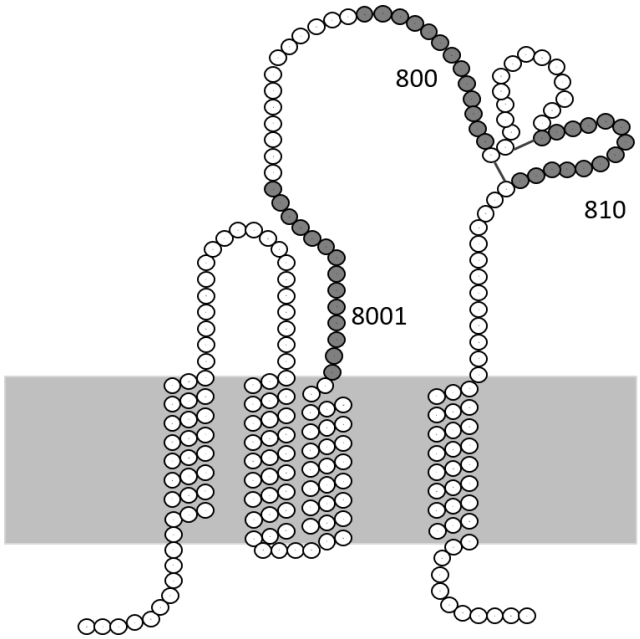


Figure 1.10: The Origin of Peptide Sequences: Peptides are generated based on the EC2 domain of CD9. 8001 is part of the stalk region of the EC2, 800 is part of the large loop, and 810 is part of the highly variable sub-loop structure of CD9. Diagram generated by Dr Daniel Cozens and reproduced with consent.

1.7 Conclusion

Bacterial skin infections are a serious problem for healthcare, in terms of patient mortality and comfort, as well as financially. Due to the rise in antibiotic resistance cause by antibiotic use and misuse, it is becoming increasingly important to find alternate treatments with which to treat or prevent infection. Various compounds that fit this description are currently being developed and trialled, and many show great potential for the future. There is room however for a greater collection of compounds with a wide range of mechanisms of action to prevent the increase in occurrences of resistance, and to treat the infections quickly, efficiently, and inexpensively. One particular approach that has not yet been fully explored is that of therapeutics that target the host rather than the pathogen, and specifically anti-adhesion therapies.

Tetraspanins are multi-functional eukaryotic membrane proteins, and have previously been shown to be involved in infection by pathogens. It has also been shown that antibodies against tetraspanins, recombinant EC2 domains and CD9 based peptides can reduce the adherence of a variety of pathogens including *Neisseria meningitidis*, *Escherichia coli* and *Salmonella enterica* to host cells. This therapy aims to disrupt receptor organization within microdomains organized by tetraspanins, and thus reduce the opportunity for bacteria to adhere to their target receptors. This approach does not put selective pressure on the bacteria, and thus is less likely to induce resistance.

1.8 Hypothesis

Tetraspanin-targeted therapies will reduce the adherence of *Staphylococcus aureus* to skin cells, showing a potential use for the treatment of wound infections.

1.9 Aims and Objectives

The aim of this study is to determine if peptides based on the EC2 domain of CD9 can disrupt the adherence of *Staphylococcus aureus* to cells found in the skin, and in a 3D model of human skin. If successful, further tests will be carried out to visualise the effect of this treatment on the cells and test for any toxicity of interfering with tetraspanin functions, for example a deceleration in wound healing or a change in cytokine signalling.

Chapter 2: Materials and Methods:

2.1 Reagents, Solutions and Buffers

2.1.1 Reagents

Trypsin-Versene

1.1L Trypsin-Versene was produced by diluting 100ml 10x Trypsin-Versene® (Lonza, Belgium) into 1L of Hank's buffered salt solution (HBSS, section 2.13). This was frozen in 20ml aliquots at -20^{o}C and defrosted prior to use.

Difco trypsin

0.1%w/v Trypsin with 0.1% w/v D-glucose (Difco) was generated in house by dilution in PBS.

Collagenase A

Collagenase A was purchased from Sigma-Aldrich and frozen in 10ml aliquots at 1mg/ml.

Saponin

Saponin from *Quillaja saponaria* bark (Sigma-Aldrich, USA), LotBCBJ8417V, was diluted to an appropriate concentration (10% w/v) using Brain Heart Infusion broth (Section 2.3.2).

Methylcellulose

For cell line and tissue assays, methylcellulose was made to 2% w/v with using Green's media (section 2.3.1). Powder was dissolved using a vortex mixer after chilling and peptide or antibiotic were added as required. For the duration of the experiment, no longer than 3 weeks, this gel was stored at 5^{o}C (Sigma-Aldrich, UK). For zebrafish work, methylcellulose was made to 5% with E3 Media.

Collagen Coating Matrix

Coating matrix kit was purchase from Life Technologies and used to coat a 24 well plate by diluting as directed and incubating for 1 hour and 37^{o}C

Dimethyl Sulfoxide (DMSO)

DMSO (Sigma) was used for freezing cells by diluting 10x in Foetal Calf Serum (section 2.3.1).

Nanocin™

Nanocin™ was obtained from Tecrea Ltd™, and diluted in dH_2O to make a stock solution. This was stored at 5^oC and added to peptide dilution for formulations. To test for particle formulation, approximately 800uL of solution was injected into the stage of a NanoSight LM10 microscope and analysed by NTA software.

Bovine Serum Albumin (BSA)

Bovine serum albumin from First Link Ltd. was diluted 1 in 5 in cell media for use as a blocking agent.

2.1.2 Antibiotics

Tricane

Tricane (Sigma-Aldrich) was prepared to a final concentration of 0.02% w/v in E3 media.

Chloramphenicol

Chloramphenicol (Sigma-Aldrich) was dissolved in ethanol to a concentration 50mg/ml. This was then stored at -20^oC in 1ml aliquots and diluted to 10μg/ml for use.

Flucloxacillin

Water soluble flucloxacillin sodium, also known as floxacillin from TOKU-E, USA was dissolved in distilled water to generate a stock at 1mg/ml. This was then diluted to the appropriate concentration in media. CAS number: 1847-24-1.

Penicillin/Streptomycin

Penicillin-streptomycin solution (Sigma-Aldrich) with 10,000 units penicillin and 10 mg/ml streptomycin p in 0.9% NaCl was generated in-house as 10mg/ml stock solutions and stored at -20^oC. It was then diluted to 100μg/ml in media for use.

2.1.3 Buffers

Phosphate Buffered Saline, pH 7.3

Phosphate Buffered Saline (PBS) was made in-house by the addition of 5 PBS tablets (containing 140mM NaCl, 8mM Na_2HPO_4, 2.7mM KCl and 1.5nM $K_2H_2PO_4$, Thermo Fisher Scientific) to 1L distilled water. This was then autoclaved at 121°C for 15 minutes.

Hank's Buffered Saline Solution (HBSS)

HBSS was purchased with or without divalent cations from Sigma-Aldrich.

B/B/N buffer

B/B/N buffer for flow cytometry staining was made in house by adding 0.2% BSA (First Link) and 0.1% Sodium Azide to HBSS with divalents.

2.1.4 Dyes

DAPI staining solution

DAPI stain as generated by the addition 0.1% Triton X-100 (Sigma-Aldrich, USA), 0.02% sodium dodecyl sulphate and 0.5µg/ml of 4,6-diamidino2-phenyl-indole hydrochloride (DAPI, Molecular Probes, USA) to sterile PBS.

MTT

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT Stain) (Sigma-Aldrich, USA) was made in house according to manufacturer's instructions as a 100x stock solution and kept refrigerated until use.

Resazurin Blue

7-Hydroxy-3*H*-phenoxazin-3-one 10-oxide (Resazurin) is a weak blue dye that is converted to resorufin, a light pink dye in metabolically active cells, also known as Alamar Blue**.** Resazurin was purchased as a salt from Fisher Scientific and diluted and filter sterilised in house to make a stock solution at 1mg/ml. It was diluted in cell media to a final concentration of 50µg/ml for use.

2.2 Peptides, Antibodies and Recombinant EC2 Regions

2.2.1 EC2s

N-Terminal Glutathione S-transferase (GST) tagged recombinant extracellular EC2 proteins were synthesized in 2 *E.coli* strains: *E.coli* Rosetta-gami B (DE3) pLysS (Novagen) and *E.coli* Shuffle T7 Express lysY (New England Biolabs) by Dr Marzieh Faneaei and Dr John Palmer. The relevant tetraspanin expression plasmid was added to competent heat shocked bacteria to induce uptake.

Transformants were identified using antibiotic selection and these colonies were then grown and to an OD_{600} of 0.6-0.8. The cells were then induced for protein expression using IPTG for 4 hours, spun down, and cell pellets were kept at -80^{o}C until needed. Protein was collected from induced cells using affinity purification (glutathione-sepherase beads) followed by dialysis. LPS was removed where appropriate using Triton X-144 using a technique outlined in Reichelt et al (Reichelt et al., 2006, Fanaei, 2014, Palmer, 2016).

2.2.2 Peptides

Peptides based on various regions of the EC2 region of CD9 (Tspan 29) were synthesized using solid phase synthesis with Fmoc chemistry by SI Biologics at the University of Sheffield or GenScript UK with no differences in function or efficacy between batches.

Figure 1.10 shows the locations of the CD9 EC2 to which the peptides correspond. 8001 is based on the 'stalk' region of the EC2 loop which has an alpha helix structure. 810, corresponds to part of the EC2 sub loop created by disulphide bridge formation, and 800 is part of the larger loop of the EC2. Scrambled peptides are randomly generated from the cognate CD9 sequence (Table 2.1).

2.2.2.1 Tagged Peptides

A version of 800 peptide was then generated with a tetramethylrhodamine tag by GenScript UK. This tag has a MW of 481 Da and an excitation/emission of 557/576nm.

Table 2.1: Peptide Names and Sequences

Peptide	*Alternate names*	*Peptide Sequence*
810	L07009a, Peptide E	GPKKDVLETFTVKS
810 Scrambled	L07009b, Scrambled E	TSKEKLVGPDTKVF
8001	L09002, Peptide A	SHKDEVIKEVQEFY
8001 Scrambled	L09003, Scrambled A	EEVKKFESQHDIYV
800	L11001, Peptide C	EPQRETLKAIHYALN
800 Scrambled	L11002, Scrambled C	AYPHLENLQPEIAKT

2.2.3 Antibodies

- Anti-hapten (JC1) mouse monoclonal IgG (In house)
- Anti-CD9 mouse monoclonal IgG (602.29) (In house)
- Anti-CD9 Alexa Fluor® 647 conjugate mouse monoclonal IgG (MEM-61, ab187776) (Abcam)
- Anti-CD63 (H5C6) mouse monoclonal IgG (In house)
- Anti-CD81 mouse monoclonal IgG (1D6) (ab35026) (Abcam)
- Anti-CD151 mouse monoclonal IgG (14A2) (In house)
- Anti-Involucrin SY5 FitC direct labelled antibody. Mouse monoclonal IgG (Abcam)
- Anti-mouse FitC conjugate polyvalent produced in goat (Sigma-Aldrich, F1010, batch 019K6280)
- Anti-staphylococcal rabbit polyclonal (Ab20920) (Abcam)
- Anti-Rabbit Alexa fluor® 488 conjugated goat Secondary (Therma-fisher)

2.3 Growth Media

2.3.1 Cell and Zebrafish Media

Foetal Calf Serum (FCS)

Foetal calf serum was supplied by Source Bioscience, UK (Labtech batch No. 4-107-500/1446). It was further heat inactivated (to HI-FCS) by incubation at 56°C for 20 minutes, then frozen in 50ml aliquots until use.

Dulbecco's Modified Essential Medium (DMEM)

DMEM was made in house with 500ml Dulbecco's Modification of Eagle's Medium high glucose AQ media (Sigma Aldrich), 5ml Amphotericin B (Fungizone Life Technologies; 250

µg/ml), and 50ml Foetal Calf Serum . Media was stored at 5°C. Penicillin/Streptomycin (ICN, Gibco) was added before use at 100µg/ml. Chloramphenicol was added 10µg/ml for infection assays.

Eagle's Modified Essential Medium (EMEM)

50ml FCS was added to 500ml EMEM +1% L-glutamine (Lonza). This was sealed and stored at 5°C between uses.

Green's Medium

500ml Greens medium was made in-house with the following components: 104ml Nutrient Mixture Ham's F-12 with L-glutamine, 17ml Sodium hydrogen carbonate (NaHCO3; 7.4%w/v), 330ml Dulbecco's Modification of Eagle's Medium without sodium bicarbonate and L-glutamine, 5ml L-glutamine (200 mM), 5ml Penicillin/Streptomycin (100 µg/ml), 1.25ml Amphoteracin B (Fungizone, Life Technologies (250 µg/ml)), 50 ml Foetal Calf Serum (FCS), 2ml Adenine (46 mM), 2.5ml Insulin (recombinant human; 1 mg/ml), 0.5ml T/T (Consisting of 1.36 µg/ml 3,3,5, triiodo-L-thyronine 1.36 ng/ml and 5 mg/ml apo-Transferrin), 0.08ml Hydrocortisone (25 mg/ml), 0.025ml Epidermal Growth Factor (EGF; recombinant human; 200 µg/ml) , 0.5ml Cholera toxin (from Vibrio cholerae; 847 µg/ml)

(Gibco, BDH, ICN, Sigma, R&D Systems)

3D Priming and Barrier Medium

For the construction of epidermal membrane cultures, CNT-prime and CNT-prime 3D barrier medium were purchased from CELLnTEC and stored at -20^{o}C in 500ml aliquots.

E3

E3 media was generated in house as a 60x stock solution with 34.8g NaCl, 1.6g KCl, 5.8g $CaCl_2 2H_2O$, 9.78g $MgCl_2 6H_2O$, 6ml Methylene blue (Sigma-Aldrich) and 2L Distilled Water.

2.3.2 Bacterial media

Brain Heart Infusion (BHI) Broth

18.5 g of brain heart infusion (BHI) agar base (Oxoid) was diluted in 500ml distilled water and autoclaved at 121°C for 15 minutes. 10µg/ml final concentration chloramphenicol was added immediately before use.

Brain Heart Infusion Agar

5g bacteriological agar was added to 18.5g brain heart infusion agar base (Oxoid) and 500ml distilled water and autoclaved at 121°C for 15 minutes. 10µg/ml final concentration chloramphenicol was added after cooling before pouring into 10cm bacterial growth plates (Sterilin Ltd).

2.4 Bacterial Strains and Eukaryotic Cell Types:

2.4.1 Bacterial Strains

SH1000

SH1000 strain of *Staphylococcus aureus* is derived from RN4220 and contains a chloramphenicol resistance pSK5487 plasmid expressing GFP (Herbert et al., 2010). All culturing of SH1000 prior to the infection of cells therefore occurred in the presence of 10µg/ml chloramphenicol. *Staphylococcus aureus* was grown on Brain Heart Infusion (BHI) and was maintained as a frozen stock at -80°C attached to beads in glycerol.

S235

S235 is a virulent clinical isolate of Community Associated-MRSA (CA-MRSA) kindly donated by the Sheffield School of Clinical Dentistry.

USA300

USA300 is a very well characterised strain of community acquired multi-drug resistant *Staphylococcus aureus* used in this study for analysis of surface distribution. This was obtained with a plasmid expressing YFP and chloramphenicol resistance from Dr Andrew Liew Tze Fui.

MRSA JE2

MRSA strain JE2 is a well characterised clinical isolate of community acquired multi-drug resistant *Staphylococcus aureus*. It is derived from a community-associated strain USA300 by removing a macrolide resistance plasmid and a cryptic plasmid.

MRSA NCTC14132

MRSA NCTC14132 is a clinical isolate of Methicillin-resistant *Staphylococcus aureus* isolated locally from a patient at the Northern General Hospital in Sheffield, UK.

2.4.2 Mammalian Cells

HaCaT Cells

HaCat cells are a human keratinocyte cell line generated by heat shock (Boukamp et al., 1988) obtained from Cell Line Services GmbH. Cells were maintained as a monolayer in T-75 culture flasks using DMEM with 10% FCS (Section 2.3), without penicillin or streptomycin or fungizone. Long term frozen stocks were made by freezing in FCS with 10% DMSO before storing in liquid nitrogen.

Primary Keratinocytes/Normal Human Keratinocytes (NHKs)

Keratinocytes were isolated with ethical approval from tissues obtained from female human abdominoplasties and breast reductions. These cells were isolated as described in section 2.6 and were maintained in Green's media in T-75 flasks at 37^{o}C 5% CO2 with a layer of irradiated 3T3 cells (generated in house). These cells were kept only up to passage 3, beyond which they failed to replicate efficiently. Long term frozen stocks were made by freezing in FCS with 10% DMSO before storing in liquid nitrogen. Long term frozen stocks were made by freezing in FCS with 10% DMSO before storing in liquid nitrogen.

Primary Dermal Fibroblasts

Fibroblasts were isolated as in Section 2.6 then maintained as a monolayer in T-75 flasks in DMEM media. These cells were only used after passage three and before passage 10. Long term frozen stocks were made by freezing in FCS with 10% DMSO before storing in liquid nitrogen.

N/TERT

N/TERT immortalised keratinocytes were obtained from Professor Birgitte Lane. These cells are immortalised by removal of the pRB/$p16^{INK4a}$ cell cycle control mechanism along with an increase in hTERT expression leading to the upregulation of telomerase activity (Dickson et al., 2000). A line of N/TERTs were also used with silenced Filaggrin expression and a GFP marker.

2.5 Bacterial Techniques

2.5.1 Solid Culture of Bacteria

Viable bacteria were kept as frozen stocks at -80°C on beads coated in glycerol. Aliquots of bacteria were streaked by placing a bead onto an appropriate agar plate and removing bacteria by rolling. This was then streaked using a sterile loop. Plates were incubated at 37°C with 5% CO_2.

2.5.2 Broth Culture of Bacteria

For liquid cultures, approximately 1 colony of viable bacteria from solid culture plates were transferred using a sterile loop into 10ml appropriate broth. These were cultured in a stirrer for 2-5 hours at 37°C with 5% CO_2 in a humidified atmosphere. Absorbance at OD_{600nm} was taken to define the quantity needed for cell infection.

2.5.3 Growth Curves

To determine the OD to cfu conversion figures, growth curves were undertaken with each species of bacteria. From an overnight plate, colonies were transferred to 10ml liquid broth to an OD_{600nm} of 0.060. These were then grown in aerobic conditions, and OD600 measurements taken every hour. Additionally, every 2 hours, 20µl of sample was diluted and plated to calculate colony forming units.

2.5.4 Counting Viable Bacteria

Tenfold serial dilutions followed by plating on BHI agar was used to estimate the quantity of viable bacteria in growth culture. Agar plates were split into 6 sections onto which 3 10µl aliquots of each dilution were dropped. Plates were air dried within a class II microbiological

safety cabinet before being incubated for 12-24 hours at 37°C with 5% CO_2. The mean number of bacteria was obtained by taking the average of the 3 spots of the 2 most appropriate dilutions number of bacteria, then calculating an estimated total number of bacteria per 1ml (colony forming units/ml).

2.6 Cell Line Assays

2.6.1 Cell Line Seeding and Growth

The number of cells in 4 1mm^2 sections of a Neubauer counting chamber was determined and averaged to give an estimate of cells in 0.1mm^3. This figure was then used to determine the dilution factor required to obtain the correct seeding density per well. Keratinocytes were seeded at 3x10^5, fibroblasts at 7.5x10^4, and HaCaT cells at 3.75x10^4. Cells are seeded onto 12 mm diameter glass coverslips within 24-well cell culture plates then incubated overnight at 37°C with 5% CO_2 before use.

2.6.2 Expression of Tetraspanins on Cells

To analyse the expression of tetraspanins on the cell types used in this study, cells were seeded at a density of 1x10^5 into a 96 well U-bottomed plate. They were then spun at 400rcf for 3 minutes. The supernatant was flicked off and the cells re-suspended in PBS and washed 2 times. 50µl primary antibodies (section 2.2.3) were then added at a concentration of 10µg/ml in B/B/N buffer and left on ice for 1 hour. The cells were washed again and an equal volume and concentration of secondary antibody added for 45 minutes on ice. Cells were then washed and re-suspended in BBN before analysis by flow cytometry. Data was collected using the Attune acoustic cytometer and analysed using Attune cytometric software.

2.6.3 Adherence Assays

Using a sterile Pasteur pipette, media was removed from the wells and the cells washed twice with PBS. 1ml of 5% BSA was then added to each well and incubated for 30 minutes, ensuring no non-specific bacterial binding. The BSA was then also removed, and the cells were washed twice with PBS. Each well was treated with 250µl of relevant recombinant tetraspanin EC2: GST fusion proteins at a concentration of 500nM. Glutathione-S-transferase (GST) was used as a control. Peptide fragments based on the EC2 region of CD9 were dispensed in a similar

protocol to the fusion proteins, at a concentration of 50nM. 'Scrambled' peptides with the same composition to the active peptides but with alternate amino acid sequences were used as a control.

250µl of bacteria were incubated with pre-treated the cells for 1 hour at an appropriate MOI to give around 60% infection. Bacteria were then removed and cells washed 3 times with PBS to remove unbound bacteria. The wells were then fixed using 150µl 2% paraformaldehyde.

2.6.4 Staining

Cell coated coverslips from wells were stained using PBS containing 0.1% Triton X-100, 0.02% sodium dodecyl sulphate and 0.5µg/ml of 4,6-diamidino-2-phenyl-indole hydrochloride (DAPI). Each well was incubated with 400µl for 12 minutes at room temperature. DAPI binds to nucleic acid and stains both eukaryotic cells and bacteria (maximum excitation wavelength of 368nm, maximum emission wavelength of 461nm).

After 12 minutes, the stain was removed and the cells washed twice with PBS. The cover slides were then extracted from the plate using tweezers and mounted onto slides using Vectashield mounting medium with DAPI. These were fixed in place using nail varnish to reduce slippage and desiccation.

2.6.5 Quantitative Microscopy

A DMRB1000 fluorescence microscope (Leica, Germany) at 100x magnification was used for counting. Each coverslip was analysed by a random count of 100 cells, scoring for cells with bacteria attached, and number of attached bacteria. Cells undertaking mitosis were considered abnormal and therefore not scored.

GraphPad Prism 6 was used for the majority of adherence data analysis. Data were initially tested for skew and if this was found to be more than double the standard error, then data were considered to be non-parametrically distributed and different analytical tests were chosen. The specific statistical test used and separate n values are provided for each experiment.

2.6.6 Dose Response

To test at which doses the peptide was effective, cells were treated and infected as in section 2.6.2, but with varying doses of bacteria. These were then quantified by fluorescence microscopy and analysed using a three parameter nonlinear regression.

2.6.7 Retention

To test the retention of peptide effects in the system, cells were treated for 1 hour with 50nM peptide treatment. They were left for varying lengths of time then all infected simultaneously with S235 strain *Staphylococcus aureus*. Infection was quantified as above.

2.6.8 Scratch Assay

To measure the rates of migration and proliferation of cells when treated with peptides, the relevant wells of a 24 well plate were first coated in Collagen Coating Matrix for 1 hour with incubation. The wells were then washed with PBS before HaCaT cells were seeded at a density of $3x10^5$ cells per well. These were then left overnight to adhere and form a monolayer. After around 16 hours a scratch was generated in the monolayer using a p200 pipette tip and the wells were washed and treatment (200nM peptides, 500nM EC2s/GST, or media control) applied before photos were taken. Photos were captured at 0 and 18 hours, and any other relevant time points. The percentage coverage in the field of view was measured using ImageJ software and analysis carried out in Graphpad Prism 6.

2.6.9 MTT Assay

An MTT assay is designed to assess cell viability and under controlled conditions this can also reflect cell number. In this assay, enzymes from metabolically active cells reduce the MTT dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan, inducing a colour change in the dye which is indicative of viability (Berridge et al., 2005).

HaCaT cells were grown in a 24 well plate for 24 hours, then treated for a further 24 hours with 200nM peptides in media. Peptide was then removed and the tetrazolian MTT dye added at a concentration of 0.5mg/ml in cell media for 1 hour. Cells were then lysed with Cellusolve

(2-butoxyethanol) to release the dye, and replicate samples of each condition plated into a 96 well plate, then read using a plate reader referenced at OD_{562nm}.

2.7 Tissue Engineering

2.7.1 Ethics Approval

All work using human keratinocytes and fibroblasts was performed on samples from abdominoplasty and breast reduction, obtained with prior patient consent. The protocol was approved by the local ethics committee Sheffield NHS Trust, Sheffield, UK. Tissues and cells were stored under license number 12179 of the Human Tissue Authority (UK).

2.7.2 Isolation of Keratinocytes

A sterile scalpel was used to obtain skin pieces of approximately $0.5cm^2$ in size. These were then transferred to 10ml of 0.1% Difco-Trypsin (plus 0.1% D-glucose in PBS, pH 7.45) and incubated at 4°C overnight. The epidermis was then excised using sterile forceps and the underside of the epidermis and papillary of the dermis gently scraped with a sterile scalpel to remove basal keratinocytes. These were then centrifuged at 200g for 5 minutes and re-suspended in 10ml Greens medium. The keratinocytes were then be cultured in $75cm^2$ tissue culture flasks containing a feeder layer of i3T3 fibroblasts (obtained from communal frozen stocks) at 37°C and 5% CO_2. For storage, cells were harvested when 60% confluent during their first passage and frozen in FCS with 10% DMSO.

2.7.3 Isolation of Fibroblasts

A scalpel was used to cut the small sections of dermis obtained when isolating keratinocytes into small pieces of $<1mm^2$ in area. These were then transferred into a sterile tube containing 10ml of 0.5% Collagenase A, and incubated at 37°C and 5% CO_2 overnight. Sterile forceps were used to remove remaining pieces of dermis and the suspension was centrifuged for 5 minutes at 400g. The cell pellet was then re-suspended in 10ml of 10% DMEM medium and cultured in a $75cm^2$ tissue culture flask at 37°C in a humidified atmosphere of 5% CO_2.

2.7.4 Isolation of DED

De-epidermised acellular dermis (DED) was obtained by incubating skin for 24 hours in 1M sodium chloride and removing the epidermis manually using sterile forceps. The dermis was stored at 4^{o}C in Greens Media until use.

2.7.5 Construction and Culturing of TEskin

Small pieces of DED (2cm^2) were placed onto grids in a 6 well plate, and a 10mm diameter steel ring pressed on top to create an airtight seal. Alternatively, for infection work, DED was placed in 12 well culture plate inserts with 0.4µm pores to allow media diffusion (Griener Bio-One). Keratinocytes (between passages 1 and 3) were seeded into the centre of the ring or inside the baskets at a density of 3x10^5 cells per composite, and fibroblasts between passages 3 and 10 were seeded at a density of 1x10^5 cells per composite. The centre of the ring and surrounding well were filled with Greens media and the composites are cultured for 3 days at 37^{o}C 5% CO_2 in submerged culture. At day 3, media is replaced, so that the underside of the composites is immersed in the media however the top layer is exposed to the air (air-liquid interface, ALI). Composites are cultured for 10-14 days at air-liquid interface before wounding. For infection work, media was replaced with Greens medium not containing Penicillin or Streptomycin at least 3 days before infection.

2.7.6 Infection of TEskin

On the day of infection the skin was wounded by applying a 4mm diameter rod heated in a flame to each composite for 4 seconds. The skin pieces were then washed three times with PBS before the treatment was applied. 200nM of the relevant peptide and scrambled control were applied to the composite in 100µl Serum Free DMEM and serum free DMEM without additions was added to the remaining untreated composites.

SH1000 bacteria were grown for 2.5 hours until in exponential growth phase, then spun down and washed three times with PBS. It was then re-suspended in HBSS and the OD_{600} measured. The bacteria were then re-suspended at a concentration of 1x10^8 bacteria/ml and 100µl of this was then applied to the skin 1 hour after wounding/treatment. The sample was incubated

for 5 further hours at 37°C in a humidified environment of 5% CO_2, then washed to remove non-adherent bacteria. 24 hours after infection the TEskin was washed again and analysed.

2.7.7 Infection Analysis

The TEskin was then separated into 2 halves by cutting through the centre of the wound. Some pieces were fixed in 4% Paraformaldehyde for histology. Remaining pieces were weighed, divided into 8 pieces, and placed in 1ml 10% Saponin for 12 minutes. 20µl of each tube was then removed and serially diluted before plating to obtain viable counts. Data were analysed using a One-way ANOVA with Sidak's multiple comparison.

2.7.8 Cytokine Analysis

Cytokine samples were taken at 6 and 24 hours by pipetting the serum free media over the wound 3 times. The remaining volume was measured before placing into micro-centrifuge tubes and centrifuging at 10rcf for 5 minutes to remove any bacteria. Samples were then stored as 30µl aliquots at -20°C until analysis with minimum freeze-thaw cycles.

Cytometric Bead Arrays were run by the flow cytometry core facility at the Medical School, University of Sheffield by Susan Clark and Julie Swales according to the protocol by BD Bioscience. In essence, beads are coated in antibodies to specific cytokines and are added to the sample. The beads are then washed and a secondary fluorescent tagged antibody added to allow detection of the beads using flow cytometry. 24 hour samples were analysed for MCP-1, IL-1α, IL-6, IL-8, IL-11, TNF-α/β, IFNα and IL-10. Samples for IL-6 and IL-8 analysis were diluted 50x in serum free media before further analysis.

2.7.9 Epidermal Migration Assay

The ability of the epidermis to migrate and proliferate is indicative of its ability to heal. This was assessed by seeding $3x10^5$ keratinocytes and $1x10^5$ fibroblasts into the centre of a 10mm diameter ring on top of a de-epidermised dermal scaffold and allowing to grow submerged for 3 days and at air liquid interface for another 7 days. At 10 days after seeding the metal ring was removed and the epidermis allowed to migrate outwards onto the bare DED. The viable area of epidermis was measured by submerging in Resazurin blue stain at 50µg/ml as for 1 hour at 0, 7 and 10 days. Treatments were added daily to the top of the skin in a 2% methylcellulose gel with serum free media. Resazurin blue images were analysed for

percentage coverage using the colour threshold and area measurement tools on ImageJ and the rate of healing analysed using GraphPad Prism 6.

2.7.10 Histology Processing and Embedding

For histological analysis tissue samples were fixed in 4% formaldehyde overnight and processed using a short program on the Leica TP1020 tissue processor as follows:

1. Formalin - 15 minutes
2. Formalin - 15 minutes
3. Alcohol 70% - 15 minutes
4. Alcohol 80% - 15 minutes
5. Alcohol 95% - 15 minutes
6. Alcohol 100% - 15 minutes
7. Alcohol 100% - 15 minutes
8. Xylene - 15 minutes
9. Xylene - 15 minutes
10. Paraffin - 15 minutes
11. Paraffin - 15 minutes
12. Paraffin - 15 minutes

They were then wax embedded and sectioned using a microtome and mounted on Super Frost Plus slides by Fisher Scientific.

2.7.11 Histology Staining and Immunohistochemistry

For Haematoxylin and Eosin staining a standard protocol was followed as below. Haematoxylin and Eosin were purchased from Thermo-scientific.

- Xylene – 3 minutes
- 100% IMS – 1 minute
- 70% IMS – 0.5 minutes
- Distilled water – 1 minute
- Haematoxylin – 1.5 minutes
- Running tap water – 4 minutes
- Eosin – 5 minutes
- Tap water – 2 brief washes
- 70% IMS - brief wash
- 100% IMS – 0.5 minutes
- Xylene – Brief wash

For immunohistochemistry, wax was removed from tissue samples using xylene, and the tissue was rehydrated with alcohol and distilled water. Sections were then blocked with BSA

serum for 1 hour, washed with PBS and incubated for 2 hours with primary labelled antibodies or control JC1 antibody. Sections were then mounted using Vectashield© with DAPI.

2.7.12 Construction of Epidermal Cultures

In order to visualise the distribution of bacteria on the surface of the epidermis, just the epidermis was grown, using a method outlined by CELLnTEC. In this method, keratinocytes are seeded onto porous membranes at 3x105 cells per insert and suspended in CELLnTEC 3D priming media. After 3 days submerged growth at 37^{o}C and 5% CO_2 they are transferred to 3D barrier medium, grown overnight, and then grown at air-liquid interface for 14 days before analysis.

These cultures were infected with 7x10^{6} USA300 strain *Staphylococcus aureus* bacteria per insert in 200µl PBS for 5 hours, then washed and left for another 19 hours. Epidermal cultures were fixed for 2 hours in 4% paraformaldehyde then divided into 2 sections, half for histological analysis and half to separate from the membrane and mount for surface analysis. Surface analysis sections were suspended in DAPI staining solution for 12 minutes before mounting in Hydro-mount.

2.8 Zebrafish Methods

2.8.1 Mating and Embryo Collection

London Wild-type embryos were collected from fish using the method of 'marbling' detailed in Zfin.org (S, 2016). After 12 hours the eggs were removed from the marbling trays and transferred to petri dishes containing E3 media. Eggs at the wrong cell stage and abnormal eggs were removed and discarded. The plates were then incubated at 28.5^{o}C for 1 day (until approximately 30 hours post fertilisation).

On the day of injections, a suitable number of fish (approximately 25 per condition) were dechorionated using 2 pairs of fine tweezers and a dissecting microscope. Damaged fish and egg debris were removed into a bleach solution with a Pasteur pipette.

2.8.2 Preparing Bacteria

SH1000 bacteria used to infect embryos were grown in 50ml BHI broth until they reach OD_{600nm} of approximately 1.0 in exponential growth phase, and harvested by centrifugation

(4500g, 10 minutes). The supernatant was discarded and pellet of cells re-suspended in 4ml sterile PBS. Bacterial concentration was confirmed by serial dilutions and plating on solid brain heart infusion media.

2.8.3 Injecting Fish

Before injection, embryos were anaesthetised using 0.02% w/v Tricane in E3 media. The dish was covered immediately to prevent exposure to light. Fish were injected directly into the bloodstream with 1nl bacterial suspension (prepared as above) using a pulled glass microcapillary pipette, a pneumatic micropump (World Precision Instruments PV820), and a micromanipulator (WPI) over a dissecting microscope. At the beginning, middle and end of the injection process, the quantity of bacteria being injected was tested by ejecting the same volume into 1ml PBS, which could then be scored by viable counts. Fish that were not injected with SH1000, and fish that were injected but untreated were used as control groups.

Fish were then transferred as groups of approximately 20-25 into a 6 well plate, and the treatment added to the media. 8001 peptide was used at a range of concentrations to test the most effective, and 8001 scrambled was used as a control. The fish were then decanted individually with 200µl treated media into a 64 well plate, and scored for survival twice daily up until 120 hours post fertilization. Survival rates were plotted using GraphPad Prism 6 and statistical analysis carried out using a survival test.

2.9 Statistical Analysis

Statistical analyses were carried out using ImageJ, Excel or GraphPad Prism, and the statistical test chosen is specified in the appropriate figure legend. Data were analysed for skew using the D'Agostino & Pearson omnibus normality test. Outliers were removed using a ROUT test, Q=1%.

Chapter 3 – The Use of Tetraspanins for the Prevention of *Staphylococcus aureus* Infections

3.1 Introduction

3.1.1 Anti-Adhesion Therapies

Adhesion is the first stage of bacterial invasion. Once bacteria adhere to a host, they can exist on the surface of the skin as a community in the form of a bio-film or colony, or can cross the epithelial barrier and cause complex infection. This infection can be intracellular or extracellular, but once within the system bacteria can transfer from cell to cell or in the areas between cells, and potentially into the blood stream (bacteraemia). Once in the blood, an infection can be transported to other sites around the body, resulting in systemic infections (Cozens and Read, 2012). One area of focus for new therapeutics is the prevention of this initial stage of infection, known as anti-adhesion therapies.

Anti-adhesion therapies are a relatively new area of research focus, and have varied mechanisms of action, as outlined in the recent review by Cozens et al (Cozens and Read, 2012). They can be host targeted or pathogen targeted and some examples can be found in Section 1. Preventative therapies such as anti-adhesion therapies are becoming increasingly popular in correlation with the rise in antibiotic resistance, as they exert a weaker selective pressure on bacteria and are therefore less likely to cause resistance to develop.

3.1.2 Tetraspanins as Anti-Adhesion Therapies

It is known that tetraspanins interact with diverse molecules on the membrane of host cells to create tetraspanin enriched microdomains (TEM), and through this mechanism can create adhesion platforms for a wide range of bacterial species (Charrin et al., 2009, Green et al., 2011, Barreiro et al., 2008). It was therefore hypothesised that disruption of these TEM could reduce the adherence of bacteria to host cells, and allow the infection to be cleared naturally by the immune system or with weaker courses of antibiotics or other anti-microbials.

Hassuna et al (2009) have previously shown that the application of recombinant tetraspanin EC2 domains, and antibodies targeted to the EC2 domain disrupt bacterial adherence to mammalian cells (Hassuna et al., 2009, Green et al., 2011). This has been shown for a wide

range of bacterial species and in a variety of human and mouse cell lines (Materials and Methods, Table 3.1, (Green, 2010, Green et al., 2011, Hassuna, 2010)), and at the time of writing, research is still ongoing to determine the range of bacteria affected. The reduction in bacterial adherence with anti-tetraspanin antibodies suggests a role for tetraspanins as an anti-adherence therapy for the prevention of *Staphylococcus aureus* infections and this possibility is explored in this chapter.

Peptides are ideal molecules for therapeutics in terms of size, permeability and ease of production (O'Driscoll et al., 2013), and therefore a series of peptides based on various regions of the extracellular loop (EC2) of CD9 were developed to be tested for anti-adhesive action. It was hypothesised that some of these peptides would still exert an anti-adhesive effect, preventing the adherence of *Staphylococcus aureus* to host cells, as well as defining the specific areas of EC2 domain necessary for tetraspanin function.

3.1.3 Staphylococcus aureus Adherence Mechanisms

Section 1.3.2.1 contains an in depth literature review outlining the mechanisms by which *Staphylococcus aureus* adheres to host cells, and how these mechanisms may be linked to tetraspanin function and organisation. In brief *S. aureus* has a wide range of adhesins, including but not limited to: FnBP A and B, ClfA and ClfB, and Atl. These have been shown to bind, amongst others, host cell factors fibronectin, fibrinogen, Hsp60 and Hsc70.

As mentioned previously, CD9 is known to be involved with many of the host cell receptors exploited by *Staphylococcus aureus* to instigate an infection. Some of these assossiations are demonstrated in figure 1.4 (introduction). CD9 closely interacts with β1 integrins, as identified by co-immunoprecipitation experiments (Rubinstein et al., 1994) and this connection is necessary for Staphylococcal adherence via FnBP (Wilkinson et al., 1995). CD9 is also known to associate with a wide range of other integrins as outlined in Berditchevski et al (Berditchevski, 2001), including α6β4 in keratinocytes (Jones et al., 1996) and αvβ3 (Peddibhotla et al., 2013), which associates with both CD9 and Hsc70, another cellular receptor which *S. aureus* exploits for adhesion to host cells (Guerrero and Moreno, 2012). Fibronectin, one of the major cell receptors for *Staphylococcus aureus*, has also been shown to bind directly to recombinant CD9 and immobilised platelet CD9, suggesting a direct interaction between CD9 and Fibronectin (Hato et al., 1988). Additionally, in platelets,

fibrinogen receptors become exposed when cells are treated with anti-CD9 antibodies (Hato et al., 1988).

The reliance of multiple staphylococcal target molecules on CD9, our protein of interest, further supports the hypothesis that CD9 could be a useful target molecule for the prevention of *Staphylococcus aureus* infections, and this hypothesis is explored in this chapter.

3.1.4 A Tissue Engineered Skin Model

The primary method used to test these peptides and EC2 domains until now has been a cell line assay outlined in Green et al (Green, 2010), in which generic epithelial cells are pre-treated with the tetraspanin blocking therapies, then infected with pathogen. After 1 hour of infection, non-adherent bacteria are washed off, and remaining adherent bacteria quantified by fluorescence microscopy. As a simple system this shows the effects of treatment in a simple cell monolayer, and is a good preliminary indicator of anti-adhesive potential. Cell line models however do not take into account the more complicated structures of human tissue.

Staphylococcus aureus is found commonly on skin as a commensal organism, and is the most common cause of skin and soft tissue infections. Therefore to test if the effects of these treatments were still effective in preventing SSTIs in a simple model, cell lines that reflect this target site were chosen, specifically: primary human keratinocytes (NHKs), primary dermal fibroblasts, and HaCaT cells, a cell line generated from human keratinocytes (Boukamp et al., 1988).

A 3D tissue engineered model of human skin (TEskin) was also constructed, as developed by Shepherd et al (Shepherd et al., 2009). This model consists of primary keratinocytes and dermal fibroblasts grown on a de-cellularised dermal scaffold at air-liquid interface (ALI). The keratinocytes and fibroblasts migrate and differentiate into their appropriate layers, forming a dermis, epidermis and stratum corneum, mirroring basic skin architecture, however it does not contain any of the immune cells or other diverse cell types observed in the dermis of live skin. The stratified stratum corneum of this TEskin is impermeable to bacteria unless a break is formed by burning or wounding, which is also representative of the way opportunistic pathogens must invade the skin. This model provides a relevant and reproducible platform on which to test the peptides in a more complex setting.

3.1.5 Staphylococcus aureus Strains

For the work detailed below a range of strains of *Staphylococcus aureus* were used, reflective of the range of strains isolated from the clinic. SH1000 is a low virulence lab strain, obtained from Professor Simon Foster expressing a pSK5487 plasmid containing a gfp gene and chloramphenicol resistance, useful for visualising the bacteria infecting cells at early stages. It is a derivative of RN4220 with a restored functional *rsbU* (Herbert et al., 2010). JE2 is a virulent strain of *Staphylococcus aureus*, generated from community-associated strain USA300 by removing a macrolide resistance plasmid and a cryptic plasmid (Kennedy et al., 2008) and S-235 is a local clinical isolate of Community Associated-MRSA (CA-MRSA) and provides a virulent and clinically relevant pathogen model.

3.2 Results

3.2.1 EC2:GST Fusion Proteins Reduce the Adherence of SH1000 to HaCaT Cells

To test the hypothesis that treatment with tetraspanin derivatives would reduce the adherence of *Staphylococcus aureus* to host skin cells, the HaCaT cell line was pre-treated with 500nM EC2:GST fusion proteins and infected with S235 strain bacteria. Treatment with CD9 and CD81 EC2 reduced the percentage of cells with adherent bacteria (For the purposes of this thesis I will refer to these as infected cells), and the number of adherent bacteria per 100 cells (Figure 3.1). This reduction in infection was not seen using GST alone, used as a control, showing that this effect is specific to the tetraspanin domain. Interestingly, no effects of any treatment were observed on the number of bacteria per infected cell, indicating that the treatment is affecting a subset of the cells. Approximately 40% of cells are unaffected by the treatment. A possible explanation for the reduction in bacterial adherence in this experiment could be downstream effects of residual lipopolysaccharide (LPS) from the protein expression and purification process outlined in Chapter 2.2. Residual levels of LPS were measured and shown in Table 3.1, and range from 0.16 ng/ml to 34.5ng/ml.

Table 3.1: Remaining exotoxin LPS content (ng/ml) in 500nM EC2:GST proteins

Recombinant Protein	LPS content (ng ml-1 per 500 nM protein solution)
GST (SHuffle)	18.00
CD9 EC2: GST (SHuffle)	34.50
GST (Rosetta Gami)	3.25
CD9 EC2: GST (Rosetta Gami)	12.40
GST (Rosetta Gami), Triton X-114 wash	0.16
CD9 EC2: GST (Rosetta Gami), Triton X-114 wash	1.40

Data acquired by Dr Marzieh Fanaei using the Limulus Ameobocyte Lysate chromogenic assay.

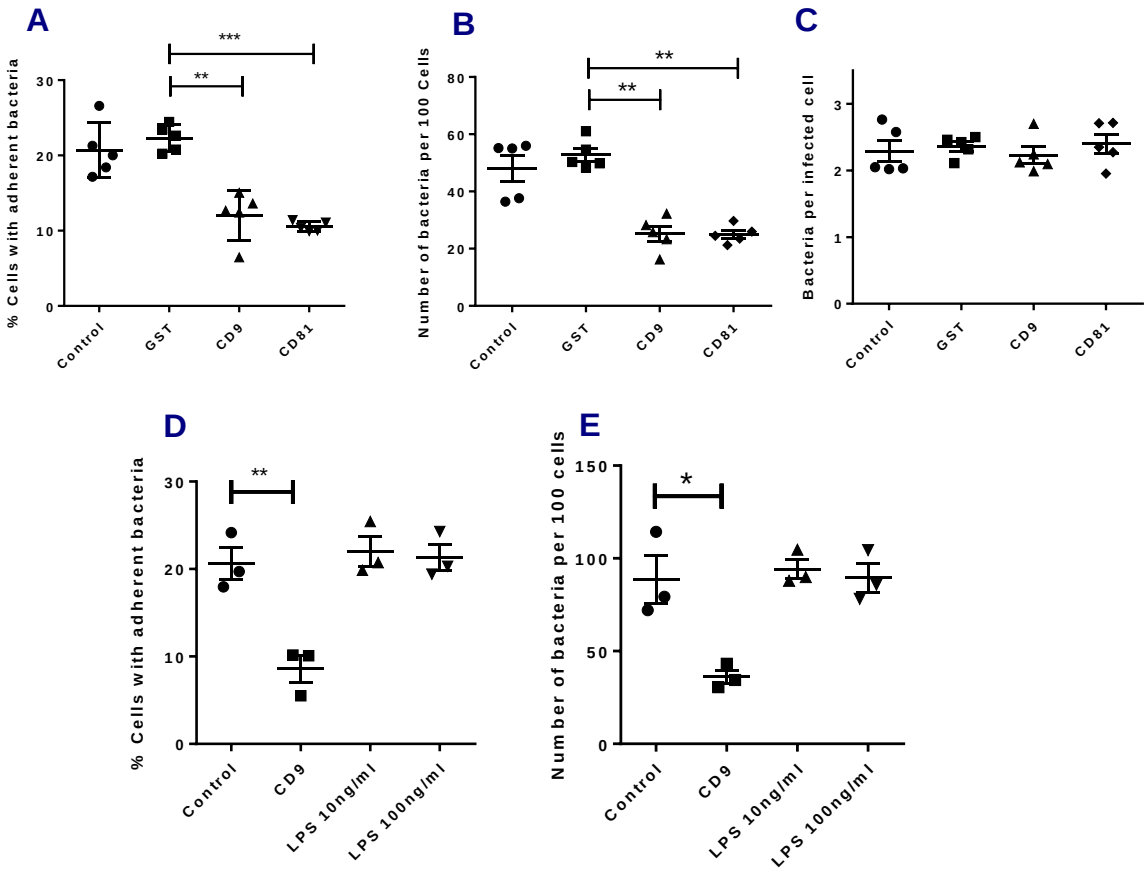


Figure 3.1: EC2:GST Fusion Proteins reduce Staphylococcal Adherence to HaCaT Cells: HaCaT cells were treated with 500nM Recombinant EC2:GST fusion proteins for 30 minutes before infection with SH1000 at an MOI of 30 and bacterial adhesion measured by fluorescent microscopy. (A and D) Change in proportion of cells with adherent bacteria (B and E) Change in total number of bacteria attached to 100 cells (A-C) Effects of recombinant EC2 domains on SH1000 adherence to HaCaT cells, n=5. (D-E) The effects of LPS on the adherence of SH1000 adherence to HaCaT cells, n=3. Data represented as mean ± standard error of the mean (SEM), and analysed by One-Way ANOVA with Sidak's multiple comparisons test, percentage data transformed Y= $\log_{10}$Y before analysis. * p≤0.05 **p≤0.01 ***p≤0.001

LPS alone was therefore tested for anti-adherence effects using the same method as the EC2 regions. At 10 and 100ng/ml, concentrations higher than those detected in the EC2 solutions, no effect was observed with LPS on the adherence of *Staphylococcus aureus* suggesting that the effect observed is due to the addition of the EC2 domains, not LPS contaminants. No attempt was made to discriminate between extracellular and intracellular bacteria, as previously no effect on these ratios has been observed with other pathogens .

3.2.2 CD9-Based Peptides Reduced the Adherence of SH1000 to HaCaT Cells

As inhibition was seen with the EC2 domains of CD9 and CD81, and because success was seen with a range of pathogens in the group, a series of peptides were developed based on the sequence of the EC2 domain of CD9 (sequences in Section 1, Figure 1.10). Here, we assess the 3 of these peptides, 800, 810 and 8001, for any inhibitory effects on the adherence of *S. aureus* to skin cells. Scrambled (SCR) peptides were also tested, which contain the same amino acids as the peptides, in a random order, as controls for non-specific effects.

Figure 3.2 shows the effects of the peptides on adherence of a low virulence *Staphylococcus aureus* strain, SH1000, to HaCaT cells. A significant reduction in adherence was observed with 810 peptide as defined by the number of bacteria per 100 cells. In other places a trend is observed however statistical significance was not achieved due to large variances in the data when plotted as a percentage of a media treated control value. Some success in reducing adherent *Staphylococcus aureus* however was observed with all peptides, and, as previously, no effect was seen on the number of bacteria per infected cell.

3.2.3 CD9-Based Peptides Have No Effect on the Adherence of SH1000 to Primary Dermal Fibroblasts

To assess if this effect is exclusive to cell lines or is also present in primary cells, primary dermal fibroblasts and epidermal keratinocytes isolated from patient biopsy were pre-treated with the peptides and infected with SH1000 strain *S. aureus*.

Primary fibroblasts from 3 donors were non responsive to the peptides, displaying no difference in the number of infected cells or the number of bacteria per 100 cells with treatment. To confirm that this was true even at higher concentrations of treatment, fibroblasts were then pre-treated with varying concentrations of 8001 peptide, which seemed

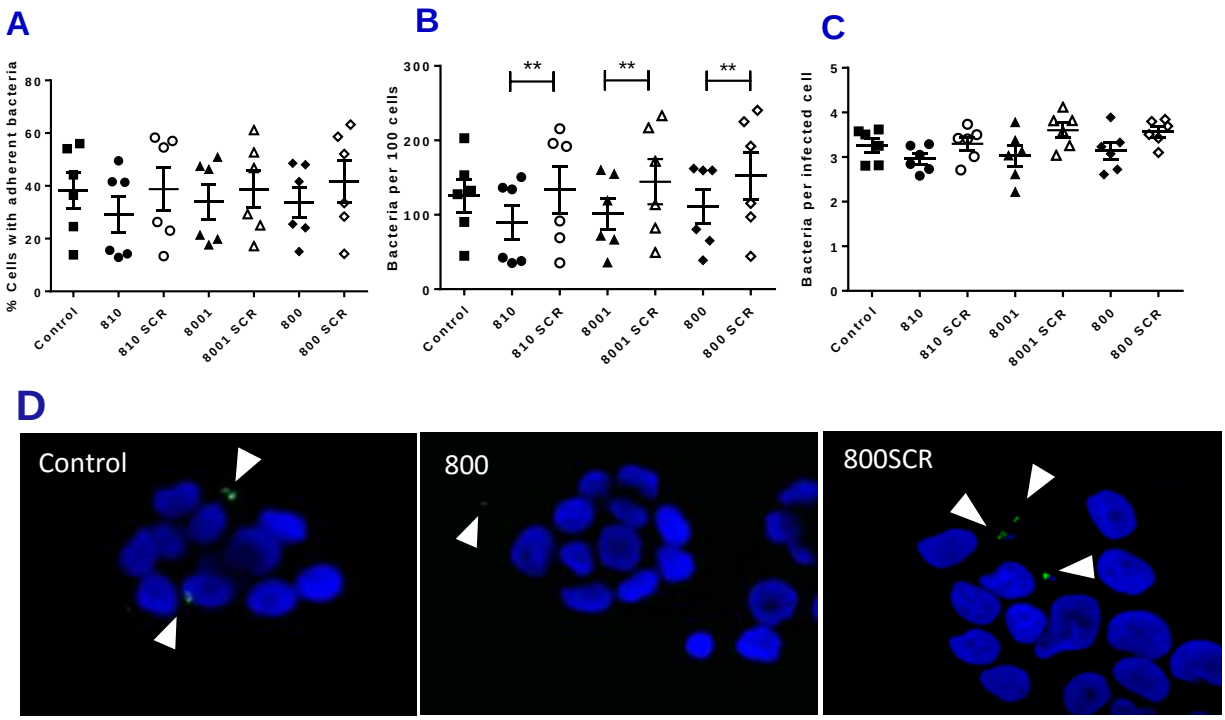


Figure 3.2: Peptide Treatment Reduces SH1000 Staphylococcal Adherence to HaCaT Cells: HaCaT cells were treated with 50nM Peptides or their scrambled controls for 30 minutes then washed three times with PBS. SH1000 was then added at an MOI of 160 and allowed to adhere for 1 hour at 37^{o}C before washing again to remove non adherent bacteria and fixing in 2% paraformaldehyde until analysis by fluorescence microscopy. (A) % of cells with adherent bacteria. (B) Number of bacteria attached to 100 cells. (C) Bacteria per infected cell. (D) Representative images of adherence of SH1000 bacteria as observed by fluorescence microscopy. Blue: Nuclei with DAPI. Green: SH1000 bacteria. n=6, data represented as mean ± SEM. One-Way ANOVA with Sidak's multiple comparisons test, percentage data transformed Y= $\log_{10}$Y before analysis. * p≤0.05 **p≤0.01

the most promising treatment from the previous experiment. These cells however also did not show a reduction in adherent bacteria in response to treatment (figure 3.3). As the peptides are effective with HaCaT cells, we deduced that the effect is cell specific and that the peptides do not affect fibroblasts. Possible reasons for this are suggested later in this chapter.

3.2.4 CD9-Based Peptides Reduce the Adherence of SH1000 to Differentiated and Undifferentiated Keratinocytes

Keratinocytes are the main cell type found in the epidermis, the top layer of skin. Upon wounding these cells are damaged and exposed to the air and are often the first cell type to become infected. Keratinocytes in different regions of the epidermis however are different, with keratinocytes close to the surface of the skin being strongly differentiated and producing high levels of keratins and structural proteins compared to basal keratinocytes, which are highly proliferative. To simulate keratinocytes from different layers, primary cells from donors were grown in high calcium media with serum (Greens + 10% FCS) or low calcium serum free media (MCDB153) for 2 days prior to testing.

Growth in high calcium media allows keratinocytes to differentiate and stratify as they would do as they cycle up through skin layers. These differentiated primary epidermal keratinocytes, unlike the dermal fibroblasts, appear responsive to treatment with peptides. 50nM peptide treatment reduced the quantity of adherent bacteria per 100 cells and a trend is observed with all peptides (figure 3.4). On these cells, 800 peptide, which is derived from the head region of EC2 adjacent to the sub-loop, was the most consistently effective. Overall, approximately a 40% reduction was seen with the peptides when compared with the scrambled controls, consistent with a tetraspanin-specific effect. As in previous experiments, no effect was seen in the number of bacteria per infected cell.

In keratinocytes grown in low calcium media, the cellular processes that lead to cell differentiation are stalled, causing these cells to remain in their proliferative state. These cells were seeded at the same density and treated and infected in the same conditions as differentiated keratinocytes (Figure 3.5). The severity of infection in these undifferentiated cells was higher than for differentiated. In control wells, the percentage of infected cells was 17% in differentiated cells, but 25% in undifferentiated cells. Additionally, upon differentiation, the

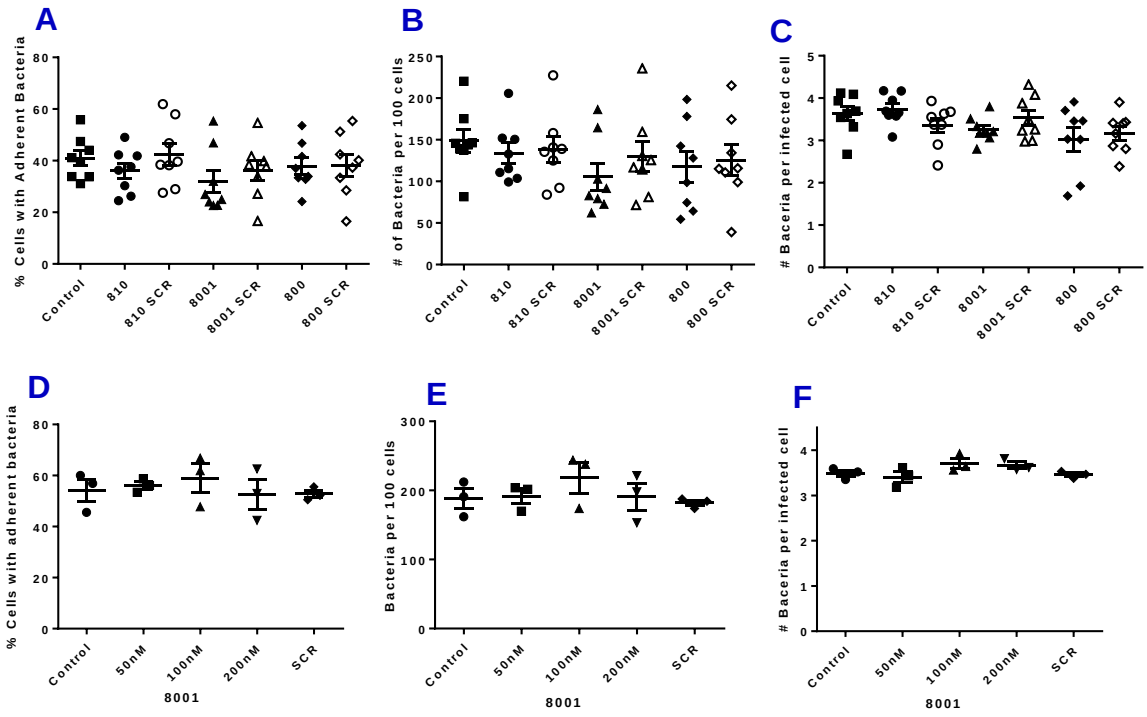


Figure 3.3: Peptide Treatment has No Effect on Staphylococcal Adherence to Primary Fibroblasts: Primary fibroblasts isolated from 3 donors were treated with 50nM peptides for 30 minutes and washed three times with PBS. SH1000 was then added at an MOI of 5 to the cells and allowed to adhere for 1 hour at 37°C. After infection the cells were fixed in 2% paraformaldehyde before analysis by fluorescence microscopy. (A-C) Cells were treated with 50nM of peptides. (D-F) Cells were treated with varying concentrations of 8001 peptide, with 200nM of 8001SCR as a control. Data represented as mean ± SEM, analysed by One-Way ANOVA with Sidak's multiple comparisons test, percentage data transformed Y= $\log_{10}$Y before analysis. A-C n=8, D-F n=3.

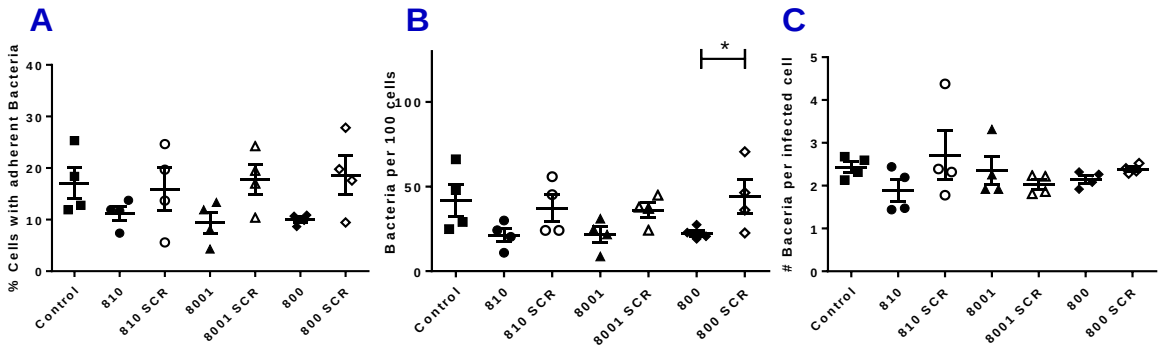


Figure 3.4: Peptide Treatment Reduces Staphylococcal Adherence to Primary Differentiated Keratinocytes: Primary keratinocytes were isolated from 3 donors and seeded in high calcium Greens media with 10% serum. After 2 days they were treated with 50nM peptides for 30 minutes. SH1000 was then added at an MOI of 30 to the cells and allowed to adhere for 1 hour at 37°C. After infection the cells were washed to remove non adherent bacteria and fixed in 2% paraformaldehyde until analysis by fluorescence microscopy. Data represented as mean ± SEM, n=4. Data analysed by One-Way ANOVA with Sidak's multiple comparison test, percentage data was transformed by $Y= \log_{10}Y$ before analysis.

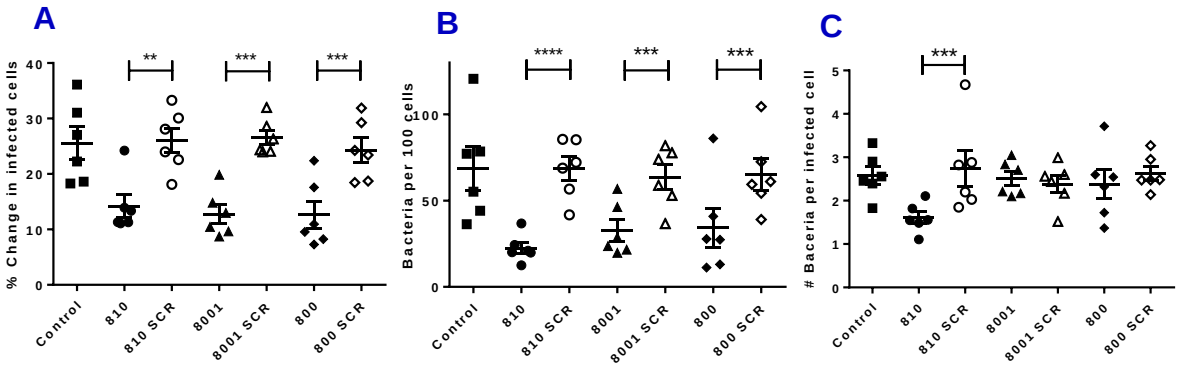


Figure 3.5: Peptide Treatment Reduces Staphylococcal Adherence to Primary Undifferentiated Keratinocytes: Primary keratinocytes were isolated from 3 donors and seeded in low calcium MCDB153 serum free media to prevent differentiation. After 2 days they were treated with 50nM peptides for 30 minutes and SH1000 added at an MOI of 30 for 1 hour at 37°C. After infection the cells were washed again to remove non adherent bacteria and fixed in 2% paraformaldehyde then analysed by fluorescence microscopy. (A) Percentage of cells with adherent bacteria (B) Number of bacteria per 100 cells. (C) Bacteria per infected cell. Data represented as mean ± SEM and analysed by One-Way ANOVA with Sidak's multiple comparison test. Percentage data was transformed by $Y= \log_{10}Y$ before analysis. n=6.

mean number of bacteria per 100 cells in an untreated well falls from approximately 68 to 42, suggesting that differentiated cells are less susceptible to bacterial infection.

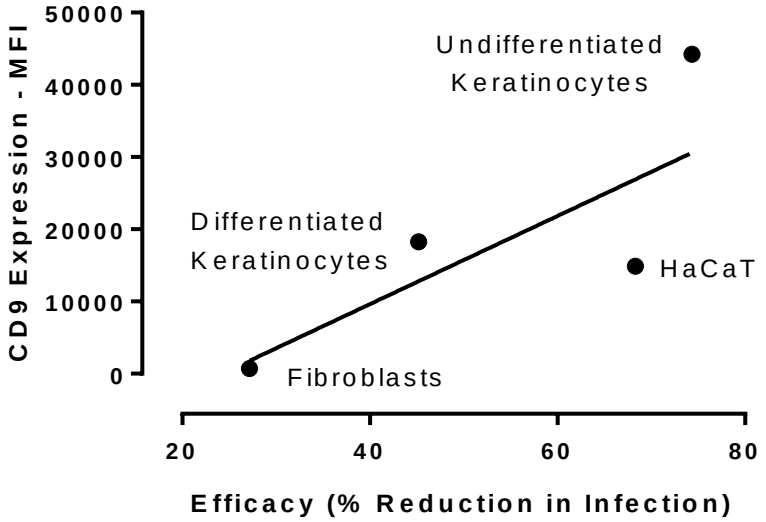
When pre-treated with the peptides and infected with SH1000 strain *Staphylococcus aureus*, all tetraspanin peptides tested showed success in reducing the percentage of infected cells, and the number of adherent bacteria per 100 cells in these undifferentiated low calcium keratinocytes. Noticeably, in undifferentiated keratinocytes, the effect with the peptides is much more significant, with a maximum 74% reduction from the scrambled control is observed with 810 peptide. In this experiment, there is a small and significant effect on the number of bacteria per cell, which is not seen significantly in other cell types infected with SH1000 bacteria. This effect can also be observed to a lesser degree with other strains of *Staphylococcus aureus*, (S235) and HaCaT cells in section 3.2.6.

3.2.5 Peptide Efficacy Correlates with CD9 Expression

Interestingly, when the efficacy of the peptides against SH1000 adherence is correlated with CD9 expression on the cell surface, as quantified by flow cytometry in chapter 4 we can see a direct correlation between the two parameters. Figure 3.6 shows that the maximum percentage reduction in adherence caused by the peptides, their efficacy, correlates directly with the expression of CD9 on the cell membrane of that cell type. As shown, cell types such as undifferentiated keratinocytes, which display the highest levels of CD9 expression, also respond the best to the peptides and have, relative to their controls, the greatest reduction in the percentage of infected cells. Conversely dermal fibroblasts displayed a very low level of CD9 and were non-responsive to peptide treatment.

3.2.6 CD9-Based Peptides Reduce MRSA Adherence to HaCaT Cells

To test if this anti-adhesive effect is still present on more virulent and clinically relevant strains of *Staphylococcus aureus*, 2 other strains were tested: JE2 and S235. JE2 is a virulent derivative of USA300 strain community associated *Staphylococcus aureus*. This strain was used to infect HaCaT cells pre-treated with 50nM peptides (Figure 3.7). A significant reduction was observed in peptide treated cells compared to those treated with scrambled control peptides, in terms of the percentage of cells with adherent bacteria and the number of bacteria per 100 cells. Approximately a 65% reduction in adherent bacteria was seen with all peptides, with the maximum effect seen with 800 peptide (64.8% reduction in infected cells,



Goodness of Fit	
R square	0.5566
Sy.x	11320
Is slope significantly non-zero?	
F	8.788
DFn, DFd	1.000, 7.000
P value	0.0210
Deviation from zero?	Significant

Figure 3.6: Peptide Efficacy is Correlated to CD9 Expression: Plotting CD9 expression as quantified by flow cytometry in Chapter 4 against the maximum efficacy of the peptides reveals that how effective the peptides are directly correlates to the levels of CD9 expressed on the cell surface. Fibroblasts which have a low CD9 expression show very low level success with the peptide treatment, whereas undifferentiated keratinocytes have high CD9 expression, and showed the greatest response to the peptides by the greatest decrease in adherent bacteria. Data is fitted to a linear regression, and is significantly non-zero.

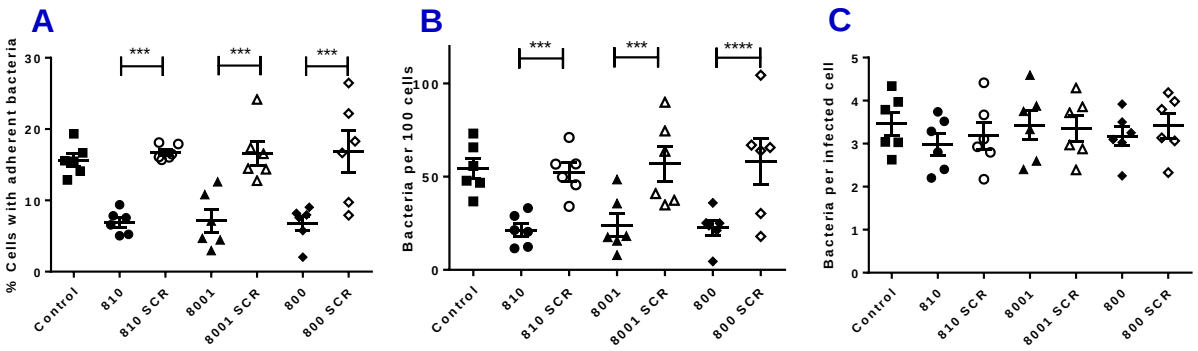


Figure 3.7: Peptide Treatment Reduces Staphylococcal JE2 Adherence to HaCaT Cells: HaCaT cells were treated with 50nM peptides for 30 minutes then washed with PBS before infection by JE2 strain *S.aureus* at an MOI of 200 for 1 hour. After infection the cells were washed to remove non adherent bacteria and fixed in 2% paraformaldehyde before staining and analysis by fluorescent microscopy. Data presented as mean ± SEM. n=6. Data analysed by One-Way ANOVA with Sidak's multiple comparisons test, percentage data was transformed by $Y= \log_{10}Y$ before analysis. * p≤0.05 **p≤0.01 ***p≤0.001 ***p<0.0001.

62.0% reduction in number of bacteria per 100 cells). No significant effect is seen on the number of bacteria per infected cell.

The S235 MRSA clinical isolate was then used to infect HaCaT cells pre-treated with 50nM peptides or scrambled control peptides (Figure 3.8). A significant effect was seen with all peptides in reducing the number of adherent bacteria and proportion of infected cells, but no difference was observed with the number of bacteria per cell. The greatest effect was seen with 800 peptide, which reduced the number of cells with adherent bacteria by an average of 77.52% and reduced the number of bacteria per 100 cells by up to 74.55%.

Due to the slightly stronger effect of 800 peptide, this was chosen as a candidate for further development.

3.2.7 Tagged Peptides Retain Function and Interact Directly with Host Cells

To visualise how the peptides interact and localise, versions of 800 peptide and 800SCR peptide were generated with tetramethylrhodamine (TMR) tags. Due to the relatively large size of the TMR tag (MW 478.97Da) comparative to the peptide size (MW 1783.02Da), these new peptide conjugates were tested for their anti-adhesive properties, thus indicating any steric hindrance or changes in net charge exerted by the tag. Figure 3.9 shows the effects of the tagged peptides versus their original counterparts in preventing the adherence of S235 strain *Staphylococcus aureus* to HaCaT cells. The fluorescently tagged peptides were shown to function to an equal, if not greater extent than the original peptides suggesting that the relatively large tag is not affecting their function or localisation.

These TMR peptides were then used to visualise peptide interactions with cells. To do this, primary keratinocytes were grown overnight, then treated with 500nM peptides for 30 minutes. They were then fixed using 2% paraformaldehyde and analysed by confocal microscopy. Peptides were observed localised on the surface of the cells (Figure 3.10). TMR peptides could not be visualised using flow cytometry due to low level fluorescence.

3.2.8 Determining the Optimum Dose for 800 Peptides in HaCaT Cells

A dose response experiment was carried out to ascertain at what doses 800 peptide treatment was effective (figure 3.11). HaCaT cells were pre-treated for 30 minutes with a

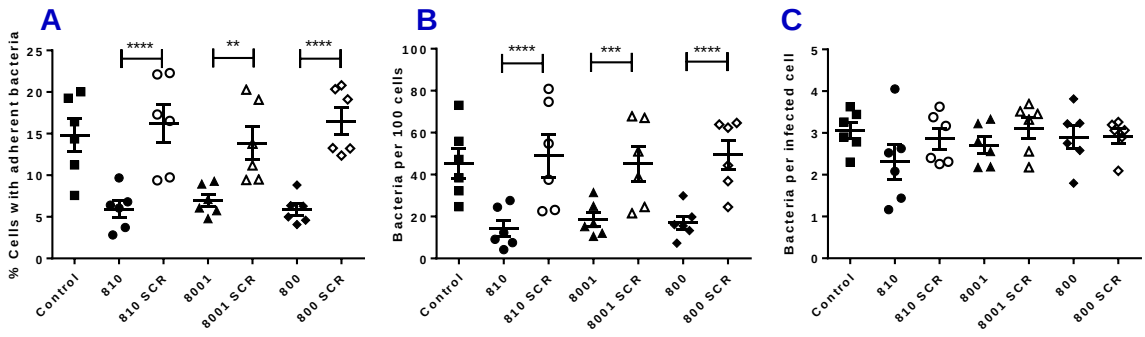


Figure 3.8: Peptide Treatment Strongly Reduces Staphylococcal S235 Adherence to HaCaT Cells: HaCaT cells were treated with 50nM peptides for 30 minutes then washed. S235 strain *S. aureus* at an MOI of 40 was then added to the cells and allowed to adhere for 1 hour. After infection the cells were washed to remove non-adherent bacteria and fixed in 2% paraformaldehyde until analysis by fluorescent microscopy. (A) Percentage of cells with adherent bacteria (B) Number of bacteria attached to 100 cells (C) Bacteria per infected cells. Data represented as Mean ± SEM. n=6. Data analysed by One-Way ANOVA with Sidak's multiple comparisons test. Percentage data was transformed by $Y = \log_{10}Y$ before analysis. * $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.001$ **** $p \leq 0.0001$

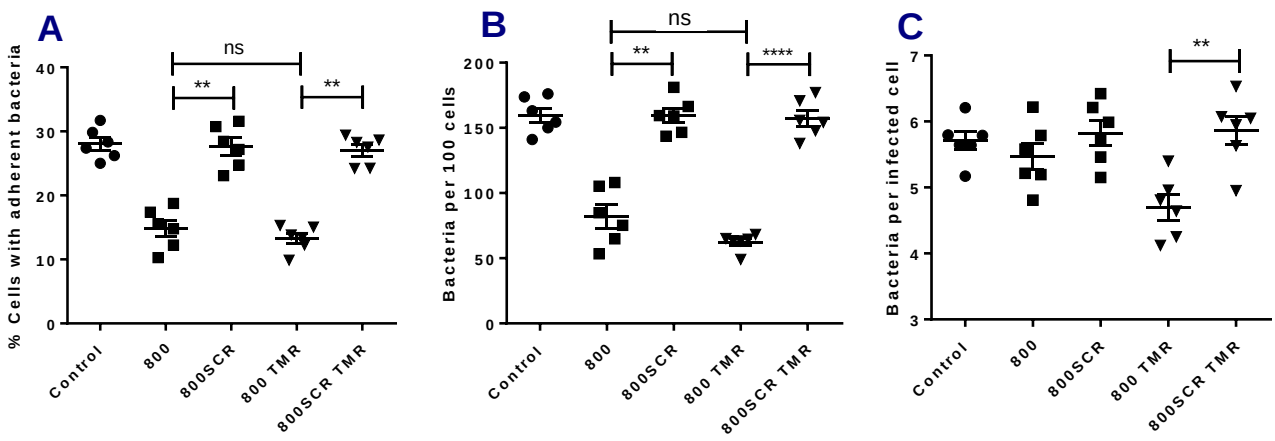


Figure 3.9: The Anti-adhesive Effect of Peptide 800 is Retained with the Addition of a Tetramethyl Rhodamine (TMR) Tag: Peptides 800 and 800SCR were conjugated to a Tetramethylrhodamine tag by GenScript UK in order to visualise their localisation. These conjugates were tested for their anti-adhesive effects at 50nM against the adherence of S235 *Staphylococcus aureus* at an MOI of 40. Data represented as mean ±SEM Data were analysed by One-way ANOVA with Sidak's multiple comparisons, and percentage data was transformed by $Y=Log_{10}Y$ before analysis. * p≤0.05 **p≤0.01 ***p≤0.001. n=6.

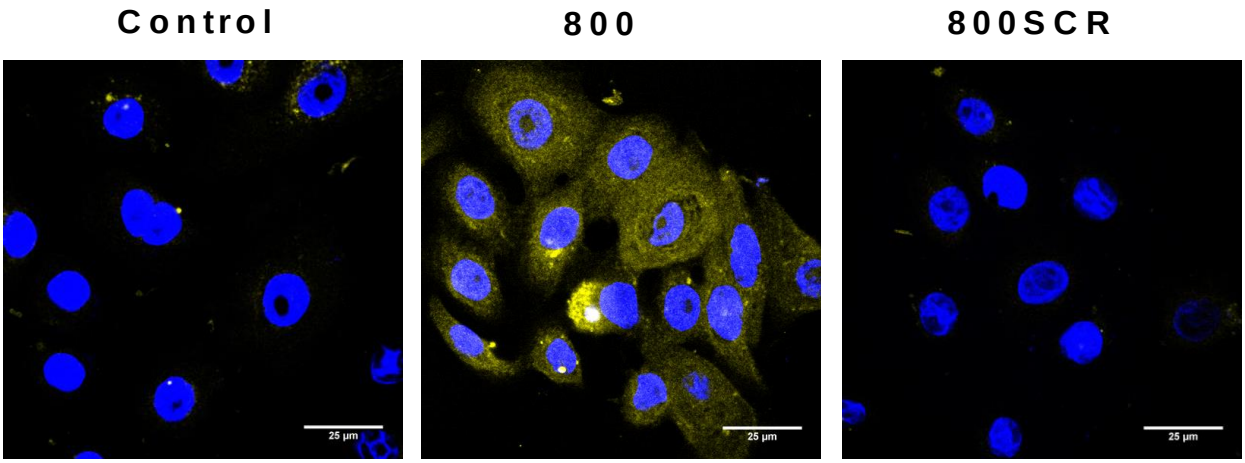


Figure 3.10: Peptide 800 Interacts with the Cell Surface: Primary human keratinocytes were seeded onto glass coverslips and grown overnight, then treated with 800 peptide or its scrambled control conjugated with a TMR tag or media only control for 30 minutes. They were then fixed in paraformaldehyde and stained with DAPI before imaging by confocal microscopy. 100x objective, scale bar 25µm. DAPI shown in blue, TMR shown in yellow.

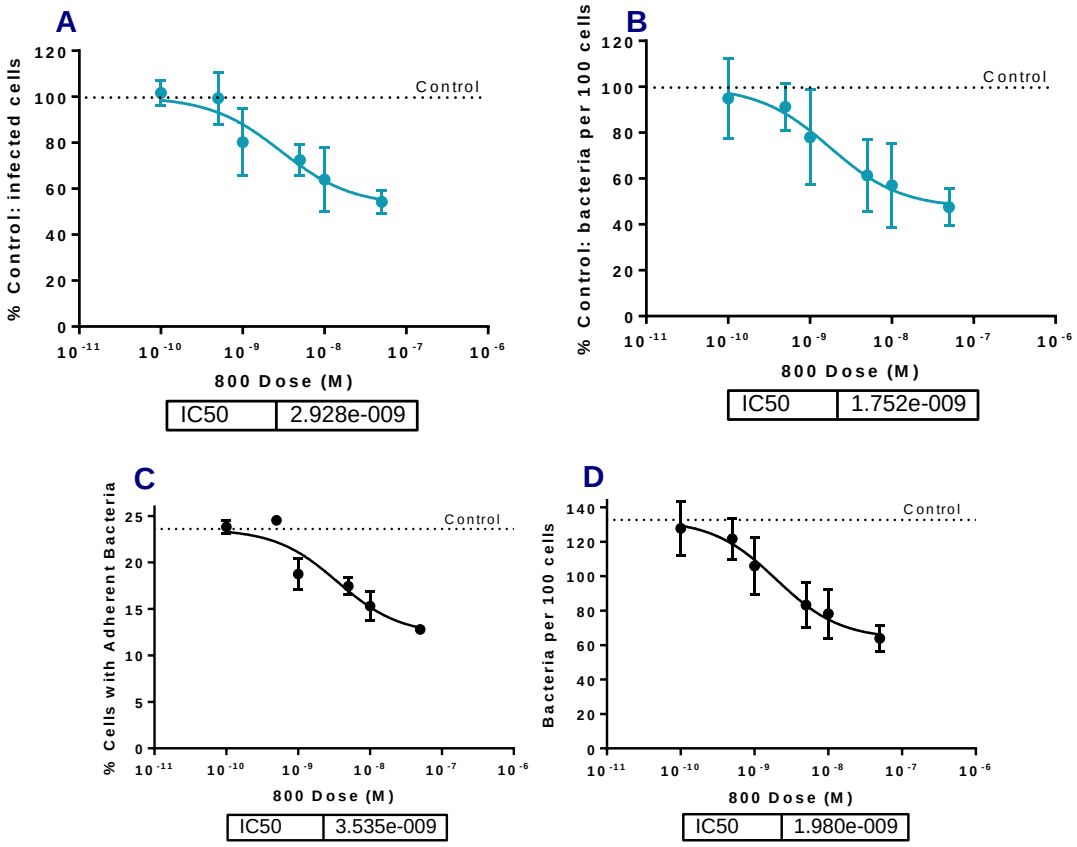


Figure 3.11: Dose Response of 800 Peptide: HaCaT cells were pre-treated with a range of peptide concentrations from 0.1 to 50nM then infected with S235 bacteria at an MOI of 40 for 1 hour. As previous, cells were fixed in paraformaldehyde, stained with DAPI and quantified by fluorescence microscopy. Data analysed by three parameter nonlinear regression, with the top values constrained at no treatment average control figures, and consequently to 100% as appropriate. Control values are indicated with a horizontal dotted line. Goodness of fit R^2 value is 0.42-0.78 suggesting a good fit for the data. When compared with other reasonable fits, this was chosen as statistically the most appropriate. (A) Change in proportion of cells with adherent bacteria (B) Change in total number of bacteria attached to 100 cells (C, D) Non-normalised data represented as mean ± SEM. n=5, duplicate. (A and B) represented as mean ± SD.

range of peptide doses then infected with S235 strain for 1 hour. Cells were then washed to remove non-adherent bacteria and infection quantified by fluorescence microscopy. For each curve a three parameter non-linear regression curve was fitted, and an IC_{50} value calculated. The IC_{50} indicate that the peptides retain 50% function to concentrations as low as $1x10^{-9}$ to $4x10^{-9}$ M. The inhibitory peptide effect is lost at concentrations below $5x10^{-10}$M.

3.2.9 Determining the Duration of the Anti-Adhesive Effect of 800 Peptide

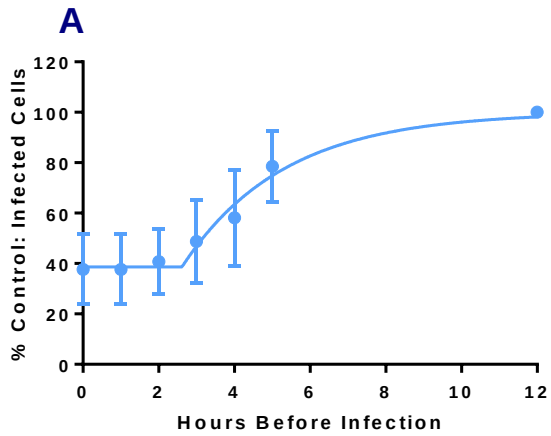
To determine the persistence of the effect of 800 peptide after wash-out, HaCaT cells were treated with 800 peptide or media as a control for an hour, then washed. They were then left in media for 0-6 hours before being infected with S235 strain for 1 hour (Figure 3.12). The data show that a full effect of the peptide in reducing the adherence of bacteria to cells is observed for up to 2.6 hours. For example, when the peptide is added for 1 hour, washed off and replaced with media, then infected 2 hours later, we can still see a significant reduction in the number of cells with adherent bacteria and number of bacteria per 100 cells. The effect begins to be lost after the cells are incubated in media for more than 2 hours, as shown by the one-phase dissociation kinetics following the plateau, the model statistically shown to best fit the data, however the effect is not completely lost until approximately 8 hours after wash-out.

3.2.10 Effects of Peptides on Bacterial Growth and Viability

S235 strain *Staphylococcus aureus* were grown in the presence or absence of 200nM peptides over 48 hours to determine if the tetraspanin therapy was having any effect on bacterial viability, growth or proliferation (Figure 3.13). No reduction was seen in these factors over a 48 hour period, confirming that the peptides work by targeting the host as opposed to the pathogen.

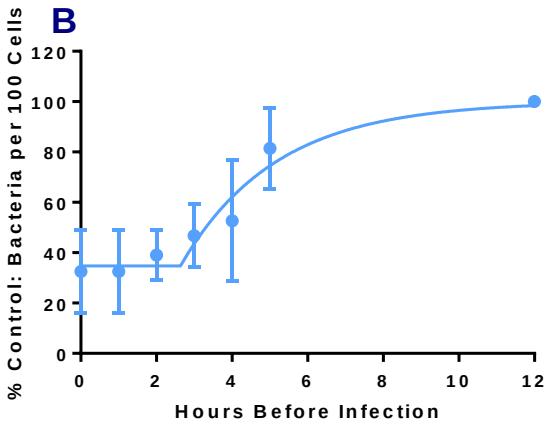
3.2.11 Construction of a 3D Tissue Engineered Model of Human Skin (TEskin)

Although cell lines are a good preliminary method for determining the effects of the tetraspanin based treatments in a simple system, they cannot tell us much about how the therapy would work in a bigger system such as skin. To this end, a 3D tissue engineered skin model was constructed (Shepherd et al., 2009), and optimised as a system to represent



Goodness of Fit	
Degrees of Freedom	34
R square	0.5738
Absolute Sum of Squares	7233
Sy.x	14.59

Best-fit values	
X0	2.615



Goodness of Fit	
Degrees of Freedom	34
R square	0.5635
Absolute Sum of Squares	9142
Sy.x	16.40

Best-fit values	
X0	2.635

Figure 3.12: Anti-Adhesive Effects of 800 Peptide are Retained for More Than 2 Hours: HaCaT cells were treated with 200nM 800 peptide for 1 hour at different time points, then washed off and re-suspended in media until infection. S235 strain *S.aureus* was then added to all wells at the same time at an MOI of 40 for 1 hour, then washed off, fixed in paraformaldehyde, and adherent bacteria quantified by fluorescence microscopy. (A) Shows the change in the proportion of infected cells. (B) Shows the change in the number of bacteria per 100 cells. Data normalised as a percentage of a no treatment control figure and plotted as Mean ± SD. n=3, duplicate. Data is modelled as a plateau followed by one phase association.

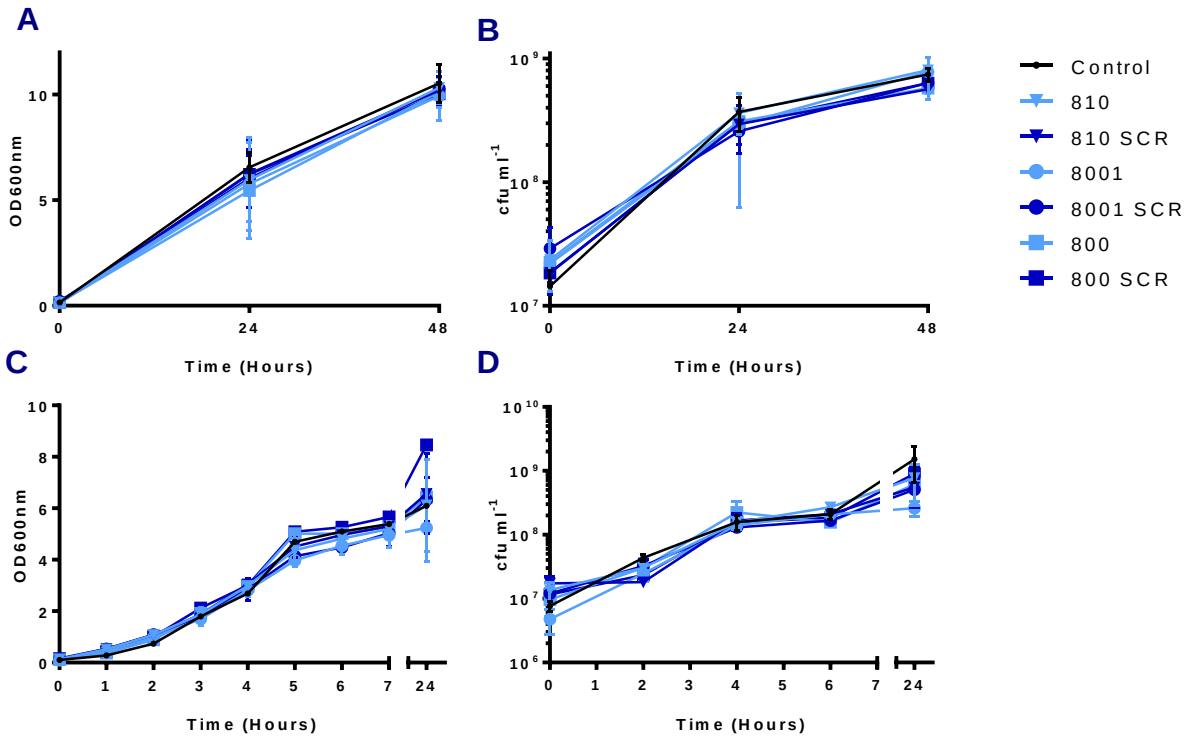


Figure 3.13: Peptides Do Not Impact Bacterial Viability: S235 strain *Staphylococcus aureus* was grown for 48 hours in the presence of 200nM peptides or their scrambled controls. Optical density measurements were taken at 600nm, and colony forming unit measurements were taken by serial dilution followed by viable counts. n=3, data analysed by two-way ANOVA with Sidak's multiple comparisons, no significance found. Data displayed as mean ± SEM. (A and B) Growth over 48 hours. (C and D) Growth over 24 hours. (A and C) Optical density of bacteria. (B and D) Viable counts.

Staphylococcus aureus infected wounds in human skin. TEskin mimics the tissue structure of normal adult human skin and can be used to analyse the penetration of peptides and bacteria, and the effectiveness of the peptides in a more relevant model. Figure 3.14 (A) shows a cross section of TEskin after 14 days growth at air-liquid interface (ALI) stained with haematoxylin and eosin. The cells migrate and differentiate within the decellularised dermal scaffold to form a fibroblast-seeded dermis, an epidermis consisting of granular, basal and spinous keratinocytes, and a fully stratified stratum corneum. When the model is wounded, much of the epidermis is lost (B) and the dermis is exposed. At the periphery of the wounded area, lower levels of the epidermis are exposed leaving undifferentiated keratinocytes susceptible to infection.

After 1 hour of 800 peptide treatment, bacteria are added to the top of the wounded TEskin and allowed to adhere for 5 hours. The TEskin is then washed to remove non-adherent bacteria, and the remaining bacteria allowed to grow and infect until 24 hours after wounding, when it was washed again. The TEskin is then fixed, and bacteria can be visualised by indirect antibody staining and the nuclei by DAPI staining (Figure 3.15). Even after 24 hours, infecting bacteria are localised to the surface of the skin, with some infecting the remaining epidermis, but very few penetrating deeper into the dermis.

3.2.12 Peptide 800 treatment Reduces Staphylococcal Adherence to TEskin

TEskin was wounded with 3 seconds of burning, pre-treated for 1 hour with 200nM 800 peptide, and then infected with S235 bacteria. At 6 hours post-burning, non-adherent bacteria were washed off, and the remaining adhered bacteria allowed to proliferate for a further a further 18 hours before the skin was treated with saponin, to isolate the bacteria viable cell counts (Figure 3.16). Treatment with 800 peptide reduced the quantity of adherent bacteria to around 52% of the untreated control value. This reduction in bacteria indicate a less severe infection in the pieces of TEskin that were treated with peptide, suggesting a possible role for the peptides as an anti-adherence therapy candidate for development. This reduction in infection was not reflected in the 800SCR peptide, suggesting a specific effect.

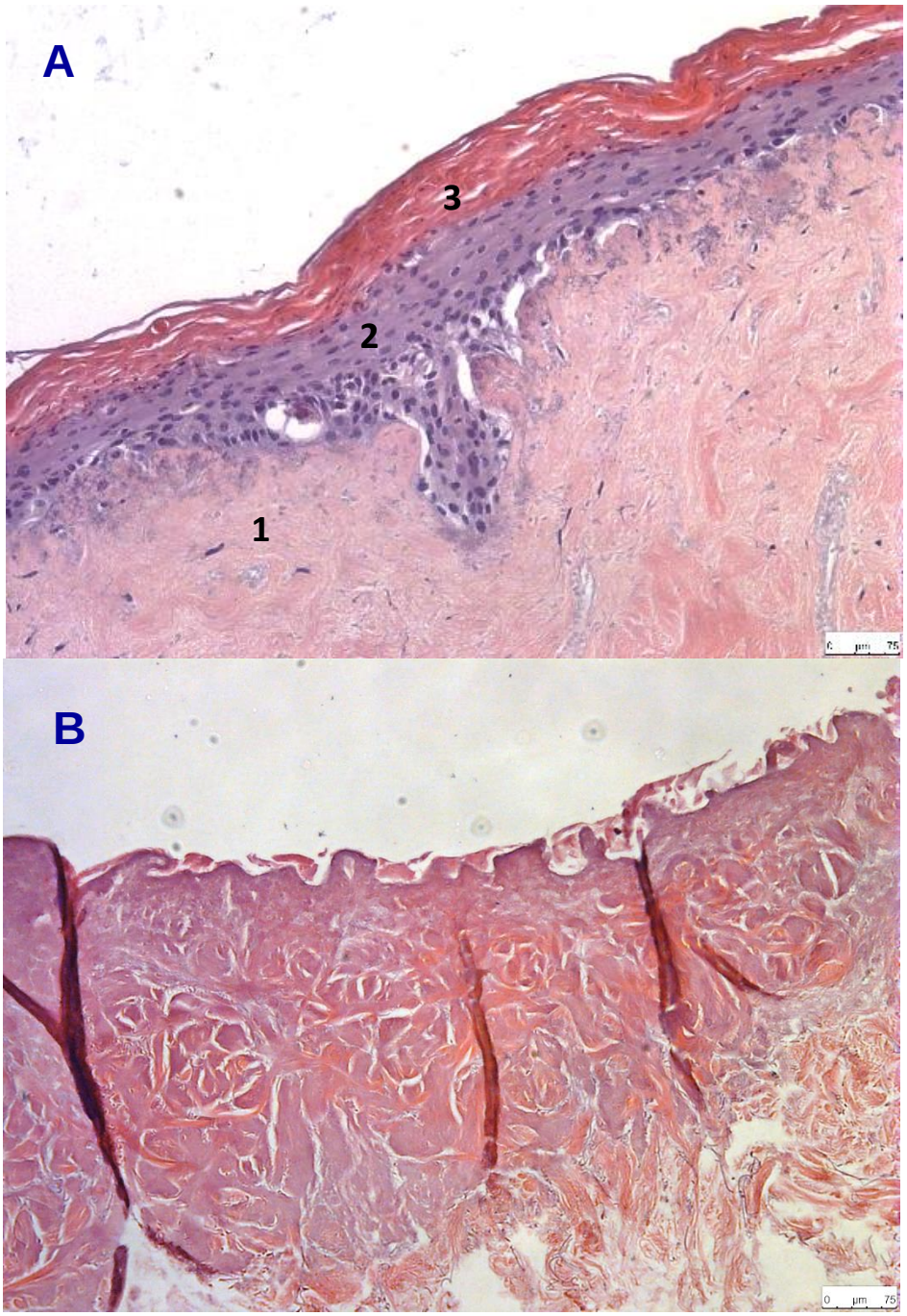


Figure 3.14: Construction of a 3D Tissue Engineered Model of Human Skin (TEskin): In order to test the peptide effects in a more relevant model, a tissue engineered model of human skin (TEskin) was generated as shown in section 1. This model is made of primary keratinocytes and fibroblasts from human biopsies, seeded back on decellularised dermis (DED). The cells migrate to their appropriate layers, with fibroblasts seeding the dermis (1), and keratinocytes forming the epidermis (2), migrating upwards and stratifying to produce the stratum corneum (3). A small amount of damage in the form of keratinocytes detaching from the basement membrane is observed, caused by the process of sample processing and embedding. (B) After 2 weeks growth at air-liquid interface a wound is generate in the TEskin by burning, which removes the epidermis and damages the tissue, allowing for penetration by the bacteria. Paraffin wax embedded TEskin sections of 8µm stained with haematoxylin and eosin, 20x magnification, scale bars 75µm. Small dust particles are visible in the background of the slide.

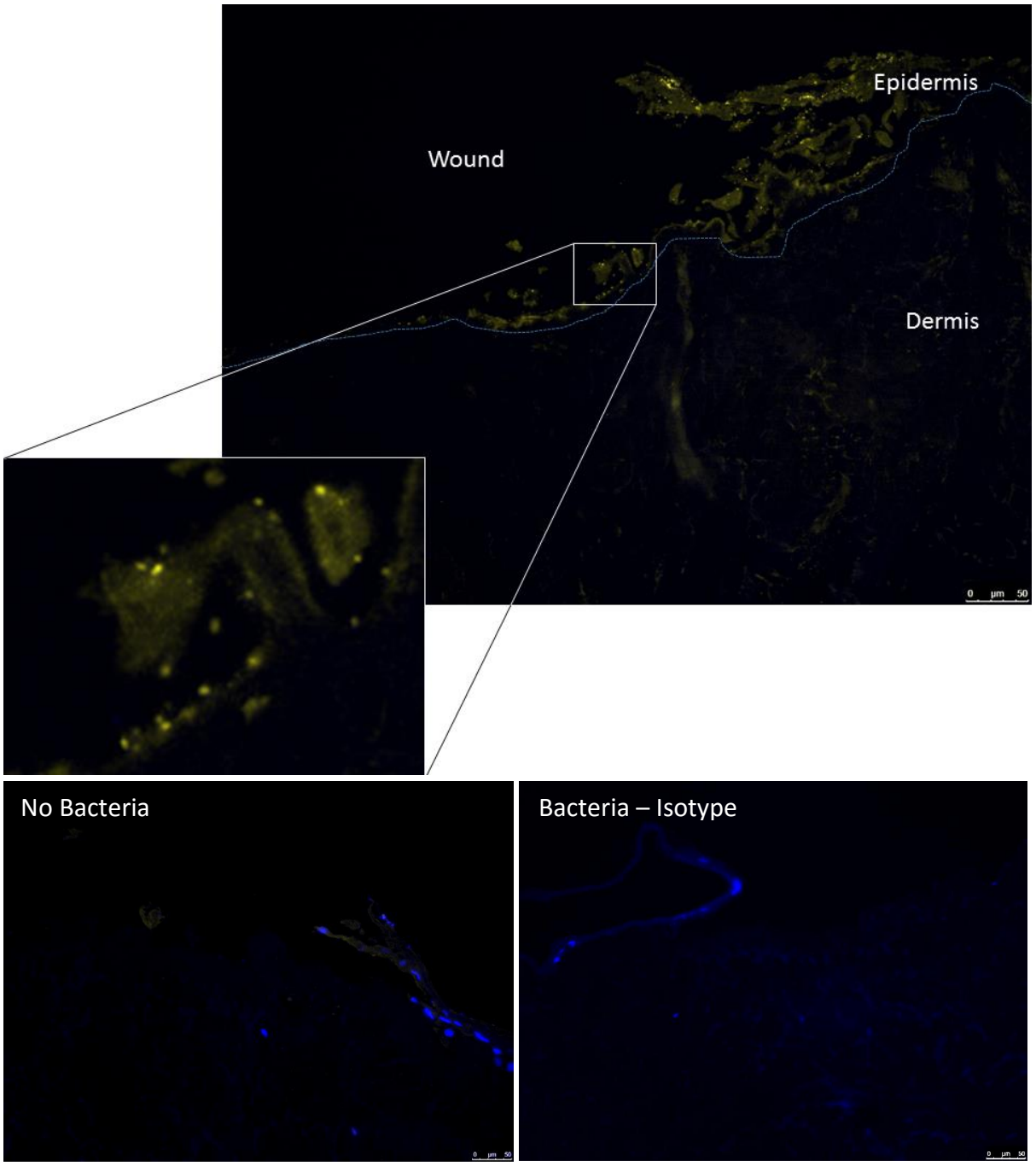


Figure: 3.15: Bacterial Infection of a TEskin Wound: TEskin was wounded by burning, infected for 24 hours with S235 stain *Staphylococcus aureus*, then washed to remove non-adherent bacteria. Samples were fixed, sectioned, and stained indirectly using a rabbit anti-staphylococcal antibody and an Alexa Fluor® 568 tagged anti-rabbit secondary (yellow) then mounted in Vectashield® mounting medium with DAPI as a nuclear stain (blue). The blue dotted line indicates the approximate location of the dermal:epidermal junction, or basement membrane. Scale bars: 50µm.

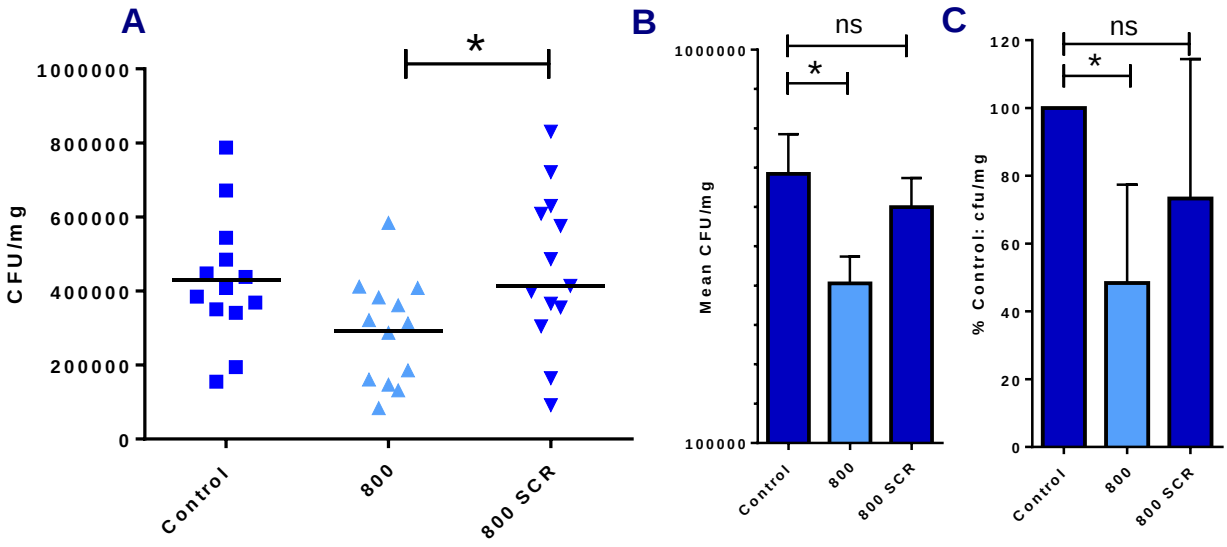


Figure 3.16: Peptide Treatment Reduces Infection of a TEskin Model: TEskin was constructed as in section 2 using tissue culture inserts. It was then burned to generate a wound, treated with 200nM peptide, then infected with $1x10^7$ S235 strain *Staphylococcus aureus* per TEskin. 24 hours after the initial wound was generated, each TEskin was cut in half, weighed, and viable counts taken to ascertain proportion of adherent bacteria in the tissue. (A) All replicates bacterial isolated per mg of tissue. (B) Mean CFU/mg. (C) Data plotted as a percentage of no treatment control figures. (A and B) Data are presented as mean ± SEM (C) Data presented as mean ± SD. Data are normally distributed as determined by D'Agostino-Pearson Omnibus Normality Test. n=3-6, triplicate, data analysed by Ordinary One-way ANOVA, with Sidak's multiple comparisons post-test. Percentage data was transformed by $Y= \log_{10}Y$ before analysis. * $p \leq 0.05$.

3.2.13 Construction of Simple Epidermal Cultures as an Alternative Model for Infection

An alternate model to the 3D DED model of TEskin is a simpler multi-layered epidermal model, which only requires keratinocytes. In this model, cells are seeded onto a semi-permeable membrane insert and allowed to proliferate into a confluent monolayer. Their media is then switched to one that allows stratification and the formation of a multi-layered epidermal structure. After 14 days at air-liquid interface, the epidermal cultures can be tested on.

Epidermal cultures made with primary keratinocytes were compared to those made with N/TERT immortalised keratinocytes at a passage number of 58, and these cultures had a similar thickness and morphology, showing multiple layers or gradually differentiating keratinocytes with a fully formed stratum corneum (Figure 3.17A). Part of each membrane culture was removed from its membrane, stained with DAPI, then mounted onto slides for viewing with confocal microscopy (Figure 3.2.17B). A 3D reconstruction shows the bacteria localised to the top of the epidermis, with a gap of cellular material not containing DNA (the stratum corneum) visible between the bacteria and the upper keratinocyte nuclei. The bacteria are not seen to penetrate further than the surface in the skin infection model. The epidermal cultures could not be wounded due to their depth and fragility.

To assess if these epidermal culture models could be used for studies of bacterial infections of a compromised barrier, cultures were also made using N/TERT cells containing silencing RNA for the knockdown of filaggrin, mimicking a mutation often found in patients with atopic dermatitis. These epidermal cultures showed a similar morphology to those made with wild type N/TERTs and with primary cells. In these cells however bacteria may penetrate more deeply into the cell layers (data not yet quantified).

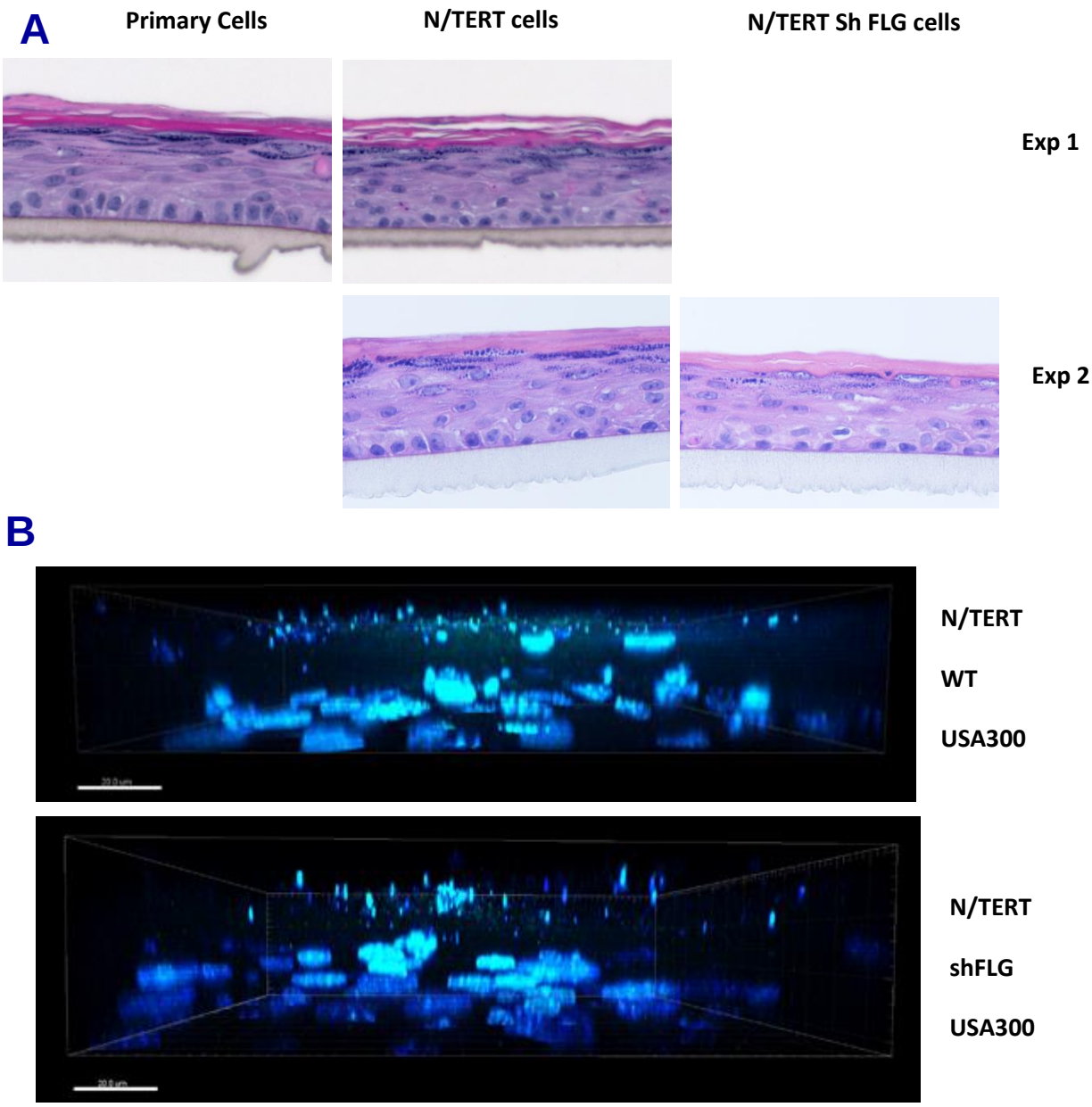


Figure 3.17: Epidermal Cultures as an Alternate Skin Model: Primary keratinocytes, N/TERT immortalised keratinocytes or N/TERTs containing filaggrin silencing RNA were grown as a monolayer on a porous membrane (Thermo scientific, Nunc, 0.4µm pore) at a density of $3x10^5$ cells per insert until a confluent monolayer was formed. They were then switch from 3D priming to 3D barrier medium (CELLnTEC), and the following day moved to air liquid interface. After 14 days' growth they were sent for histological analysis or infected with USA300 strain *Staphylococcus aureus*. (A) Histology: Samples fixed in paraformaldehyde were rehydrated and stained with haemotoxylin and eosin as previous, by the IMCB institute of molecular and cell biology, Singapore. 40x magnification. Microscopy carried out by Dr Vijaya Chandra Shree Harsha. (B) 3D reconstructions: Samples were stained by submersion in DAPI for 15 minutes, then mounted onto slides with HydroMount. Stacked images were taken with an Olympus FV1000 upright confocal microscope and reconstructed using ImageJ and Imaris software. 100x magnification. Scale bar 20µm. Based on size and stain distribution, smaller spots represent bacteria on the surface of the skin, larger patches of signal indicate cell nuclei.

3.3 Discussion

In this chapter it has been shown that peptides based on sequences of CD9 EC2 domain reduce the adherence of various strains of *Staphylococcus aureus* to HaCaT cells and human epidermal keratinocytes, and finally to a tissue engineered model of human skin. This effect is not observed with scrambled control versions of these peptide made at the same time by the same supplier, which contain the same amino acids in a random order, suggesting that the anti-adhesive effect is tetraspanin specific and not a non-specific peptide effect. This effect was also not seen when bacterial adherence was observed with flow cytometry, which used different cell and bacteria numbers, and was exposed to greater flow pressure (appendix). It has also been deduced that the peptides are causing this effect by interacting with host cells as opposed to the bacteria, as no effect of prolonged 200nM peptide treatment is seen against S235 strain staphylococcal growth or viability over 48 hours.

The correlation of CD9 expression and efficacy of treatment leads us to hypothesise therefore that the mechanism of action of the treatment is CD9 related, and that the peptides, similarly to the EC2s, have an indirect mode of action, and may work by disrupting the tetraspanin web structures by disrupting these CD9 interactions (Barreiro et al., 2008, Green et al., 2011). Previously, cholesterol depletion by Methyl-β-cyclodextrin has been linked to disruption of TEMs, and dual treatment with the peptides did not further reduce bacterial adherence, suggesting that when TEMs are already disrupted by cholesterol depletion the peptides no longer have an effect, and that therefore the peptides also work by disrupting TEMs (Cozens, 2016). To further elucidate the mechanism of action of these peptides, super-resolution microscopy could be used to visualise the distribution of TEMs on addition of peptides or EC2s, or the localisation of Staphylococcal host cell receptors with or without peptide treatment and infection.

Pre-treatment with the peptides and EC2 domains has proven effective on multiple different cell types, as outlined in table 3.1. Within this study, we have shown that the efficacy of these peptides is directly correlated with the CD9 expression on those cells, and that cells, for example fibroblasts, with low cell surface CD9 do not exhibit a strong reaction to the peptides.

CD9 however is expressed constitutively across many cell types, suggesting the peptides could have a broad range of therapeutic actions, for example in the prevention of vascular smooth muscle (high CD9 expressing cells (Dechend et al., 1999, Scherberich et al., 1998)) infection by *Chlamydia pneumoniae* (Dechend et al., 1999), or in the prevention of urinary tract infections caused by *E. coli.*

As well as reducing bacterial adherence to various cell lines, this treatment has been shown to be successful against several different bacteria, including *Escherichia coli* and *Salmonella enterica*. In this study different levels of inhibition have been seen with different strains of *Staphylococcus aureus*. The greatest reduction in bacterial load was observed with the two more virulent strains of *S. aureus*: S235 and JE2. These both originate as clinical isolates and are therefore the sort of strains that a new anti-microbial treatment must be effective against, more so than the SH1000 lab strain. Without a full genome study it is difficult to draw a conclusion as to why the treatment should be more effective against the clinical strains, which by natural selection should be some of the best at adhering to the host. We speculate that the difference in peptide efficacy could be due to a heavier reliance of SH1000 strain on CD9 independent mechanisms of adherence. Alternatively, a large build-up in mutations in a long standing lab strain such as SH1000 due to repeated subcultures could also enhance, diminish or change genes involved in adherence.

Although a significant effect was seen with the peptide, generally up to 50% of cells still have adherent bacteria despite pre-treatment. This, combined with the observation that there is no change in the number of bacteria per cell, suggests that a subset of cells exist in each population which are resistant to the peptides. These cells could be presenting surface markers more suitable to different forms of staphylococcal adherence, or their CD9 may be unavailable for other reasons related to cell cycle, as it has previously been shown that levels of tetraspanins on the cell surface changes as cells progress through the cell cycle (Green, 2010).

Dose response and retention of effect experiments show that the peptides display positive pharmacological properties. Peptide 800 is effective against S235 adherence to HaCaT cells down to a concentration of 1.8×10^{-9}M, and this effect remains up to 2 hours after the treatment is removed. This supports the conclusion that the peptides could be a suitable

candidate for clinical development, as they would still be active throughout wound dressing changes, and work potentially even when diluted with would fluids.

Another positive result indicating the potential usefulness of the peptides as a therapy is the reduction of bacterial load observed in the TEskin model pre-treated with 200nM peptide. A 50% reduction from the untreated control is observed with 800 peptide against S235 clinical isolate infection. This infection would consequently be easier to then clear with normal biological wound clearing processes, such as shear force from the wound effluent and antimicrobial peptide secretion.

In the cell line model, peptide treatment did not reduce the proportion of fibroblasts with SH1000 strain *Staphylococcus aureus*, indicating that this cell type is immune to peptide treatment. The observation that the peptide treatment was successful in the wound model therefore indicates that the therapy is having an effect in the area in which keratinocytes have become exposed to the bacteria, at the periphery of the wound, as opposed to at the centre of the wound where the material is mostly bare dermis and fibroblasts. Immunohistochemistry from the TEskin indicated however that S235 *Staphylococcus aureus* infect more strongly at the areas at the periphery of the wound, which can go some way to explaining the 50% reduction in this model.

This model is a better representation of the normal pathological niche of *Staphylococcus aureus* than monolayer cell models, with the same basic morphology as an acute burn. This model also retains good barrier function as shown previously by Shepherd et al (Shepherd et al., 2009): neither *Staphylococcus aureus* nor *Pseudomonas aeruginosa* could infect the model without a wound to act as a gateway for infection (MacNeil et al., 2011). It has been shown in chapter 4 to secrete some of the same cytokines found elevated in wounds, and it can be assumed that the keratinocytes around the wound will also secrete anti-microbial peptides.

The TEskin model presented here however has limitations in its application as a full skin model due to its simplicity. The model lacks immune cells, and many natural structural features such as hair follicles and sweat glands. Work is ongoing to improving the model to include components such as immune cells. For example, Chau et al embedded dendritic cells into an agarose-fibronectin gel and sandwiched this between an epidermal scaffold seeded with

keratinocytes and a dermal scaffold seeded with fibroblasts to form 'Immunoskin' (Chau et al., 2013). Additionally, HDMEC cells have been added to the skin grafts to introduce low levels of vascularisation, which could lead to more complex skin models (Supp et al., 2002).

Construction of the TEskin also relies heavily on a constant supply of fresh skin biopsy for the retrieval of sufficient cells and dermal scaffold material. This however has been a limiting factor of this project, with little availability throughout the research. Recent studies indicate that alternate scaffolds could potentially be used as an alternate to DED, such as collagen (Venkatesan et al., 2015, Held et al., 2015) or electrospun fibres (Blackwood et al., 2008), however unlike DED these options will not contain the complex basement membrane structure. Additionally a simple 3D membrane culture has been developed and marketed by CELLnTEC which contains just keratinocytes, but grown into multiple layers and formed into a fully stratified barrier (Section 3.2.13) (Ananta et al., 2012). Immortalised keratinocytes such as N/TERT immortalised keratinocytes can provide an equal barrier function to primary keratinocytes, without the limitations of low population doubling potential (van Drongelen et al., 2014). As of the time of submission, a fully comprehensive study comparing all the methodologies has not yet been undertaken, leaving the primary model used here as the most characterised and consistent model used within the lab.

The use of immortalised cells for the construction of 3D skin models however has opened up new avenues for studying the effect of mutations on development and retention of an active skin barrier. For example, Atopic Dermatitis (AD), also known as Eczema, is characterised by itchiness, dryness of the skin and a pre-disposition to bacterial infections. This disease phenotype is caused by a variety of genetic and environmental factors, and is frequently characterised by mutations in the filaggrin gene expressed in keratinocytes. Mutations mirroring AD can therefore be introduced into N/TERT keratinocytes using siRNA much more simply than into primary cells, and these immortalised AD cells can then be used to study barrier function in a 3D model, as seen in section 3.2.13 (van Drongelen et al., 2013). As AD sufferers are susceptible to infection this model could highlight some differences in skin architecture and methods of invasion by pathogens such as *Staphylococcus aureus* or *Pseudomonas aeruginosa*.

Overall, data presented here shows that pre-treatment with all peptides reduced the adherence of Staphylococci to host cells and that peptide 800 can reduce the adherence of S235 strain to TEskin. This effect however is limited to around a 40-60% reduction, which is insufficient alone for the removal of pathogens such as MRSA. Therefore, work was carried out in chapter 5 to improve the efficacy of the peptide, and to test for any synergistic effects with MSSA treatment antibiotics. Another avenue of exploration is testing for any obvious side effects on cells, as targeting a multifunctional tetraspanin such as CD9 could have effects on metabolism, cell proliferation, migration, and wound healing.

Chapter 4: Expression of Tetraspanins and Toxicity of Peptides

4.1 Introduction

4.1.1 Tetraspanins

As outlined previously, tetraspanins are a superfamily of membrane proteins expressed in a range of eukaryotes, with 33 known members in humans. Tetraspanins associate with each other in the membrane via membrane-proximal palmitoylation sites as well as with other cell components including proteins and lipids, in order to form specialised microdomains known as TEM (tetraspanin enriched microdomains) (Charrin et al., 2002). TEM have been implicated in many cell functions, including cell adherence and fusion, membrane trafficking, endocytosis and motility (Berditchevski and Odintsova, 2007, Hemler, 2005).

4.1.2 Tetraspanin Expression

Tetraspanins have been observed in nearly all cell types in most genotyped eukaryotic species. They are mostly membrane localised, but additionally, they are also commonly found in exosomes. Some tetraspanins, such as CD81 and CD9, are known to be expressed constitutively, whereas others are highly regulated, based on cell type and conditions. For example, the spinal cord expresses high levels of CD9, CD81 and CD151, but little CD63 whereas, conversely, CD63 is highly expressed in platelets, in which only mid-levels CD151 are observed, and CD81 is not detected at all. Other tetraspanins are expressed in just one specific cell type such as CD37 which is expressed almost exclusively in lymphoid cells (Wilhelm et al.,

2014, Kim et al., 2014, Wang et al., 2015). Human tissue expression patterns for common tetraspanins CD9, CD63, CD81 and CD151 studied here are outlined in Figure 1.6. These 4 tetraspanin proteins have been identified on nearly all *Homo sapien* tissue types in varying levels (Wang et al., 2015).

Previously it was shown that tetraspanins interact in clusters with other tetraspanins and partners, however with the development of super resolution microscopy this view has been challenged. A recent report published by van Spriel et al contradicted the previous understanding of TEM, by demonstrating that each TEM is composed of predominantly of one type of tetraspanin and its partner molecules, and there are interactions between these and other tetraspanin abundant microdomains, however there is not a large amount of mixing of tetraspanins within domains, as was previously thought (introduction – figure 1.8) (Zuidscherwoude et al., 2015). This study however was only conducted on membrane fractions rather than intact cells and therefore is not yet widely accepted.

4.1.3 CD9 Functions and Keratinocytes

The majority of the work in this study focuses on the tetraspanin CD9. One of the most characterised tetraspanins, CD9 has a wide variety of implicated functions outlined in section 1.6.1, including immune functioning, cell motility and adhesion.

CD9 has also been shown to affect certain facets of keratinocyte function. For example, keratinocyte migration, a critical process in wound healing, can be up-regulated by anti-CD9 antibodies (Jiang et al., 2013). CD9 has also been implicated in the juxtacrine growth and proliferation of keratinocytes, with anti-CD9 antibody ALB6 reducing this growth by up to 50% (Inui et al., 1997). Additionally CD9 is seen to be upregulated in squamous cell carcinomas suggesting a role in tumour expansion and invasion in skin cancers (Ach et al., 2010).

4.1.4 Tetraspanins and Immunity

The immune system is a complex system for protection against invading pathogens and is outlined in Figure 1.4. Tetraspanins are known to be heavily involved in the immune system, in immune cell migration (Gartlan et al., 2013), the organisation of immune signalling complexes on the cell surface (Unternaehrer et al., 2007), antigen presentation(Jones et al., 2012), and the secretion of signalling molecules (Zumaquero et al., 2010, Murayama et al.,

2008, Levy and Shoham, 2005), with CD9 being involved directly with many of these processes. Due to these numerous interactions, molecules that interfere with normal CD9 function could have detrimental effects on normal immune function. To test for these effects, we can test for differences in the expression of cytokines, the signalling molecules of the immune system.

4.1.5 Tetraspanin-mediated Toxicity

Along with the immune system, targeting tetraspanins could interfere with other aspects of normal host function such as cell migration, wound healing and cell metabolism.

Some preliminary data on peptide effects on cell metabolism can be ascertained from simple cell line assays. Various metabolic stains can be used to indicate cell viability or quantity such as MTT, Sulforhodamine B, and Resazurin Blue. Scratch assays, in which cells are grown to a confluent monolayer and then scratched with a 200µl pipette tip, producing a 'wound' which cells can migrate to cover, indicate effects on both cell proliferation and migration. The addition of a proliferation inhibitor allows distinguishing between the two. An alternate assay for migration is a membrane migration assay which measures the rate at which cells migrate through a membrane.

Additionally, the 3D tissue model outlined in section 1.5.2, can be used to test for toxic effects in a more complex system, in which the 2 major skin cell types interact with each other as well as with the external environment, however it will only provide information on the response of keratinocytes, fibroblasts and extracellular matrix. This model can be used to study cytokine production, as well as presenting a more complex model for wound healing that the scratch assay mentioned above.

Another model system used here to study peptide toxicity and anti-adhesive effects is that of the Zebrafish, *Danio rerio,* whose early immune system is similar to our own (Nuesslein-Volhard et al., 2002). These fish have previously been used as a model to test the effects of small systemic drugs and other therapies. Prajsnar et al have expanded the range of this technique and developed a model in which the fish are injected with *Staphylococcus aureus* to establish a late term systemic infection, and then treated with potential therapeutic agents (Prajsnar et al., 2008). This is a simple and rapid way to detect any whole-organism toxicity

associated with the compound being tested; however, it is not a full reflection of a human system.

4.2 Results

As mentioned above, tetraspanins are expressed on a wide range of cell types. Here we look at the expression levels in the main cell types in skin and in HaCaT cells, a human epidermal keratinocyte cell line, using flow cytometry and fluorescence microscopy.

4.2.1 Microscopic Analysis of Tetraspanin Expression

Figure 4.1 shows the expression of 4 tetraspanins, CD9, CD63, CD81 and CD151 as viewed by fluorescence microscopy. Cells were grown overnight and labelled with the antibodies stated in section 2 with a FitC labelled anti-mouse secondary antibody. JC1 was used as an isotype control and shows no staining. CD9 is highly expressed on keratinocytes and HaCaT cells but in low abundance on fibroblasts. CD63 is highly expressed on fibroblasts, but not on other cell types. The HaCaT keratinocyte cell line shows expression patterns similar to that of keratinocytes. This would indicate that these cells are a reasonable model for studying tetraspanin function in human keratinocytes.

4.2.2 Flow Cytometric Analysis of Tetraspanin Expression

The expression of tetraspanins was then quantified by flow cytometry, with the same antibodies as above (Figure 4.2). CD9 was highly expressed on keratinocytes, and not expressed on fibroblasts; CD63 is highly expressed on fibroblasts but expressed in low level on keratinocytes. CD81 and CD151 were expressed most on HaCaT cells, and least on keratinocytes. From this data, a difference in tetraspanin expression was observed between keratinocytes from the same donor in their proliferative state (grown in low calcium medium), and in their differentiated state (grown in high calcium media, figure 4.3). Analysis by two-way ANOVA showed that CD9 expression was significantly reduced in differentiated keratinocytes, suggesting that, in skin, CD9 is not evenly distributed across all layers of the epidermis.

To check if the expression levels of tetraspanins were consistent across donors, primary fibroblasts from 4 donors were stained for tetraspanin expression and analysed by flow cytometry (figure 4.4). Fibroblasts were chosen for this assay as they were the most readily

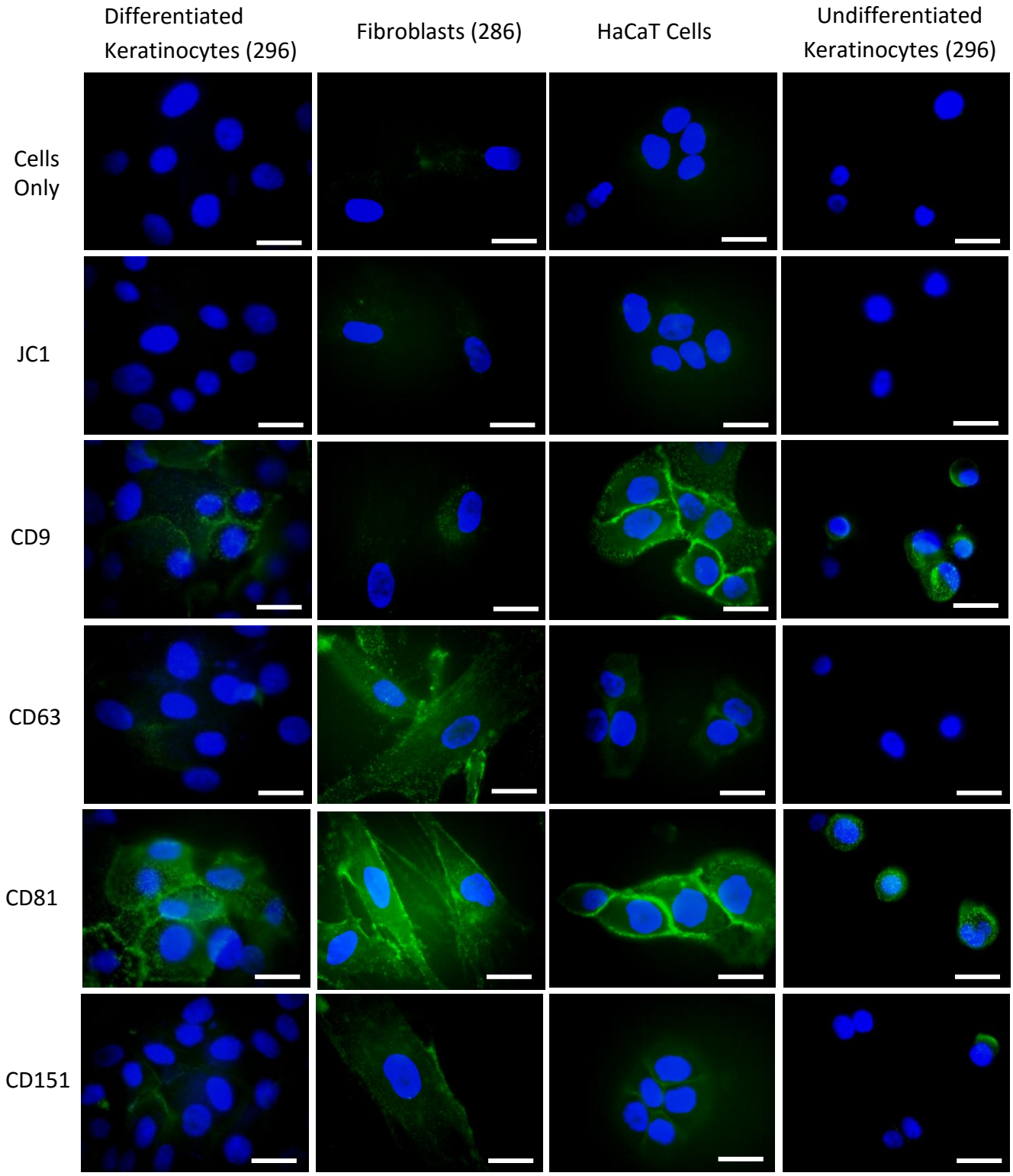


Figure 4.1 Expression of Tetraspanins by Microscopy: Representative images showing the expression of tetraspanins on HaCaT cells, primary dermal fibroblasts and undifferentiated vs differentiated primary epidermal keratinocytes. Tetraspanin expression was observed by indirect antibody staining (outlined in section 2) and visualised by fluorescence microscopy, shown in green. All cells are mounted in Vectashield with DAPI to visualise nuclei (blue) No staining was observed in the JC1 isotype control wells. 100x magnification. Scale bars = 25µm.

available cell type, and the cell type most consistent for growth rate so that cells could be harvested simultaneously. The fibroblasts viewed here share the same pattern with those analysed above in figure 4.2, with very low CD9 expression, high CD81 expression, and mid-levels of CD63 and CD151 expression. This pattern was observed in fibroblasts from all 4 donors. The levels of each specific tetraspanin however varied significantly between donors, for example donor 196 had consistently low tetraspanin expression, whereas donor 109 had higher levels of both CD63 and CD81.

4.2.3 CD9 is Expressed in the Epidermis of TEskin

In order to further hypothesise about the efficacy of the peptide treatment in skin, the expression of CD9 in the skin model was analysed by indirect staining. TEskin was grown for 14 days at air liquid interface then fixed with 3.8% formalin overnight. It was then processed into paraffin blocks and sectioned into 8μm samples. Involucrin, a marker of differentiation was used to help visualise the layers of epidermis. This is primarily expressed in higher, more terminally differentiated layers of keratinocytes. Here, CD9 is also seen to be similarly expressed, with the highest expression being observed in spinal and granular layers of the epidermis of TEskin. Figure 4.5 also shows high CD9 expression observed in sub-epidermal structures such as hair follicles.

4.2.4 Peptides Do Not Have a Negative Effect on Cell Metabolism

As mentioned previously, CD9 is a highly multifunctional molecule, and therefore anti-adhesion therapies targeting CD9, as described in chapter 3, could have a large range of side effects in human cells. A series of tests was therefore undertaken to test for any toxicity of peptide treatment on cell metabolism, proliferation and migration.

Firstly, an MTT stain was used to test for effects on cell viability metabolism. This assay uses the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), which in actively metabolising cells is reduced by oxidoreductase enzymes to its purple form, formazan. This assay gives an indication of any treatment induced effects on cell metabolism (Figure 4.6). After 24 hours' peptide treatment, no reduction in cell metabolism was measured in this assay with any treatment or scrambled control peptide. No effect on cell phenotype was observed by light microscopy (data not shown).

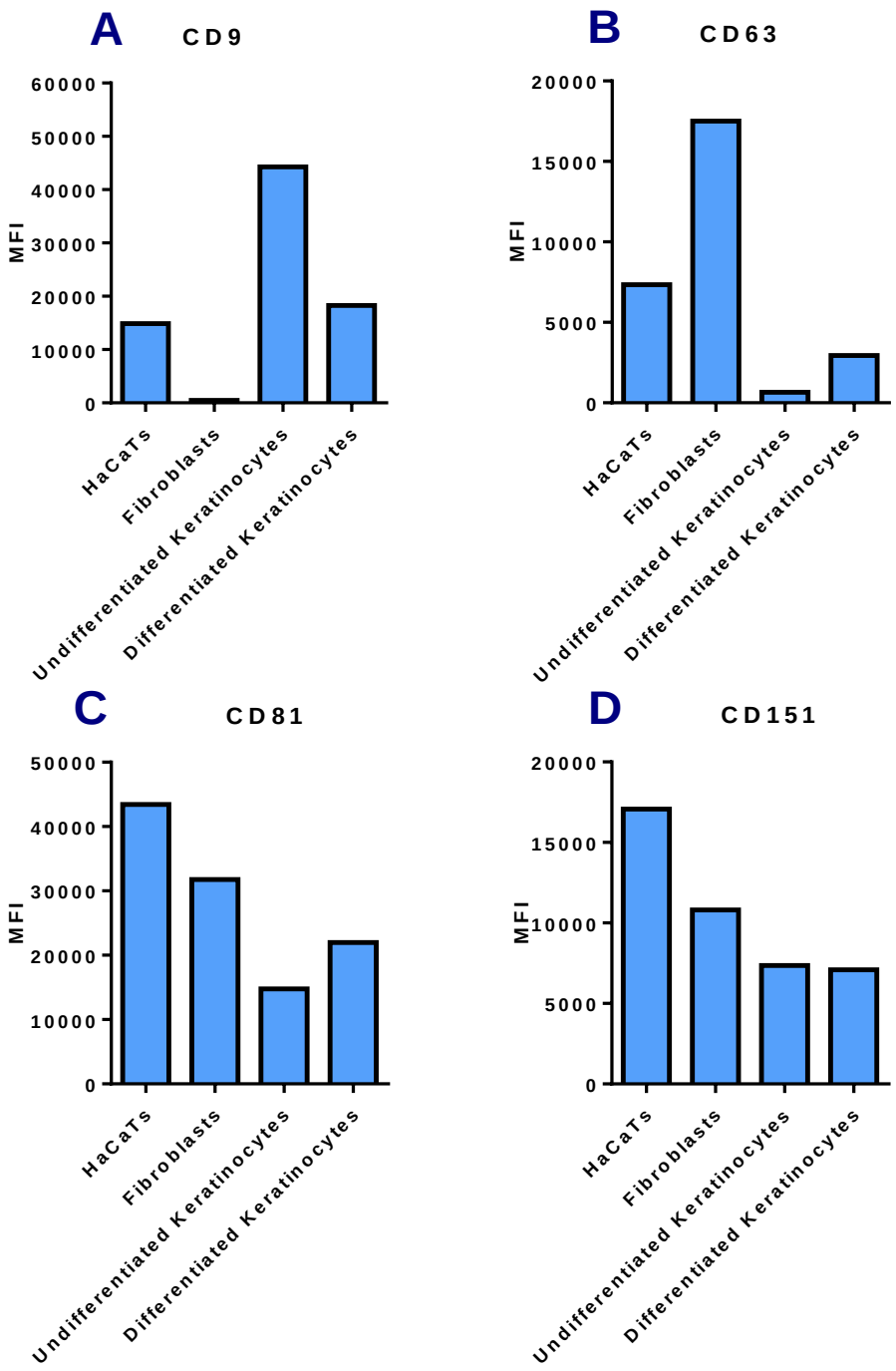


Figure 4.2: Expression of Tetraspanins Observed by Flow Cytometry: The levels of tetraspanin expression were measured by indirect antibody staining as previously described, and quantified by flow cytometry on the Attune Acoustic Focusing Cytometer. Isotype and secondary only controls gave comparable signals to the unstained control, which was subtracted from the positive values prior to analysis. Cells were not gated to distinguish singlets/doublets, due to the small proportion of differentiated keratinocytes existing singularly. (A) CD9 expression, (B) CD63 expression, (C) CD81 expression, (D) CD151 expression. n=1. Gating of cells is demonstrated in Supplementary Figure 3.

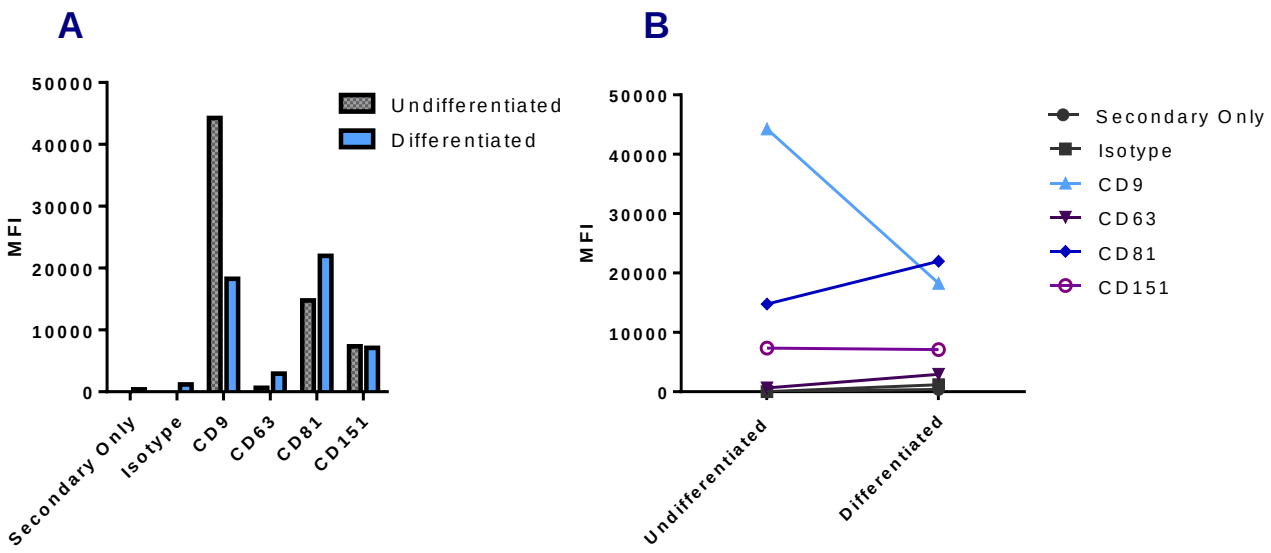


Figure 4.3: Stage of Cellular Differentiation Affects the Expression of Tetraspanins: Cells from the same donor were grown in low calcium (undifferentiated) or high calcium (differentiated) media for 2 days, then stained for tetraspanin expression as previous. Live stained cells were run through the Attune Acoustic Focusing Cytometer, and analysed for size and fluorescence. n=1.

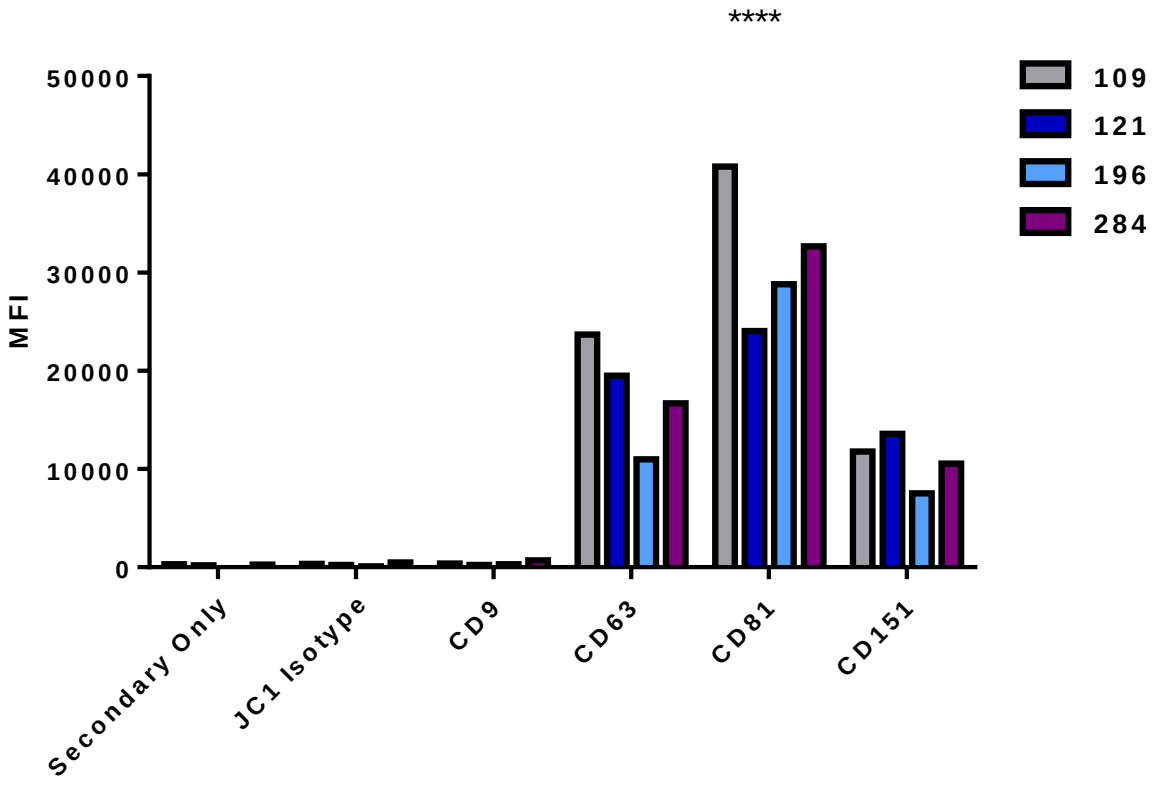


Figure 4.4: Cell Donors Express Differing Levels of Tetraspanins: Primary dermal fibroblasts from different donors (109,121,196,284) were stained for their tetraspanin expression using antibodies detailed in Section 2. n=1.

4.2.5 CD81 EC2 Domain May Have an Effect on Cell Migration, While Peptides Do Not

In 2000, a role for tetraspanins in cell migration and wound healing was first observed by Penas et al (Penas et al., 2000), and later studies found that blocking CD9 with antibodies actually increased HaCaT migration in a cell scratch assay (Jiang et al., 2013, Jiang et al., 2014). Treatment with 500nM EC2:GST fusion domains had no significant effect on cell migration and proliferation when compared to the GST control (figure 4.6). CD81 however did have a small negative effect on scratch healing when compared to the media only control, and also showed a larger range of healing speeds. None of the peptides had any effect when compared to their scrambled controls at 200nM or when compared to a media only control. A small shift in the healing rate of cells treated with 800 peptide is not significant but was deemed worth exploring further as this peptide was selected as an anti-adhesion therapy due to its consistent anti-adhesive effects against virulent strains of *Staphylococcus aureus*.

4.2.6 800 Peptide Does Not Affect Epidermal Migration in TEskin

To determine if 800 peptide can affect the migration and proliferation essential for wound healing, the TEskin model was constructed with the keratinocytes and fibroblasts grown onto DED but contained in the centre of a metal ring until fully formed and stratified. This ring was removed after 7 days at air liquid interface, and the live keratinocytes and fibroblasts allowed to re-epithelise the bare dermis, representative of the exposed dermis of a wound. The area of viable cells was measured by Resazurin Blue staining and ImageJ analysis at 0, 7, and 10 days after removal of the metal ring (Figure 4.8). TEskin was treated with 200nM peptides every two days throughout the experiment. No negative effects on the essential wound healing process were observed in this more complex model at any of the time point observed, and therefore 800 peptide was deemed to not have a negative effect on cell migration and proliferation.

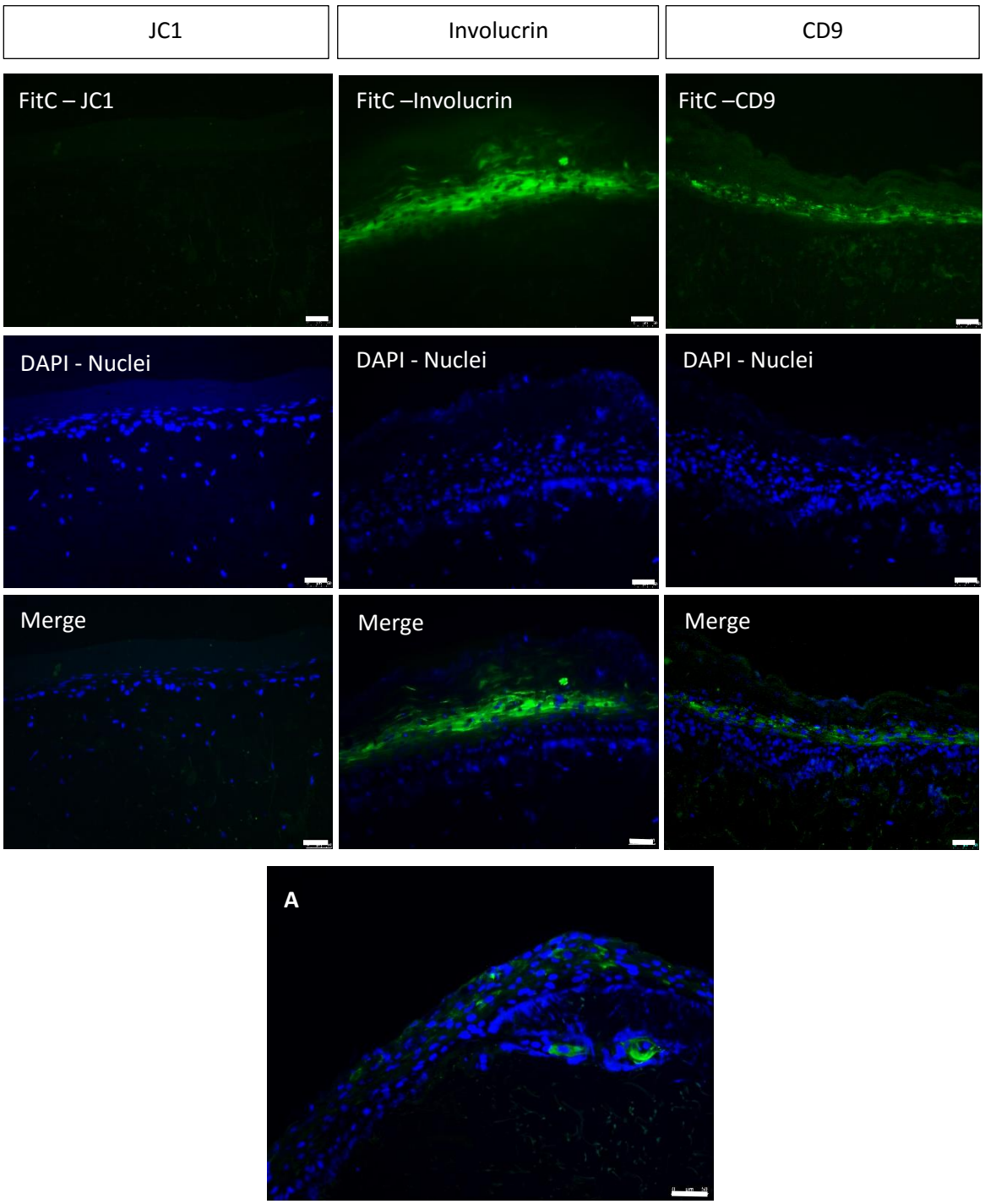


Figure 4.5: CD9 Distribution in TEskin: TEskin was grown at ALI for 14 days, then fixed, processed and paraffin embedded. Sections were then indirectly stained for Involucrin, a marker of keratinocyte differentiation, CD9, or with a JC1 isotype control antibody. DAPI was also used to visualise cell nuclei. The top of the image is the surface of the skin piece, the bottom of the image is the dermis, and so lower cell layers are fibroblasts and basal keratinocytes, and upper layers are stratified terminally differentiated keratinocytes. No staining is observed with the isotype control. 20x objective, exported with LASAF and optimised uniformly with ImageJ. Scale bars 50µm.

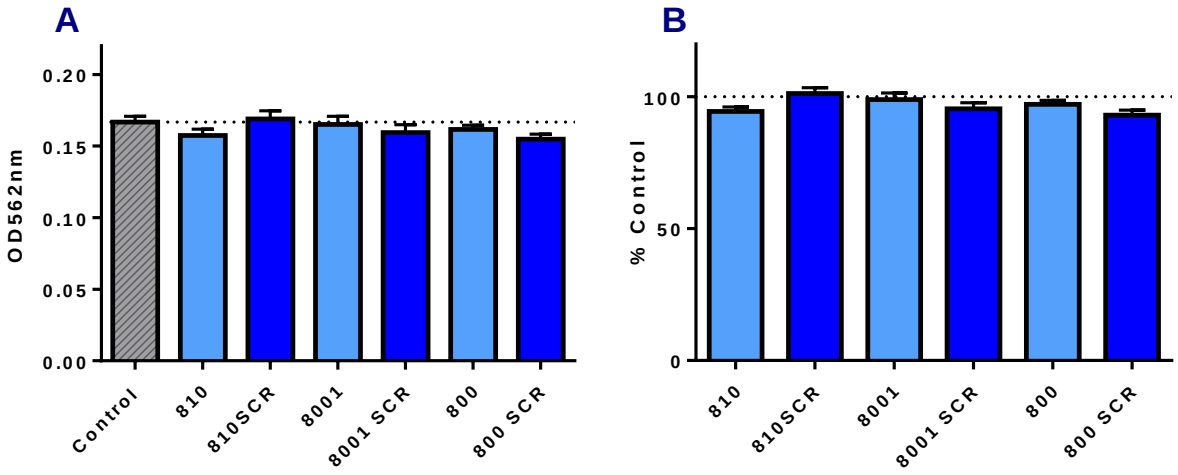


Figure 4.6: Peptide Treatment Does Not Affect Cell Viability: HaCaT cells were seeded at a density of 100,000 cells/well and grown overnight. Wells were then treated with 200nM Peptide for 24 hours, before MTT dye was added. Cells were lysed with 2-ethoxyethanol and the dye transferred to a 96 well plate for analysis by reading at 562nm. (A) Raw data presented at mean ± SEM (B) Normalised as a percentage of the control, presented as mean ± SD. Analysed by One-Way ANOVA with Sidak's multiple comparison post-test. Percentage data transformed as $Y=\log_{10}Y$ prior to testing by ANOVA. n=3.

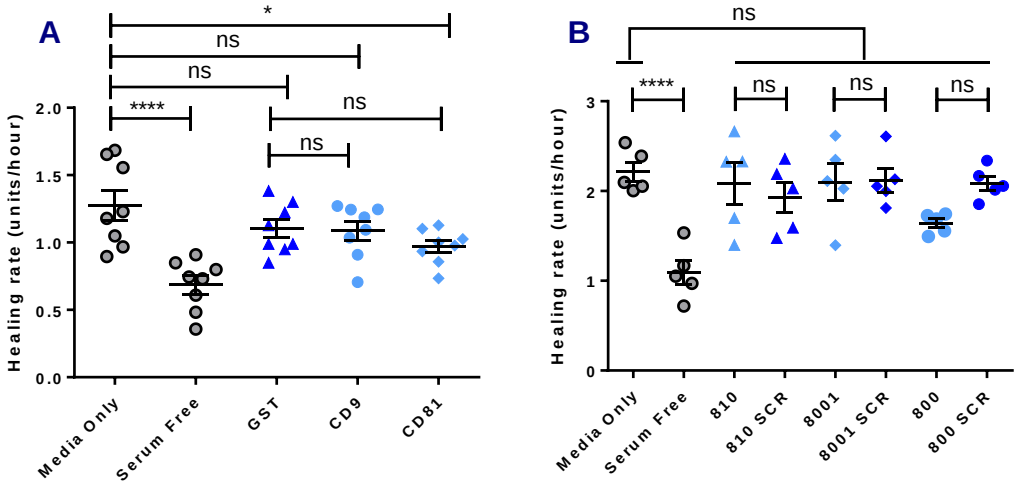


Figure 4.7: Tetraspanin Peptide Treatment Does Not Have Significant Effects on Cell Migration and Proliferation in a Monolayer Scratch Model: HaCaT cells were seeded to form a confluent monolayer. A scratch was then made using a sterile p200 pipette tip, and 200nM peptides and 500nM EC2:GST fusion proteins added. The area of the scratch was measured at 18 hours to deduce the rate of migration and proliferation. Serum free media was used as a positive control. (A) The effects of EC2 domains (B) The effects of 200nM peptides or scrambled peptides. Data presented as mean ± SEM. * p≤0.05. (A) n=4, duplicate. (B) n=5, duplicate. Data analysed by One-way ANOVA with Sidak's multiple comparisons.

4.2.7 Peptides Do Not Affect Cytokine Production by TEskin

Tetraspanins are involved in cell signalling and immunity, and therefore treatment with the peptides could affect immune functions carried out by the skin. To test for any obvious effects on immunity, a cytometric bead array (CBA) was performed to see if any changes in cytokine or chemokine production in the 3D skin model were observed with and without treatment. Samples were collected 24 hours post wounding, in serum free media.

CBAs are a quantitative technique which detects cytokine/chemokine levels in a sample by flow cytometry. Beads coated with anti-cytokine/chemokine antibodies are added to samples to trap the target of interest, then fluorescent secondary antibodies to the cytokines/chemokines are added so that the beads can be detected by the flow cytometer. The Median Fluorescence Index (MFI) values are compared to a standard curve to determine the concentration of cytokines in the samples.

In a preliminary experiment, a small number of samples were screened for the cytokines known to be secreted by keratinocytes and fibroblasts, to assess which could also be detected in wound effluent from TEskin (Figure 4.9). Detectable levels of interleukins (IL)-11, IL-6, IL-8, CCL2 (MCP-1) and IL-1α were secreted by the TEskin, however IL-11 was excluded from further analyses as the levels observed were comparatively very low.

Wound effluent was therefore collected at 24 hours post wounding/treatment from TEskin in various conditions: wounded and mock-wounded, infected or sterile, and with or without peptide treatment. Figure 4.10 shows that differences in cytokine levels occur in response to wounding and to wound infection by S235 strain *Staphylococcus aureus*. 24 hours after burning TEskin we can see a significant increase in levels of IL-6 and IL-8 secreted into the wound compared to the levels secreted by intact unwounded TEskin. A possible increase in IL-1α levels are also seen however this did not reach significance. When burned TEskin was infected with S235 bacteria, a significant increase in IL-1α was observed after 24 hours, along with a significant reduction in MCP-1 chemokine. No change was observed with IL-6 or IL-8.

To assess if treatment with 800 peptide, had any effect on the cytokine response, CBAs were performed on samples of each condition, with 200nM peptide on the skin throughout the 24 hour incubation. No effect was seen on cytokine secretion by TEskin with 200nM 800 peptide treatment (Figure 4.11, 4.12). There is however a small significant increase in the secretion of

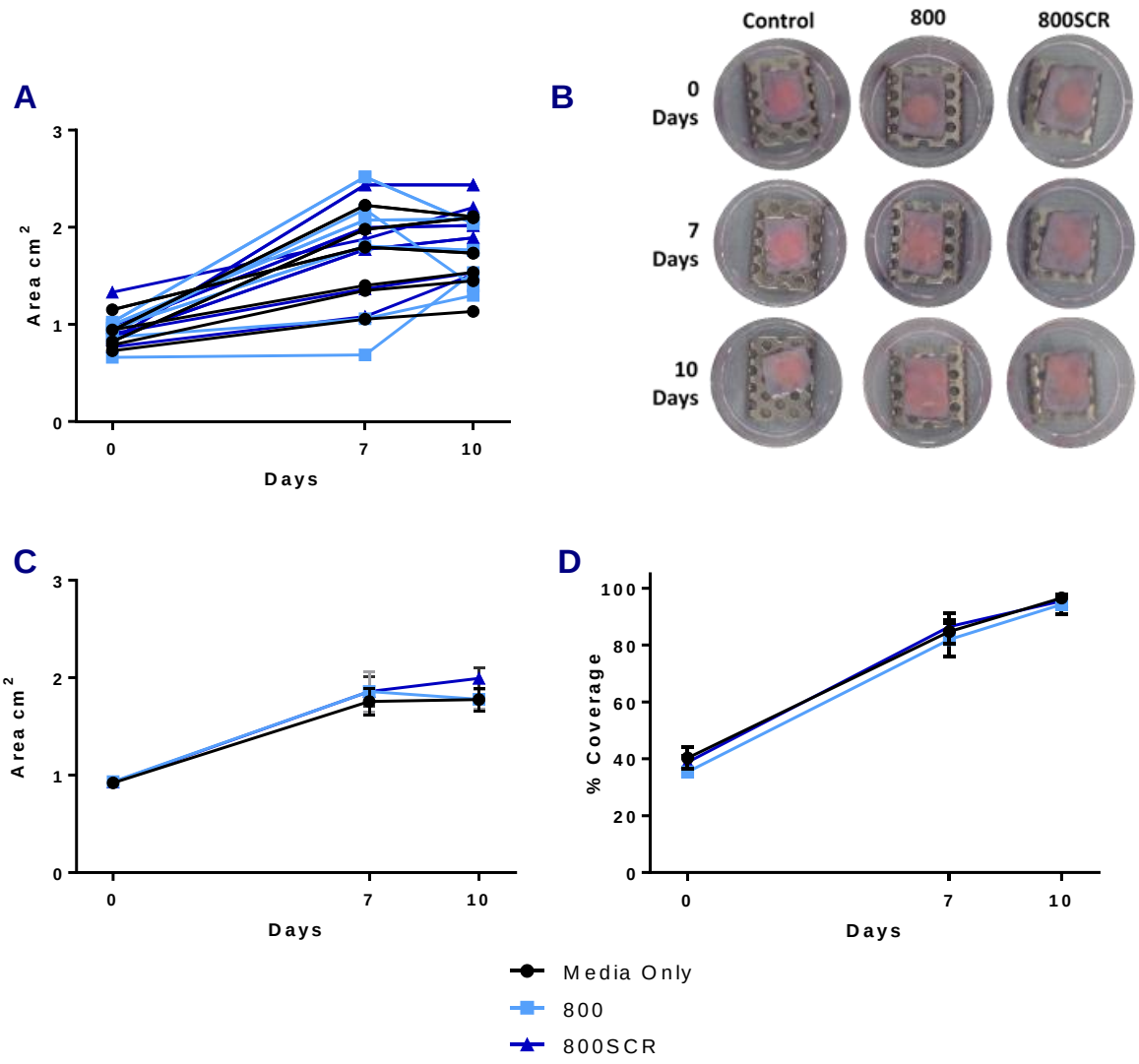


Figure 4.8: Peptide Treatment Did Not Affect Epidermal Migration in the Tissue Engineered Skin Model: Keratinocytes and fibroblasts were seeded into the centre of a 10mm diameter metal ring and grown for 3 days in submerged culture and at air liquid interface for 7 days, before the ring was removed. 200nM peptide treatment or media control was then applied to the surface of the skin every day for 10 days and the epidermis allowed to migrate outwards. Resazurin Blue stains were performed at 0, 7 and 10 days to measure the area of metabolically active viable skin cells. Conditions were judged to be not significantly different by Two-way ANOVA, and percentage data was transformed by $Y=\log_{10}Y$ before analysis. The data is presented as mean ± SEM. (A) Each TEskin piece is plotted individually. (B) Representative images of skin pieces at 0, 7 and 10 days. (C) Mean summary data. (D) Mean percentage coverage. n=3.

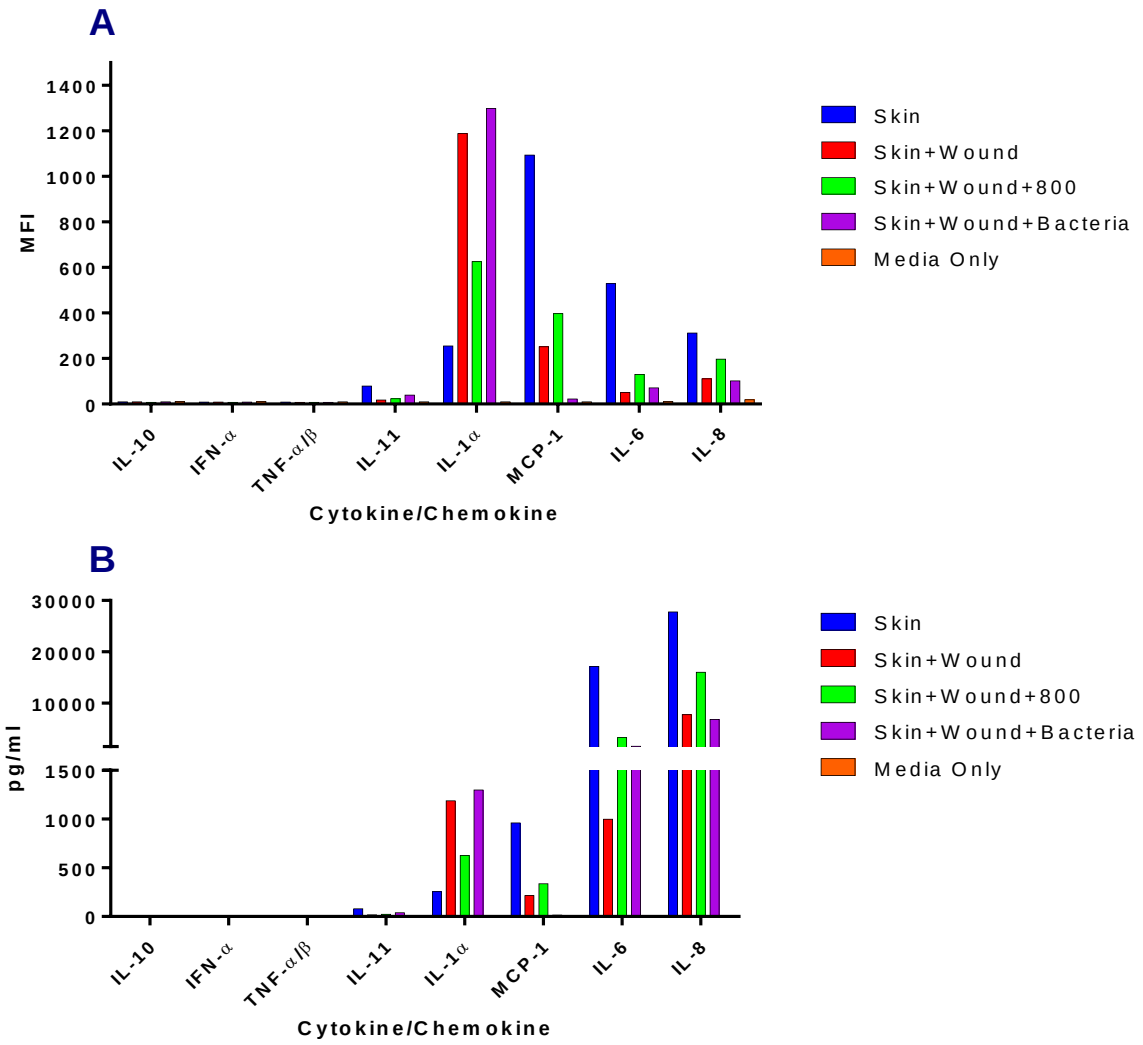


Figure 4.9: The Production of Cytokines by TEskin: Wound supernatant was collected 24 hours post-wounding/treatment in serum free media and tested with a cytometric bead array for a range of cytokines and chemokines. MFI figures were converted to pg/ml using a standard curve based on beads combined with pre-defined quantities of cytokines. Media only controls were subtracted from data values. n=1, duplicate.

IL-6 with peptide 800SCR treatment. This could be due to contaminant in the 800SCR peptide or non-specific effect of the scrambled sequence.

4.2.8 Peptide Treatment Does Not Affect the Development or Survival of Zebrafish

To test for any toxic effects on an *in vivo* model, peptide 8001 was injected at a final concentration of 50nM-150nM into London Wild Type zebrafish embryos against a PBS control, and allowed to develop for 76 hours. Zebrafish were scored for survival every 12-24 hours. No effect on the survival of zebrafish was seen with any peptides or their scrambled control suggesting that at this concentration neither the peptides, nor any remaining by-products of the peptide production process, are having any effect on the growth or viability of the zebrafish.

To test if the peptides had any effect on zebrafish viability during an infection, we used the *Staphylococcus aureus* zebrafish infection model as published in Prajsnar et al (Prajsnar et al., 2008). In this model, treatments are co-injected with bacteria directly into zebrafish and their survival scored for up to 5 days (Figure 4.13). We saw no significant difference in survival rates of fish injected with 8001 peptide, however when treated with a final concentration of 50nM8001SCR peptide a significant increase in fish mortality was observed. Injections were performed by Dr Nelly Wagner and Mr Alex Williams.

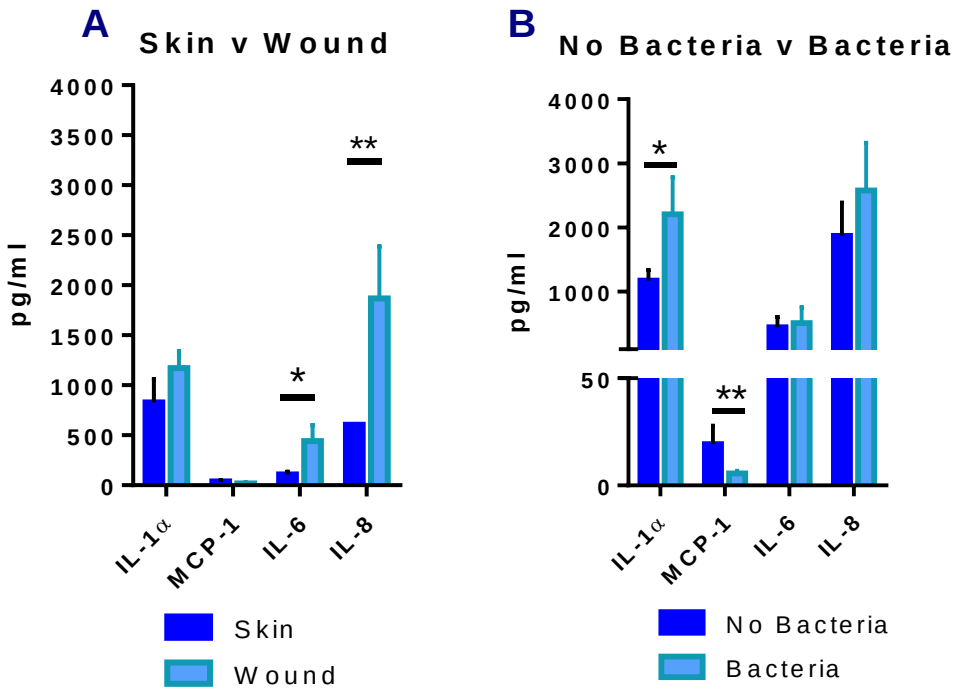


Figure 4.10: Wounding and Infection Exert Different Effects on Cytokine Production in TEskin: (A) Cytokines were collected from the supernatant of unwounded TEskin and TESkin 24 hours after burning. (B) Cytokines were collected from wounded skin with or without infection with S235 strain *Staphylococcus aureus* 24 hours post-burning. n=3, triplicate, data analysed by Unpaired T-test * p≤0.05 **p≤0.01, Data presented as mean ±SEM

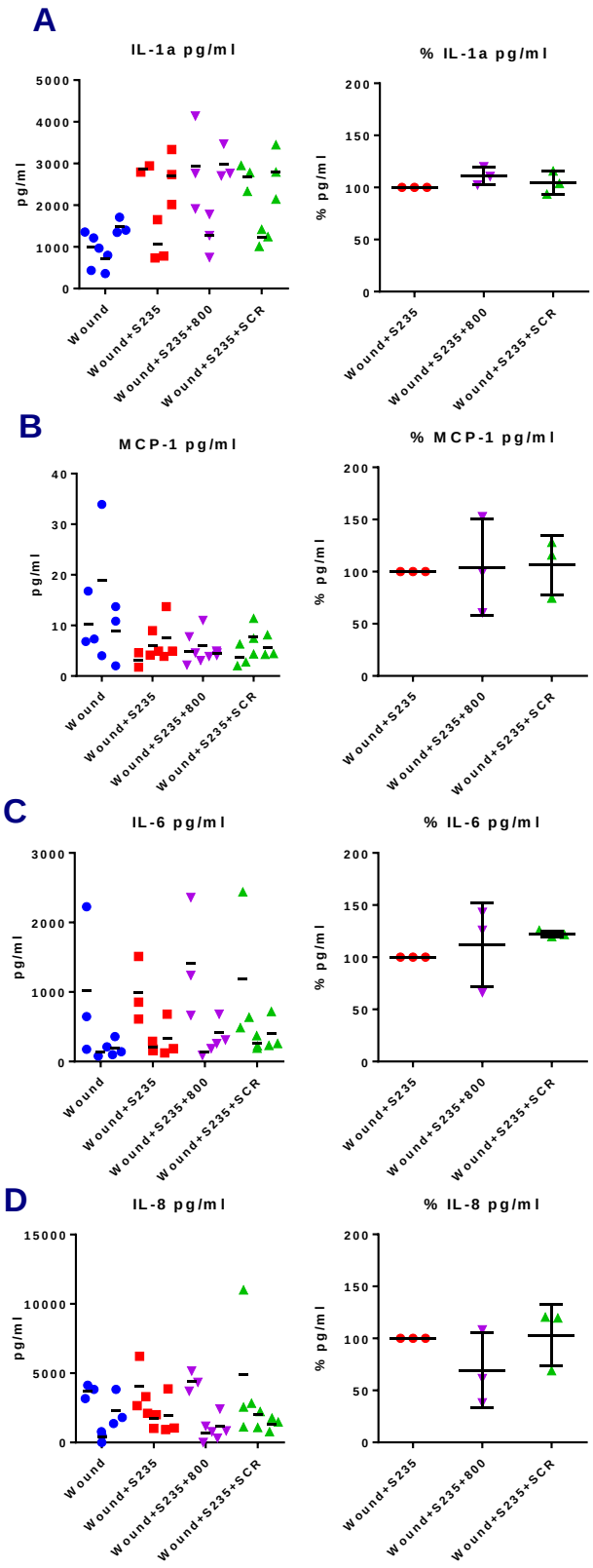


Figure 4.11: Cytokine Levels Change in Response to Infection But Not Peptide Treatment: (A) IL-1α levels (B) MCP-1 levels. (C) IL-6 levels D) IL-8 levels. n=3 triplicate Data analysed by One-way ANOVA with Sidak's multiple comparisons. Percentage data was transformed as $Y=\log_{10}Y$ before analysis.

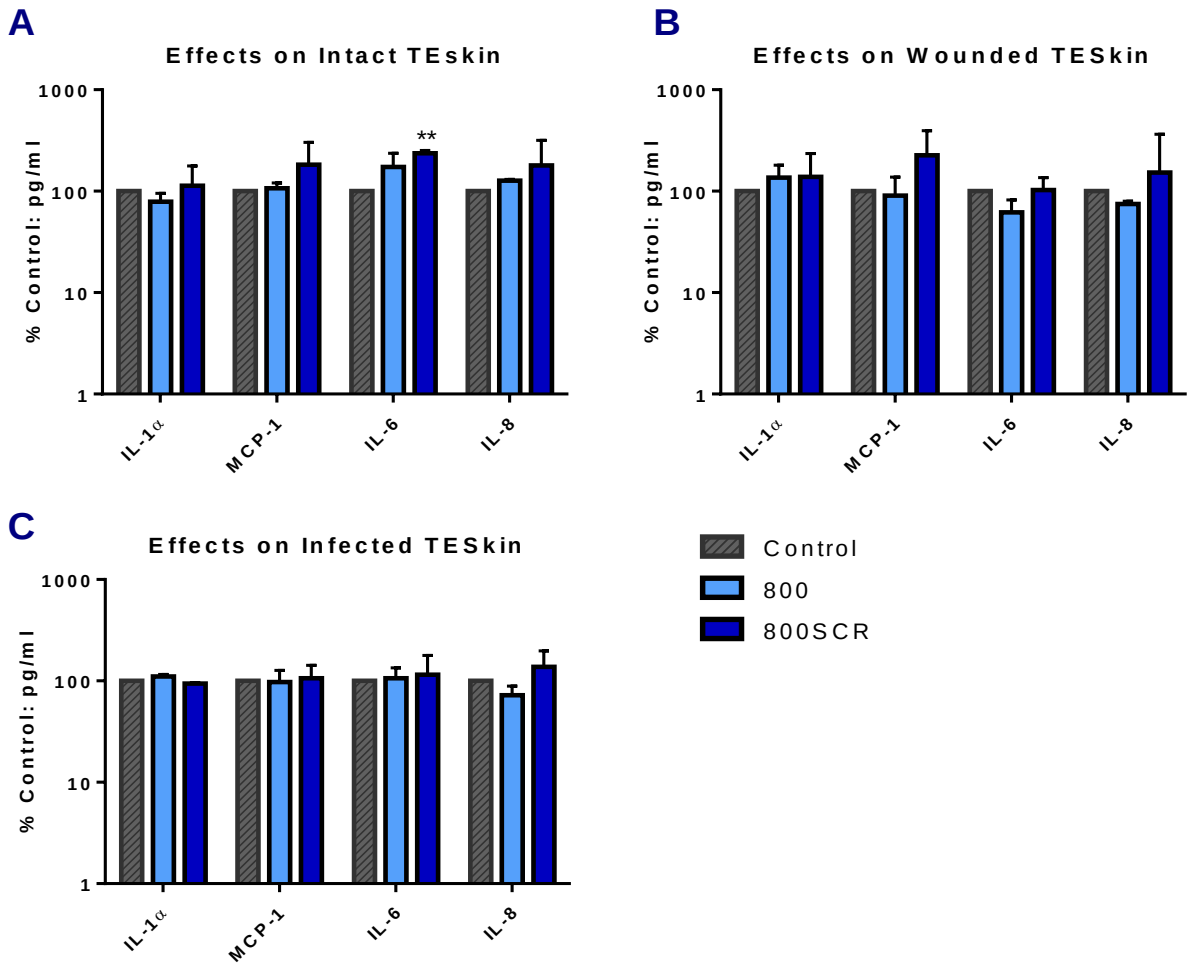


Figure 4.12: Peptide 800 does not Influence the Cytokine Response of TEskin: A) Effects of peptide treatment on intact TEskin. (B) Effects of peptide treatment on wounded TEskin. (C) Effects of peptides on infected TEskin. Data is normalised as a percentage of untreated control and transformed by $Y=Log_{10}Y$ before analysis by Two-way ANOVA. Presented as Mean ± SD * $p \leq 0.05$

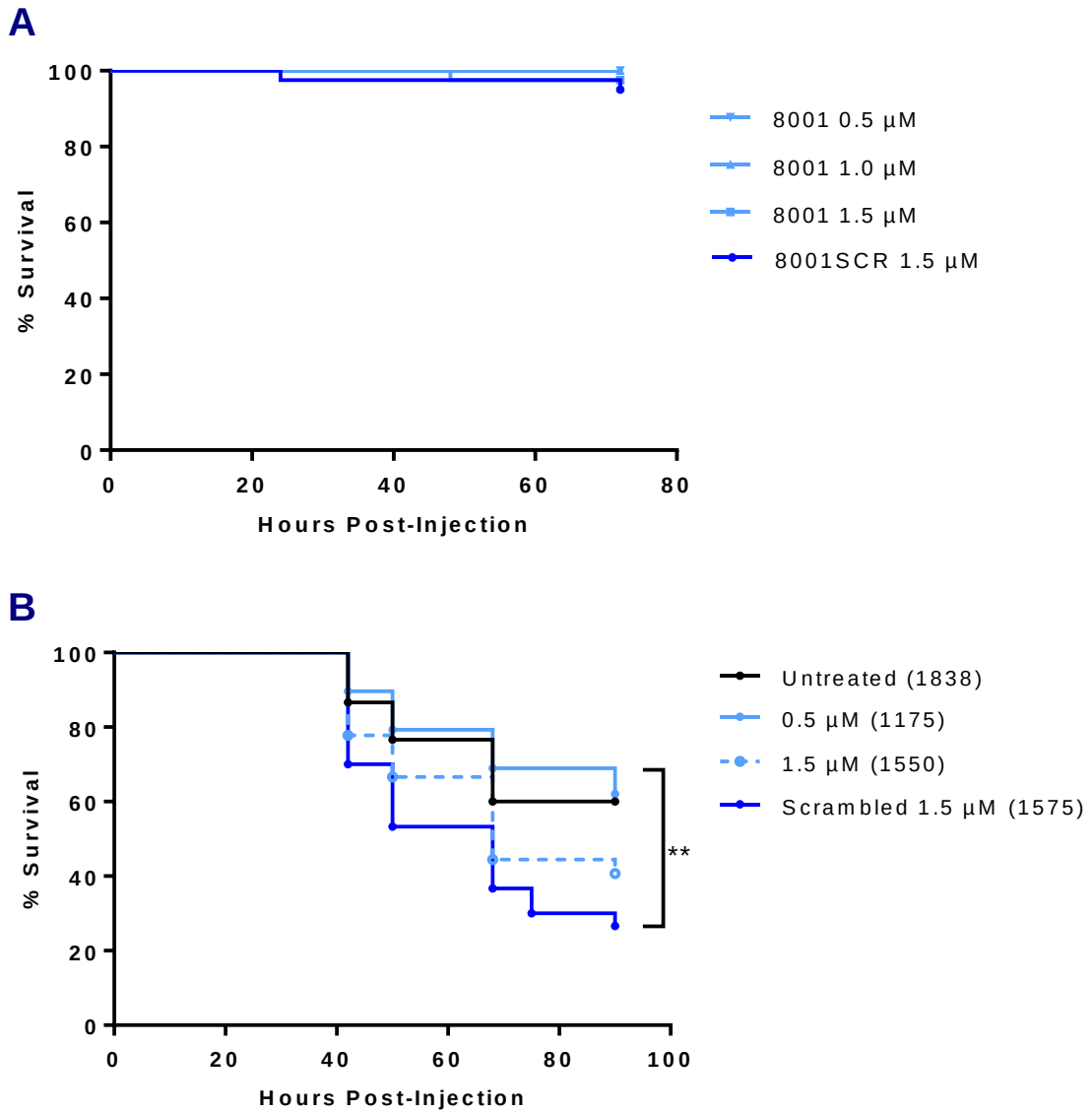


Figure 4.13: Peptide Treatment Alone Does Not Affect the Survival of Zebrafish Embryos, but may Affect Their Susceptibility to Infection: A) Viability: 8001 and 8001SCR 25-30 embryos per group were injected with 1nl peptide to a final internal concentration of 50 to 150nM, and scored every 12-24 hours for survival. (A) Fish are injected with just peptide. (B) Zebrafish were co-injected with peptide and SH1000 strain *Staphylococcus aureus*, to a final concentration of 50-150nM, and the cfu counts of SH1000 bacteria indicated by the bracketed numbers in the associated key. Log-

4.3 Discussion

Here we have shown that tetraspanin expression levels vary between cell types, donors, and the differentiation state of cells. We have also shown that peptide treatment has no major effects on cell metabolism, migration, proliferation, or the production of cytokines in response to wounding and infection.

The expression of various tetraspanin proteins on single cell types from the skin has not before been extensively studied. Data from the PaxDb database shows mid-levels of CD9 and CD63 expression with low levels of CD81 expression in the skin (Kim et al., 2014, Wilhelm et al., 2014). How these levels vary between all the different cell types however is poorly characterised. Here we looked at the expression of CD9, CD63, CD81 and CD151 by flow cytometry and microscopy to determine the relative expression levels in the cell types relevant to this study.

Keratinocytes showed high levels of expression of CD9, whereas fibroblasts showed very low expression levels. Interestingly, fibroblasts showed very high levels of CD63 expression at the cell surface, which was unexpected as CD63 is seen to be mostly expressed in intracellular vesicles. HaCaTs showed a similar level of CD9 expression to primary differentiated keratinocytes and so, HaCaTs are an acceptable model for primary keratinocytes in the context of CD9-targeted therapies. These cells however do exhibit higher levels of CD81 and CD151 than any other cell type studied here, which suggests that they should be treated with caution when used as a model for other systems, as many other proteins could be differently expressed.

Protein levels are known to be expressed differently between individuals, based on gender, age and tissue localisation as well as environmental factors (Sprenger et al., 2013). Co-culture of keratinocytes and fibroblasts also extensively changes the gene expression of fibroblasts, suggesting that cells in a 3D model will differ from those in monolayer culture (Nowinski et al., 2004). The differences in tetraspanin expression based on the differentiation state of cells should also be considered in other cell line studies. As keratinocytes become more differentiated as they move into the upper layer of the epidermis, we hypothesised that CD9 will be located in the basal to spinous layers of the epidermis and will be expressed less in granular layers and in the stratum corneum. In the TEskin model assessed here however we

see CD9 expressed in higher levels of epidermis. This distribution partially disagrees with online databases which place CD9 in the basal levels of skin, however all these data are collected from skin biopsies, whereas the distribution of proteins in TEskin is known to differ slightly. This discrepancy highlights another drawback of the TEskin model as well as the difficulties of using cell line models which only reflect one cell state.

As mentioned previously, CD9 is a highly multi-functional molecule and targeting it could affect cell function. If the peptides were to be used therapeutically, as outlined in chapter 3, they would have to be tested for toxicity against host cells. Therefore, tests were performed that could detect perturbation of normal cellular activity. No major effects on cell viability were observed with an MTT test. Also, no negative effects were seen on cell proliferation and migration a cell monolayer scratch model with CD9 EC2 or the majority of the CD9 peptides. There was however an effect with CD81 EC2 domain, which reduced the scratch healing time. CD81 is a tetraspanin commonly found associated with CD9, and in multiple systems they have been shown to have contradicting functions. Therefore, if CD9 downregulation can increase wound healing, as seen in Jiang et al (Jiang et al., 2013), it would not be surprising to see a decrease in migration speed with CD81 downregulation. In this study however we only see the CD81 decreasing migration speed without the converse increase by CD9 blocking, possibly due to the EC2 domains not being as effective as blocking antibodies. As MTT tests give an indication of cell number, and this assay showed no peptide effects, we can assume that the effects here are in relation to cell migration as opposed to proliferation.

There was also a small but not significant effect of 800 peptide on the migration of HaCaT cells. This reduction in cell migration could be a detrimental side effect of peptide 800 treatment, and therefore it was important to test for this effect using an alternate assay, namely epidermal migration in the TEskin model. This assay showed no reduction in migration or re-epithelisation time with 800 peptide treatment. Although fresh peptide was added to the top of the skin every 2 days and the overall healing rate was not reduced. As mentioned previously, the TEskin model is simplistic and therefore limited, especially for a biological process as complicated as wound healing, however it is more representative of cell migration on skin than a cell monolayer model. More variance exists in the data obtained from this model, due to the difficulty in measuring viable area, and differences in the growth rate of primary cells.

The immune response of the skin is a highly complex process and is frequently the first line of defence against infections. The cytokines and cells involved in this process are outlined in figure 1.2. We have shown in this study that the TEskin model produces sufficient quantities of cytokines to be measured by CBA analysis, despite the lack of immune cells in the model. The functions of these detected cytokines are outlined in table 4.1 (Grone, 2002, Grossman et al., 1989, Sasaki et al., 2003, Cornelissen et al., 2010, Giustizieri et al., 2001, Sato et al., 1999, Turksen et al., 1992, Sugawara et al., 2001, Luckett and Gallucci, 2007, Mohamadzadeh et al., 1994, Yen et al., 1996). Upon wounding, we can see a significant increase in interleukins (IL)-6 and IL-8, despite the reduction in viable cells upon burning. In our TEskin we can also see a small but non-significant increase in IL-1α levels upon wounding, with data variance contributing to lack of significance. No increase in MCP-1 levels were observed, suggesting that the increase in MCP-1 levels seen in skin in other studies (Tekstra et al., 1999, Cornelissen et al., 2010, Kiang et al., 2010) may be primarily due to other cell types. Previously, cytokines IL-1α, IL-6 and IL-8 have been shown to increase within 24 hours of wounding of bio-engineered skin substitutes, however the model used in that study differed from the TEskin tested here, as it uses a collagen matrix rather than de-epidermised dermis as a scaffold and allows less time for stratification at air liquid interface (Falanga et al., 2002). Clinical data has also been collected that suggests a difference between mechanical wounding and burn wounding on cytokine response, including an increase in Macrophage chemotactic protein (MCP)-1, IL-6 and Tumour Necrosis factor (TNF)-α in burn wounds as opposed to scalpel wounds (Schwacha et al., 2010).

Additionally, we observed a significant change in cytokine production in response to burned TEskins infection with S235 strain *Staphylococcus aureus*, with an increase in IL-1α and a decrease in MCP-1. Previous studies have highlighted that in skin and in keratinocytes alone IL-1α is upregulated upon staphylococcal infections (Donnarumma et al., 2004), and the bacterial endotoxin lipopolysaccharide (LPS) alone increases the secretion of IL-6 and IL-8 from keratinocytes (Sasaki et al., 2003, Schwacha et al., 2010). Again a small increase in IL-8 is observed in infected TEskin, however this is not statistically significant compared to the uninfected control. No difference in IL-6 levels are observed, however opposing research has found that virulent strains of *Staphylococcus aureus* can in some situations reduce IL-6 production (Tajima et al., 2007). Differences in cytokine levels compared to the literature

Table 4.1: The Function of Cytokines Detected from TEskin

Cytokine	Keratinocytes	Fibroblasts	Function	Sources
IL-1α	Express	Express	Pro-inflammatory Chemotactic for keratinocytes Induces keratin 6 expression Decreases adherence of certain bacteria to keratinocytes	A. Gröne et al, 2002
IL-6	Express	Express	Pro-inflammatory Stimulates keratinocyte proliferation Modulates fibroblast migration Role in wound healing	Grossman et al, 1989, Sato et al, 1999, Turksen et al, 1992, Sugawara et al, 2001, Luckett et al, 2007
IL-8	Express	Express	Pro-inflammatory Neutrophil attractant Produced in response to external chemical stimuli Observed in auto-immune-mediated diseases	Mohamadzadeh et al, 1994, Yen et al, 1996
MCP-1	Express	Express	Regulates cell traffic in both homeostatic and inflammatory conditions Attracts eosinophils, basophils, monocytes, and TH2 cells	Giustizieri et al, 2001

IL-1α, IL-6, IL-8 and MCP-1 were found to be expressed in fibroblasts and keratinocytes, and some basic functions of these cytokines/chemokines were elucidated from a search of available literature.

predicted values could be explained by differences in the model system use, the reduction in number of viable cells as a consequence of infection, or potentially staphylococcal suppression of the immune response.

In some systems, correlations between expression of tetraspanins and cytokines have been observed. For example, CD37 has been shown to inhibit IL-6 production when interacting with Dectin-1 (Meyer-Wentrup et al., 2007) and that this mechanism can be induced by *Toxoplasma gondii* infection (Yan et al., 2014). Additionally, CD63 deficient mast cells have reduced degranulation, leading to a loss of TNFα and IL-6 secretion (Kraft et al., 2013). Here we assessed if treatment with tetraspanin peptides affected the production of cytokines by TEskin. Analysis by One-way ANOVA showed that 200nM peptide 800 treatment had no effect on the production of cytokines in intact, wounded or infected TEskin. Peptide 800SCR, which

was assessed here as a negative control, had a small effect on IL-6 production in intact TEskin, however, as observed, there are large levels of variance in the data, perhaps caused by differences between donors and in cell viability and this could contribute to a false positive. Additionally, as seen in chapter 6 the sequence of 800SCR peptides is also found on other human proteins, which could be involved in IL-6 secretion. The production and testing of an alternate 800SCR peptide with a different amino acid sequence would show if this data is valid.

In addition to cell line and TEskin models described here, a zebrafish model was also used to look for any toxic effects of the peptides. The zebrafish embryo model was chosen as *Danio rerio* has a similar immune system to humans, whilst not being limited by the same ethical constraints as other *in vivo* models. Peptide 8001 rather than 800 was used for this study as it was carried out prior to all cell line assays that determined to use peptide 800. Additionally, the peptide 8001 has good sequence homology to CD9b in tetraspanins (11/12 identical or chemically similar residues). Peptides were injected to a final concentration of 50nM-150nM, based on the assumptions about the volume of a zebrafish embryo made initially by Leung et al in 1998 (Leung et al., 1998). In this model, no toxicity was seen in the fish when injected with 8001 and 8001SCR peptides, however, no increase in the survival of SH1000 *Staphylococcus aureus* infected zebrafish was observed. This could be due to the peptide concentration being too low, or because the peptides had to be injected at the same time as the pathogen instead of being administered as a pre-treatment. The peptides could also be being degraded before they can act. Additionally, this model mirrors the late stages of *Staphylococcus aureus* infection when the pathogen reaches the blood stream. Upon injection all the bacteria are taken up by the fish phagocytes, and the organisms survive until the *S. aureus* escape and cause downstream terminal infections (Prajsnar et al., 2008, Prajsnar et al., 2012). At this stage, bacterial adherence is not necessary for fish mortality. In thus model, 8001SCR peptide actually had a negative effect on zebrafish survival, perhaps due to contaminants in the peptide, or because of interference with other proteins that 8001SCR has homology with in zebrafish, as outlined in table 4.2 (Altschul et al., 1990). Additionally, the volume of liquid injected into fish has been shown to have a strong effect on zebrafish viability, and therefore small variances in the needle sizes used in for each condition could lead to higher mortality in the 800SCR condition (Schubert et al., 2014).

Table 4.2: Sequence Homology Between Peptides and Zebrafish Proteins

	Description	Max score	Total score	Query cover	E value	Ident	Accession
8001	tetraspanin-2	31.2	31.2	85%	0.022	67%	NP_001018160.1
	vacuolar protein sorting-associated protein 45	28.6	28.6	71%	0.15	80%	NP_001243585.1
	CD9 antigen	26.1	26.1	100%	0.93	50%	NP_998593.1
	sorting nexin-5	24.8	24.8	78%	2.4	53%	ABD34790.1
	inhibitor of growth protein 2	24.4	24.4	71%	3.3	70%	NP_001002448.1
	PREDICTED: CD9 antigen isoform X1	24	24	100%	4.6	43%	XP_005164541.1
	DNA damage-binding protein 1	24	24	64%	4.7	78%	AFI92852.1
	TATA element modulatory factor	23.5	23.5	78%	6.5	52%	NP_001003522.2
8001SCR	PREDICTED: protein MTO1 homolog, mitochondrial isoform X2	24.8	24.8	50%	2.5	86%	XP_009304746.1
	PREDICTED: starch-binding domain-containing protein 1 isoform X2	24	38.6	85%	4.6	67%	XP_005161545.1
	PREDICTED: zinc finger protein 208 isoform X3	24	96.1	64%	4.7	47%	XP_009289660.1
	general vesicular transport factor p115	23.1	34.4	85%	9	75%	NP_956449.1
	semaphorin 4d	22.7	22.7	64%	12	78%	NP_001038473.1
	Ddx46 protein	22.7	39.9	42%	12	100%	AAI33101.1
	protein tyrosine phosphatase type IVA 2 isoform 2	22.3	22.3	50%	17	86%	NP_001019269.1

A protein search was performed using the Basic Local Alignment Search Tool to test sequence overlaps between the peptides and proteins in the host organism. Last accessed: 16/12/15

Overall, we have shown that tetraspanin expression varies between cell types, donors, and between the specific proliferative state of the cell. Additionally, we have not found any debilitating toxicity of the peptides in cell lines, TEskin or in a zebrafish model.

Chapter 5 – Development of Peptides as Anti-Microbial Therapeutics

5.1 Introduction

Tetraspanin based peptides have been shown to have broad range anti-adhesive activity with no apparent toxicity to host cells. The maximum reduction in adherent bacteria observed in response to treatment with the peptides is around 60%. This reduction would theoretically aid the host's natural ability to clear the infection, reducing the likelihood of skin and soft tissue infections and sepsis. For immunocompromised patients, or for patients with chronic long-term wounds however, a 60% reduction would likely not be sufficient to fully clear an infection, and consequently we have attempted to improve the efficacy, retention and permeability of the peptides, exploring 2 common avenues by which the success, duration or delivery of treatments can be improved: combination therapy and drug delivery platforms.

5.1.1 Current Wound Treatment

Various methods are currently utilised to prevent infection or treat ongoing infections of wounds. Systemic drug delivery is commonly used, in which antibiotics are taken orally or intravenously. Vancomycin has been used extensively as an effective systemic treatment against multi-drug resistant *Staphylococcus aureus* (MRSA) infection, however, as with many other antibiotics, strains of *S. aureus* have been identified which are resistant to vancomycin, with up to 74% of soft tissue infection isolates having this resistance. Recent guidelines by the Infectious Disease Society of America recommend clindamycin, trimethoprim-sulfamethoxazole, tetracycline (minocycline or doxycycline) or linezolid for the treatment of suspected community acquired MRSA infected wounds (Stevens et al., 2014). Antibiotics generally inhibit essential processes found in bacterial metabolism, such as cell wall synthesis and type II fatty acid synthesis. Due to the rise in resistance to these drugs, encouraged partly by incomplete courses of treatment and the over-use of antibiotics, alternative treatments are being increasingly frequently sought (Alanis, 2005). Other categories of drugs are required which target other aspects of bacterial virulence, such as adherence, toxin production and biofilm formation (Kollipara et al., 2014).

Wound infections may also be treated using locally applied wound dressings, which generally have antibacterial and antifungal activity. Advantages include a smaller chance of adverse reactions and more specific targeting of the therapy. Local therapy also prevents interference

with commensal organisms usually found in the stomach and other surfaces. Modern antimicrobial components of wound treatments include silver (Maneerung et al., 2008, Wilkinson et al., 2011), manuka honey (Mavric et al., 2008, Cushnie and Lamb, 2005, Al-Waili et al., 2011, Kwakman et al., 2010), polyhexamethylene biguanide (PHMB) (Mueller et al., 2013, Lee et al., 2004, Huebner and Kramer, 2010), topical oxygen (Woo et al., 2012, Greif et al., 2000), and antiseptics such as chlorhexidine (NHS, 2011, Breuer et al., 2002). These therapies are capable of reducing the bacterial burden, however they are not normally as fast and effective as antibiotic therapy and some, such as silver, can be expensive for the long-term treatment of chronic infections. These treatments have also been known to cause side effects and can be toxic to the host. For example, silver treatment can cause cell cycle arrest and DNA damage after long term exposure (AshaRani et al., 2009).

5.1.2 Anti-adhesion Therapies in Development

There are currently a number of anti-adhesion therapies being developed to treat bacterial skin and soft tissue infections (Krachler and Orth, 2013, Cozens and Read, 2012). The major advantage of anti-adhesion therapy is that it exerts weaker selective pressure on the bacteria relative to antibiotics and thus does not encourage the development of more virulent strains (Krachler and Orth, 2013). One example of an anti-adhesion therapy in development are the proanthocyanidins, extracted from cranberry juice, which are being developed for the directed inhibition of urinary tract infections (Shmuely et al., 2012). These molecules have also been shown to inhibit the binding of *Helicobacter pylori* (Burger et al., 2000) and *Porphyromonas gingivalis* (Labrecque et al., 2006), making them a potentially wide spectrum treatment. Alternatively, biphenyl mannosides are known antagonists of FimH, a major binding factor of *E.coli*, first identified over 40 years ago (Hartmann et al., 2012). These saccharides have been used to successfully inhibit the adhesion of *E.coli* to host cells (Hartmann et al., 2012).

Peptide based adhesion inhibitors are much rarer, with issues frequently arising during early stages of their development with downstream toxicity and alterations in host cell signalling and metabolism (Krachler and Orth, 2013). Peptides that mimic host receptors and those that competitively inhibit bacterial adhesins can easily activate signalling pathways downstream and cause unwanted negative side effects in the host. Despite this, there are a number of

peptide based anti-adhesion molecules in various stages of development. For example, p1025, developed by Kelly et al, is a peptide designed to prevent tooth cavities by inhibiting the adherence of *Streptococcus mutans.* It acts by preventing the binding of surface protein streptococcal antigen (SA) I/II to salivary receptors on the tooth surface and has shown success at preventing the re-colonisation of teeth by *S. mutans in vivo* (Munro et al., 1993, Kelly et al., 1999). Another series of peptides in development are MAM-7 based inhibitors, which have been shown to inhibit the adherence of *E. coli*, *Yersinia pseudotuberculosis*, *Vibrio cholerae*, and *Vibrio parahaemolyticu*s to host cells (Krachler and Orth, 2011, Krachler et al., 2011).

One of the main problems facing anti-adhesive therapies, and peptide therapies specifically, is their retention and penetration into a wound. Peptides in a wound setting are also susceptible to degradation by matrix metalloproteases (MMPs) secreted by the wound to aid wound healing. Although tightly controlled, these enzymes degrade foreign matter as well as ECM components such as collagen and elastin to allow re-epithelisation (Sinclair and Ryan, 1994). Due to the nature of skin as a barrier, many peptides also cannot penetrate deeply into the skin to act on deeper levels of cells. Both of these problems have been addressed using nanoparticle delivery systems, such as liposomes and vesicular systems, and more recently nanostructured lipid carriers, polymer-based nanoparticles and magnetic nanoparticles (Desai et al., 2010).

The efficacy of peptide adhesion inhibitors could also be improved by increasing their stability. Peptide stabilisers that induce peptides to form alpha-helical type structures have previously been tested to look for any increase in the efficacy of the tetraspanin-based peptides. At low concentrations these stabilisers, provided by Professor David Fairlie from the University of Queensland, Australia, combined with the peptides to further reduce the proportion of adherent bacteria on host cells. Additionally, cyclisation of 8001 and 810 peptides, and an addition of tyrosine and aspartate to the termini of these peptides, improved their inhibitory concentration (Cozens, 2016).

5.1.3 Nanocin™

Nanocin™ is a polymer based nanoparticle drug delivery system first synthesised as a stand-alone antimicrobial therapy over 80 years ago, now developed by Tecrea™. This delivery

system has been shown to deliver small molecules such as drugs, dyes and DNA into cells and deep into tissues (Good and Ridden, Ridden et al., Tecrea, 2016, Cook and Ridden, 2014) and has been used safely in the clinic for over 30 years. It has also been shown at high concentrations to have inherent antimicrobial action against intracellular *Staphylococcus aureus* as well as *Leishmania major*, and unpublished data suggested that it functions by condensing bacterial chromosomes (Good, 2016/2017). Here we assess whether packaging tetraspanin-based peptides into nanoparticles using Nanocin™ technology can improve their function in reducing staphylococcal adherence to host cells and tissues. All work outlined relating to Nanocin™ was undertaken in collaboration with Blueberry Therapeutics.

Additionally, we test if combining the peptides with an antibiotic to which MRSA are resistant can improve the functionality of either. Flucloxacillin is a β-lactam antibiotic, and functions by inhibiting bacterial cell wall synthesis similarly to penicillins (Sutherland et al., 1970) (Sutherland et al., 1970). Contrary to other penicillins, flucloxacillin cannot be degraded by pathogen secreted beta-lactamase, which renders most penicillin members useless. It however is not frequently used to treat methicillin-resistant *Staphylococcus aureus* (MRSA) infections, as these pathogens have resistance to flucloxacillin caused by a small mutation in their bacterial penicillin binding protein which inhibits targeting of the drug (Guenthner and Wenzel, 1984, Hartman and Tomasz, 1984). Flucloxacillin is only therefore capable of eliminating MRSA infection at high concentrations *in vitro* (Guenthner and Wenzel, 1984).

5.2 Results

5.2.1 Peptide Treatment May Improve Staphylococcal Susceptibility to Flucloxacillin Treatment

Here, NCTC15132 strain MRSA was used as a model for MRSA infection (Figure 5.1). NCTC15132 is a local clinical isolate of methicillin resistant *Staphylococcus aureus* used previously in the lab to test for synergistic effects with the peptides, and previous data has suggested a possible beneficial combinatory effect of flucloxacillin with peptide 8001 (Cozens, 2016). To explore this further, flucloxacillin at various doses was added to MRSA, and it was observed that concentrations as low as 0.01µg/ml reduced the number of viable bacteria. At a flucloxacillin concentration of 1µg/ml approximately 50% of the bacteria were no longer

viable, however even at a concentration as high as 100µg/ml 16±1.2% of bacteria were still viable, a concentration much higher than the calculated bioavailability of orally administrated flucloxacillin (Paton et al., 1982).

This MRSA strain was then used to infect HaCaT cells, with or without peptide pre-treatment. After infection the cells were washed and treated with flucloxacillin for 3 hours with agitation. In this system, a synergistic effect was observed, where pre-treating with the peptide then treating with antibiotic led to a greater reduction in the bacterial load at 0.01 and 1 µg/ml of flucloxacillin (Nauta and Mattie, 1975). This synergistic effect was not seen at high concentrations of flucloxacillin. Almost 99% of control bacteria were eradicated with a combined treatment of 50nM 800 peptide and 1 µg/ml antibiotic, whereas there were still 12.6±5.7% viable bacteria with just 1µg/ml antibiotic treatment.

When the TEskin model described in Chapters 3 and 4 was pre-treated with peptides then infected with MRSA, a more complex trend is observed. In this model TEskin was burned, then treated for 1 hour with the tetraspanin-based peptide 800. MRSA was then added and after 5 hours TEskin was washed to remove non-adherent bacteria, and left to infect until 24 hours post burning, as previously. Finally, flucloxacillin was added on top of the TEskin for 3 hours before bacterial isolation. Previous experiments demonstrated that peptide 800 treated skin has a reduced bacterial load (Chapter 3), however in this experiment, the peptide applied with the flucloxacillin does not reduce the number of adherent bacteria. Furthermore, at high flucloxacillin concentrations (10µg/ml), pre-treatment with 200nM 800 peptide led to a significantly greater number of viable bacteria than flucloxacillin only treated TEskin. The quantity of bacteria in TEskin pre-treated with peptide then treated with 10µg/ml flucloxacillin is comparable to control figures and those treated with low concentrations of flucloxacillin.

5.2.2 Formulation of Nanoparticles

Previous research has shown that small peptides and molecules can be packaged into nanoparticles by the addition of Nanocin™ from Tecrea Ltd™. To test if the peptides were suitable for nanoparticle generation, they were mixed with Nanocin™ at ratios of 1:1, 1:2 and 1:3 peptide:Nanocin™, and their size and distribution measured by a NanoSight LM10. The NanoSight and its associated software use a laser to assess the size and intensity of particles,

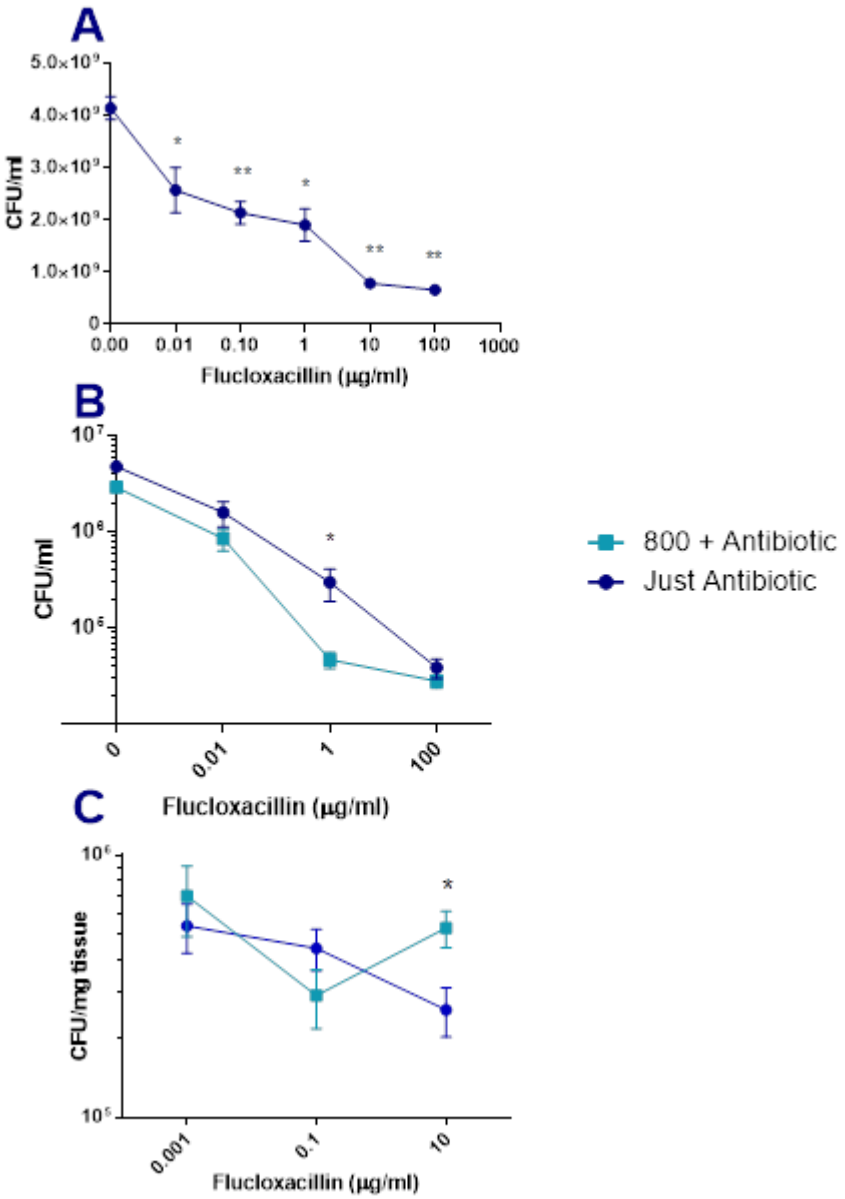


Figure 5.1: Flucloxacillin has Varied Effects in Combination with 800 Peptide: (A) The effects of flucloxacillin alone on the growth of NCTC15132 strain of *Staphylococcus aureus*: Bacteria were treated with varying concentrations of flucloxacillin to determine their sensitivity. n=3. (B) The effects of peptide and flucloxacillin treatment on bacteria adhered to HaCaT cells were determined by treating HaCaT cells with peptide or a media control before infecting them with NCTC15132 bacteria. *S. aureus* were recovered using saponin and plated for viable counting. n=6. (C) The effects of peptide and flucloxacillin treatment on *S. aureus* adhered to TEskin. n=3. Data were analysed by multiple T tests. * p≤0.05 **p≤0.01 ***p≤0.001, presented as mean ±SEM

making calculations of size based on the Brownian motion of the particles using the Stokes-Einstein equation (Figure 5.2).

Nanocin™ alone does not form nanoparticles unless combined with suitable small molecules and no particles were detected in the Nanocin™ only solution. Small particles were detected in the peptide only suspensions, which were thought to be peptide aggregates and are commonly observed in standard peptide suspensions. When combined with the Nanocin™ in a 1:3 ratio, the size distribution of the particles changed significantly, with the mean size decreasing from 307nM for 800 and 303nM for 800SCR in the peptide only solutions, to185nM for 800 and 266nM for 800SCR diameter in the Peptide:Nanocin™ solutions. This is indicative of the formulation of nanoparticles. The detected particle concentration also increases from 0.83 to 2.15 $x10^8$ for 800 peptide, and from 0.18 to 1.21 $x10^8$ for 800 scrambled peptide when combined with the Nanocin™. The 1:1 ratio and a 1:2 ratio were also measured however these formulations resulted in a lower concentration of nanoparticles and a less obvious particle formation, and the 1:3 ratio was therefore chosen for further testing.

5.2.3 Nanocin™:Peptide Formulations Retain Anti-adhesive Effects

The formulations were then tested for effects on the adherence of S235 to HaCaTs (Figure 5.3). A reduction in the proportion of adherent bacteria comparable to that of 800 alone were observed with the two 800:Nanocin™ conjugates in terms of percentage of cells infected and the number of bacteria per 100 cells, indicating that combination with Nanocin™ (and therefore possible intracellular delivery) did not affect the inhibitory activity of the peptides. Nanocin™ alone at the equivalent concentrations had no effect on bacterial adherence, nor did treatment with the 800SCR:Nanocin™ formulation, showing that the effect is still peptide specific.

5.2.4 Peptides and Formulation Show No Negative Effects on Cell Viability and Epidermal Migration

An MTT assay was used as in chapter 4 to assess any effects of the peptides on cell metabolism. A one-way ANOVA demonstrated statistically that none of formulations nor Nanocin™ alone have any drastic effects on cell metabolism (Figure 5.4). TEskin was then used to perform an epidermal migration assay as in chapter 4, in which keratinocytes and fibroblasts are allowed to migrate onto bare dermis to re-epithelise as in a wound. In this

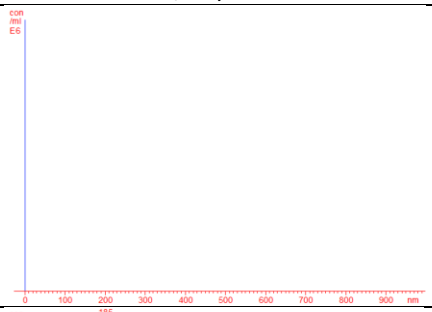
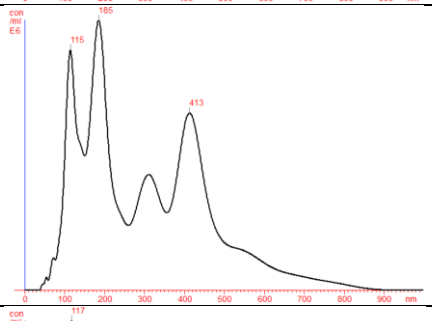
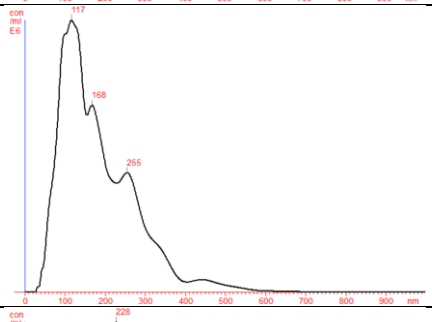
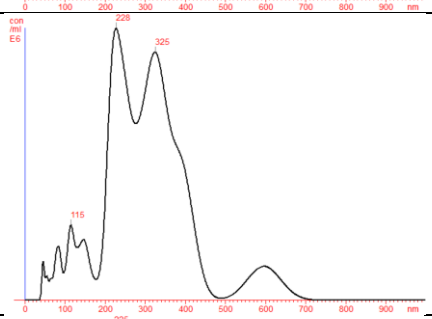
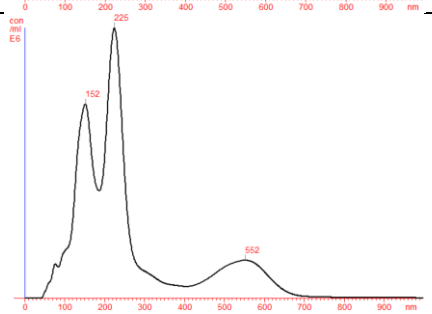
Formulation	Size Distribution (x-particle size. Y-Concentration/ml)	Concentration	Size Distribution
Nanocin™ Only		0.00x10^{8} particles / ml	Mean: 0nm, Mode: 0nm, SD: 0nm
800 Peptide		0.83x10^{8} particles / ml	Mean: 307nm, Mode: 185nm, SD: 160 nm
800: Nanocin™ 1:3		2.15x10^{8} particles / ml	Mean: 185nm, Mode: 117nm, SD: 95 nm
800SCR Peptide		0.18x10^{8} particles / ml	Mean: 303nm, Mode: 228nm, SD: 113 nm
800SCR: Nanocin™ 1:3		1.21x10^{8} particles / ml	Mean: 266nm, Mode: 225nm, SD: 153 nm

Figure 5.2: Formation of Peptide Nanoparticles with Nanocin™ Technology: 800 and 800SCR peptides were combined with existing pharmaceutical technology, Nanocin™ by Tecrea™ to form nanoparticles. A NanoSight was used to visualise and quantify particle formation. Data displayed here was obtained 3 hours after formulation. Particle size was calculated by tracking individual particle tracks and analysing this using the Stokes-Einstein equation, which relates the Brownian motion of particles to their size in suspension.

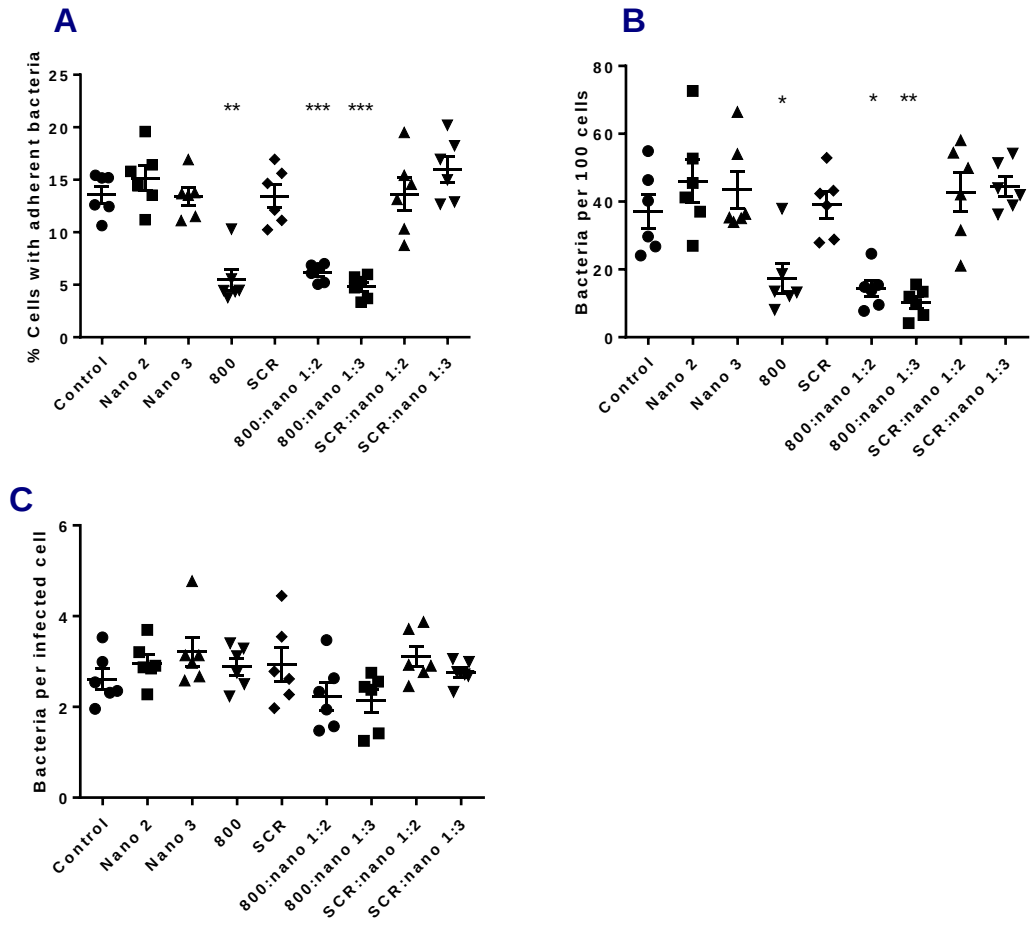


Figure 5.3: Anti-adhesive Effects of Peptide:Nanocin™ Formulations: The peptide:Nanocin™ formulations were tested against S235 *Staphylococcus aureus* adherence to HaCaT cells, as in chapter 3. Data were analysed by One-way ANOVA with Sidak's multiple comparisons* p≤0.05 **p≤0.01 ***p≤0.001. n=6, percentage data was transformed by $Y=Log_{10}Y$ before analysis. Data presented as mean ±SEM. 'Nano' denotes Nanocin™.

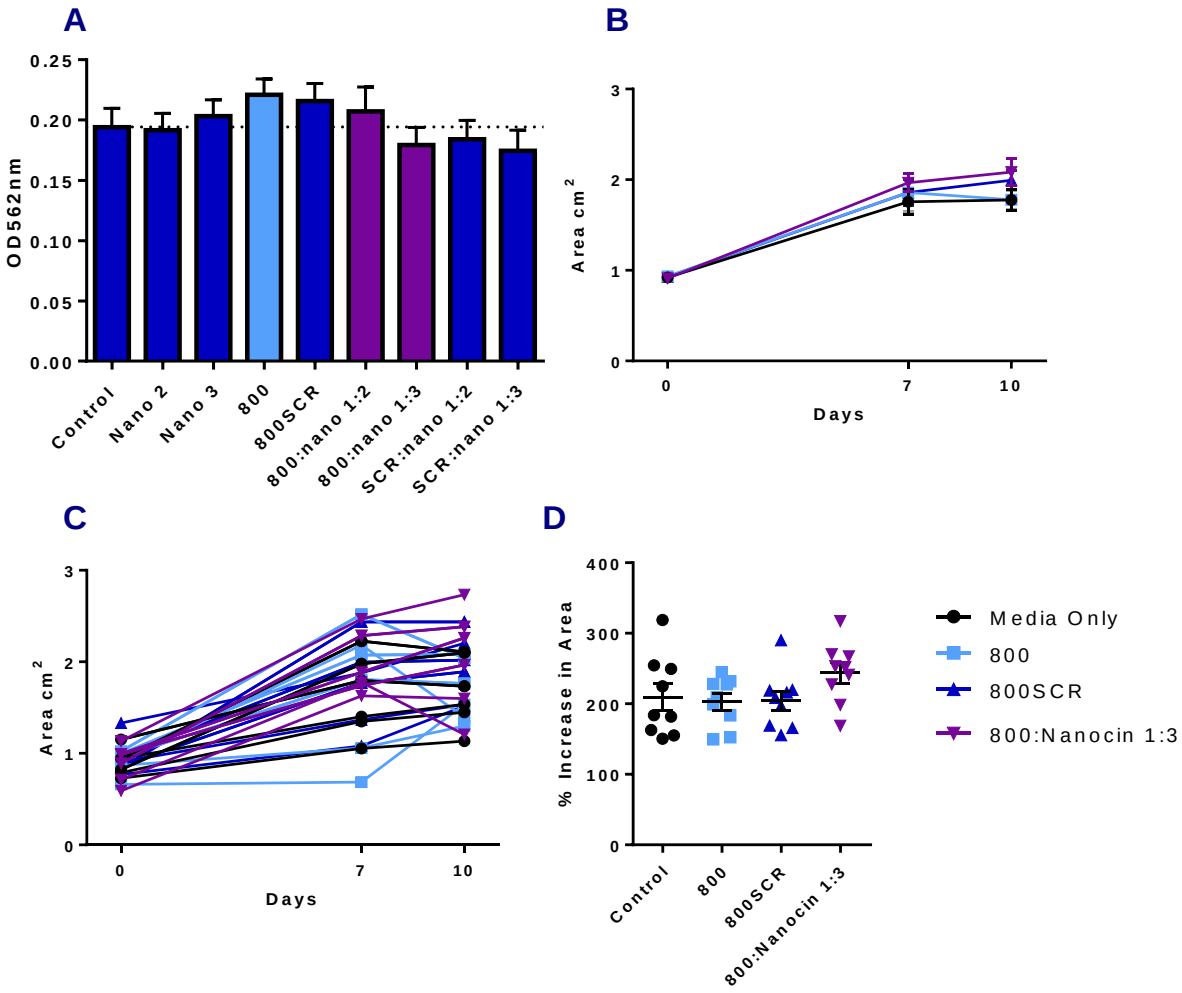
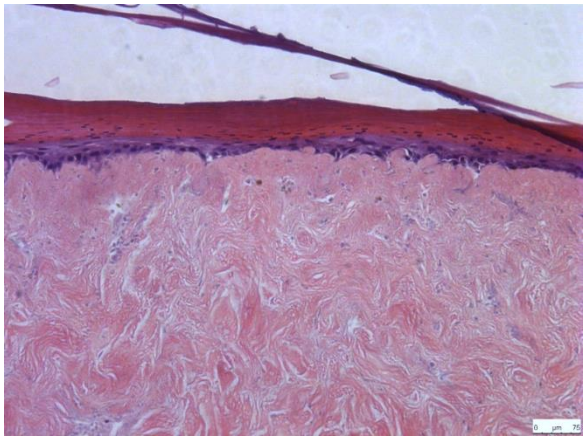
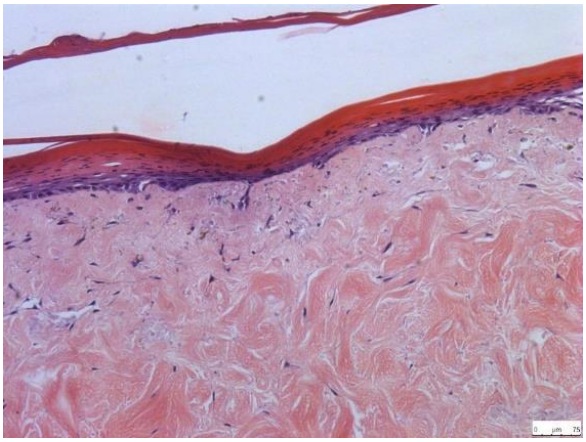


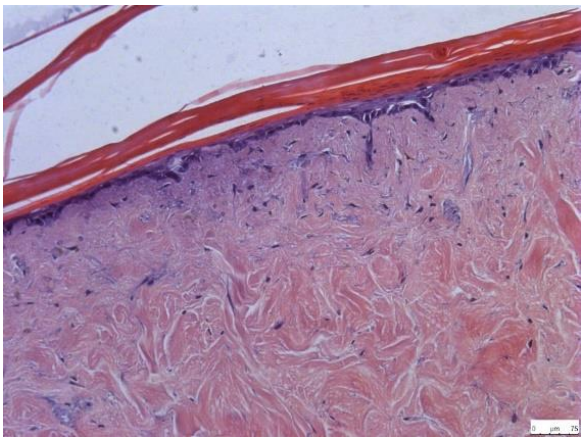
Figure 5.4: Nanoparticles Do Not Influence Cell Viability or Epidermal Migration: (A) An MTT assay was performed in which HaCaT cells were treated with peptides or conjugates for 24 hours then dyed with MTT for 1 hour before the quantification of metabolic products by OD_{562}. Data analysed by One-way ANOVA, n=3, duplicate. Dotted lines denote media only control figures. (B-D) Epidermis was allowed to migrate across bare scaffold to simulate the epidermal migration which occurs during wound healing. TEskin was treated with peptide or formulation at 200nM basal concentration every 2 days for 10 days. (B) Mean area of metabolically active epidermis per repeat. (C) Area of metabolically active epidermis of each replicate. (D) The percentage of increase in metabolically active area over the ten days of observation. Data analysed by One-way ANOVA, n=3. Percentage data were transformed by the function $Y=Log_{10}Y$ before analysis.



800



800SCR



800:Nanocin™

Figure 5.5: Peptide Treatment Does Not Affect the Structure of TEskin: TEskin was treated with 800 peptide, 800SCR or 800:Nanocin™ 1:3 formulations throughout 10 days of epidermal migration as in figure 5.4. Skin was then paraffin embedded, sectioned and stained with haematoxylin and eosin which stains nuclei and other negatively charged material blue (haematoxylin), and acidic or positively charged structures pink to red (eosin).

experiment, TEskin pieces were treated with peptides, Nanocin™ or media only as a control every 2 days during migration. The 800:Nanocin™ nanoparticles had no effect on the speed of epidermal migration across a bare dermal scaffold over 10 days. Histological samples also showed no difference in epidermal/dermal organisation in TEskin treated with peptide or Nanocin™ over 10 days (Figure 5.5).

5.2.5 Dose Response and Retention of Peptides and Formulations

To explore any changes in pharmacokinetics after formulation with Nanocin™, a dose response assay was carried out (as in Chapter 4) to assess if packaging of the peptides into nanoparticles increases or decreases their efficacy at lower concentrations. Figure 5.6 shows that the inhibitory concentration (IC_{50}) of 800 peptide alone when formulated into Nanocin™ nanoparticles is improved by around an order of magnitude from 1.5-3.5x10^{9} to 2.4x10^{10} M. This improvement is observed in terms of the percentage of cells with adherent bacteria and the number of adherent bacteria per 100 cells. The number of bacteria per infected cell remains constant throughout the conditions as observed previously (data not shown).

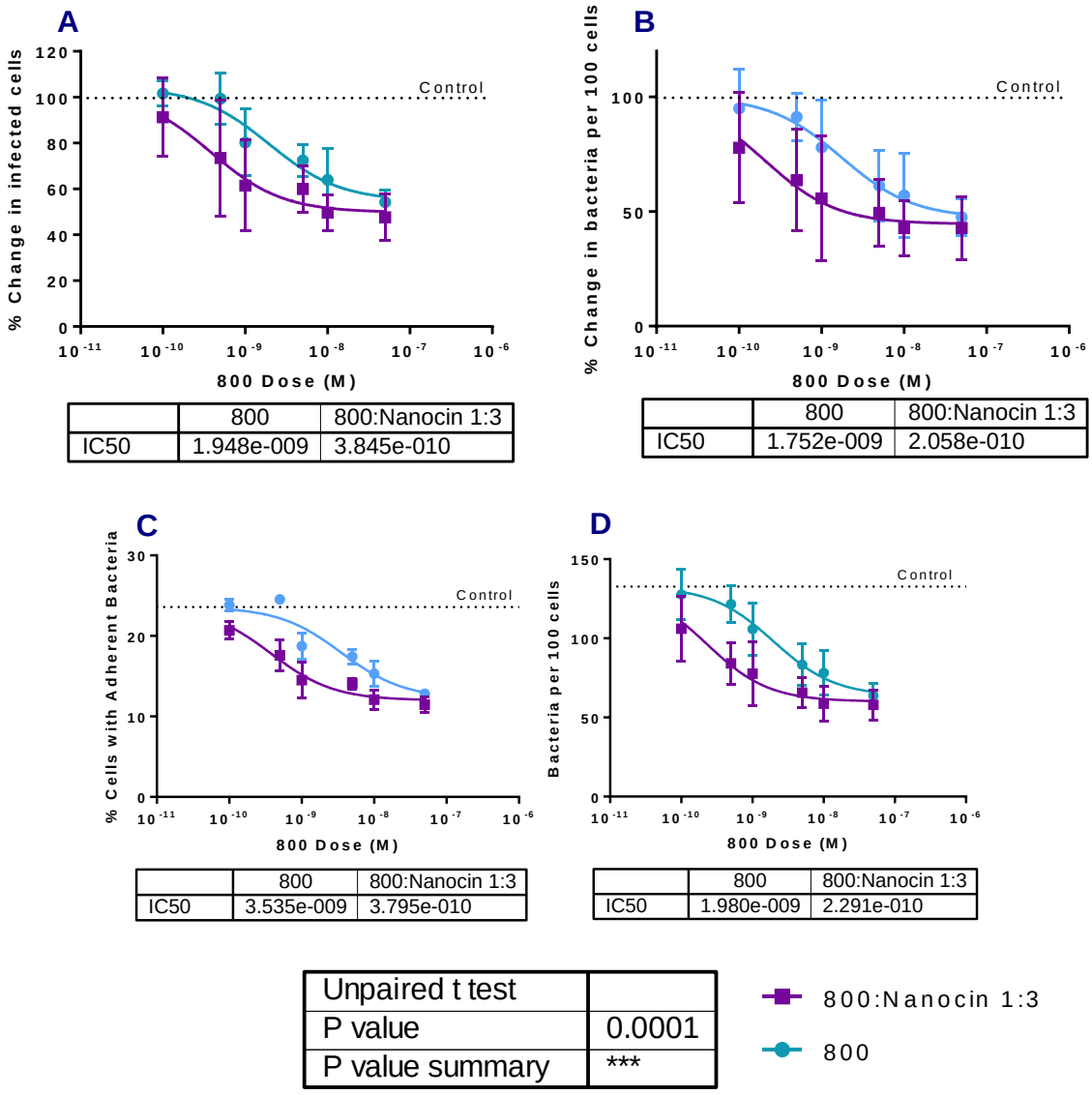
The duration of the anti-adhesive effects of the peptide was also increased by formulation with Nanocin™ (Figure 5.7). These data were best fitted with a plateau followed by one-phase association, which is reflected in their K^2 figure. A full 60% reduction in cells with bacterial adherence is maintained for 2.6±0.5 hours with peptide alone, which increases to 3.7±0.5 hours when packaged into nanoparticles. There was also a difference in efficacy as shown by the significant reduction in the IC_{50} figures of the peptide:Nanocin™ formulation.

5.2.6 The Effects of Nanoparticle Formulations on Cytokine Production

To assess if treatment with the nanoparticles affected a normal cytokine response to a wound infection the cytokine response in response to staphylococcal wound infection was measured in TEskin as in chapter 3 (Figure 5.8). Peptide:Nanocin™ nanoparticles, 800 peptide alone and a serum free media control were left on the TEskin throughout the experiment. Samples were taken at 24 hours post wounding and analysed by cytometric bead array. Outliers were then identified by the ROUT method and removed, and a one-way ANOVA performed to test for any changes in production of MCP-1, IL-1α, IL-6 or IL-8 secretion. No effect was seen on the levels of cytokines tested secreted into the wound effluent of TEskin with any of the treatments. Additionally, no effect was observed with the scrambled control of the peptide.

5.2.7 Nanoparticles Abolished Peptide Function in TEskin

TESkin was burnt and treated with the peptide and nanoparticles as previously described. After 1 hour of pre-treatment, S235 bacteria at $3x10^6$ per piece of TEskin was added and allowed to adhere for 5 hours. The skin was then washed to remove non-adherent bacteria and the remaining allowed to grow until 24 hours post-wounding, when it is washed again



	800	800:Nanocin 1:3
IC50	1.948e-009	3.845e-010

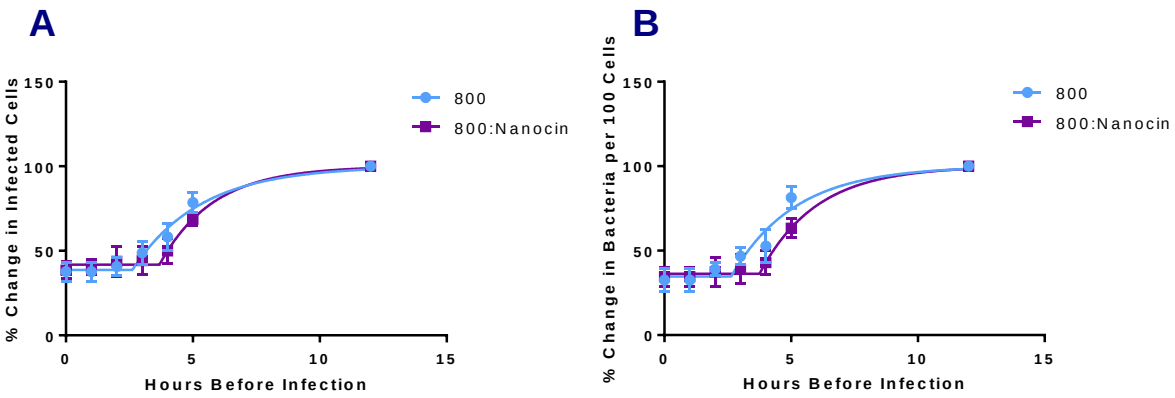
	800	800:Nanocin 1:3
IC50	1.752e-009	2.058e-010

	800	800:Nanocin 1:3
IC50	3.535e-009	3.795e-010

	800	800:Nanocin 1:3
IC50	1.980e-009	2.291e-010

Unpaired t test	
P value	0.0001
P value summary	***

Figure 5.6: Dose Response Curve of the Nanocin™ Formulation: Dose response showing the effectiveness of 800 peptide vs 800:Nanocin™ nanoparticles against the adherence of S235 *Staphylococcus aureus* to HaCaT cells as in chapter 4. Varying doses of peptides or formulation were added to the cells for 30 minutes prior to 1 hour of infection with bacteria. (A and B) Data normalised as a percentage of the no treatment control figure, presented as mean ±SD. (C and D) Non-normalised data represented as mean ±SEM. Log-inhibitor vs response – three parameter non-linear regression. The two conditions are statistically different based on an unpaired t-test comparing the log IC_{50} figures for both conditions; p=0.0001.



	800	800:Nanocin
Best-fit values		
X0	2.615	3.688
Y0	38.69	41.87
Plateau	= 100.0	= 100.0

	800	800:Nanocin
Goodness of Fit		
Degrees of Freedom	34	33
R square	0.5738	0.4428

	800	800:Nanocin
Best-fit values		
X0	2.635	3.755
Y0	34.74	36.35
Plateau	= 100.0	= 100.0

	800	800:Nanocin
Goodness of Fit		
Degrees of Freedom	34	34
R square	0.5635	0.4313

Unpaired t test	
P value	0.0010
P value summary	**

Figure 5.7: Nanoparticle Formulations Retain Their Effects for Longer: The duration of the anti-adhesive effect of 200nM peptides to *S.aureus* S235 strain was compared to that of an equal concentration 800:Nanocin™ formulations. Treatment was added to the cells at different time points for 1 hour then washed off. Cells were incubated in media for varying lengths of time, then infected with S235 bacteria for 1 hour before fixing Bacterial adhesion was quantified by fluorescence microscopy. Data are modelled by a plateau followed by one-phase association non-linear fit. Data presented as mean ±SEM. Curves compared by t-test were considered statistically significantly different p=0.001.

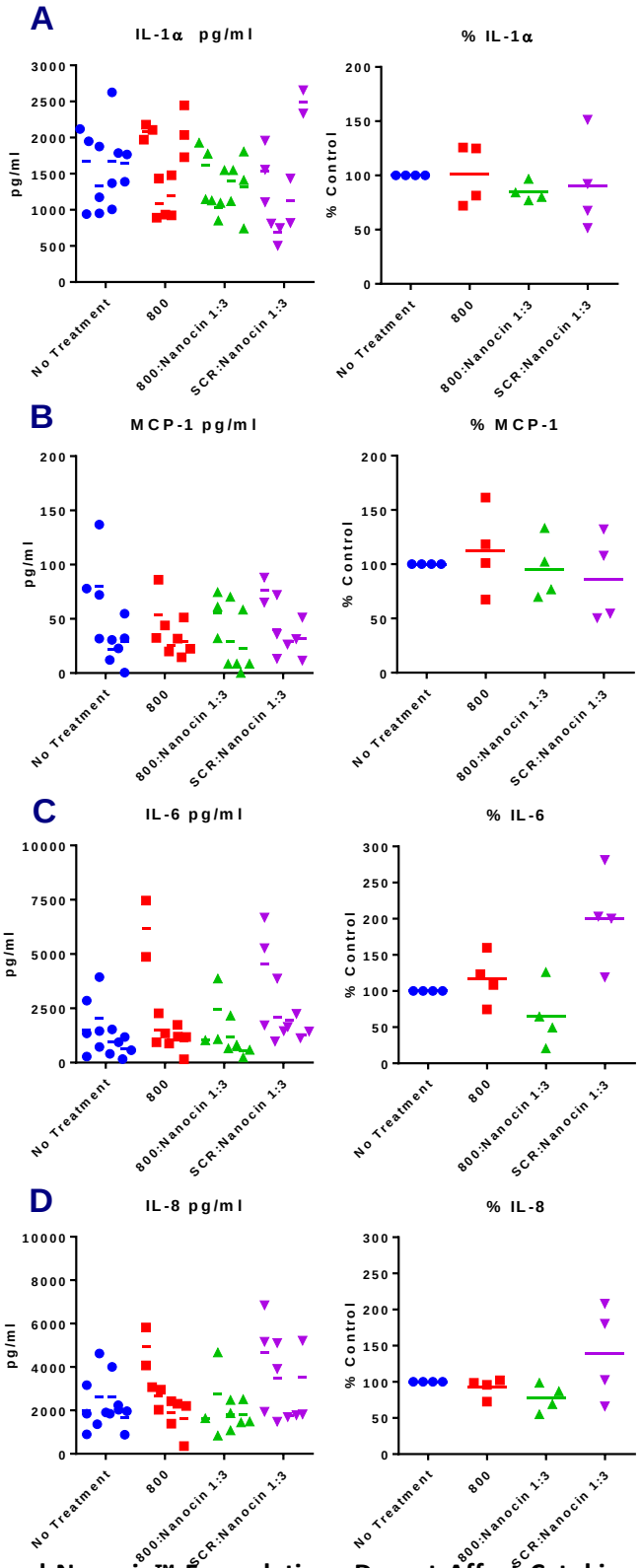


Figure 5.8: Peptide and Nanocin™ Formulations Do not Affect Cytokine Production by TEskin: Skin was wounded by burning then treated with 800, 800:Nanocin™, SCR:Nanocin™ for 1 hour. S235 *Staphylococcus aureus* was then added for a further 6 hours before the treatment and bacteria was washed off and replaced with serum free media. Cytokine samples were taken from the serum free media on top of the skin and stored at -20°C until analysis by cytometric bead array. Outliers were removed by ROUT test, Q=1%. Data were analysed for skew, and non-parametric columns were compared to the control column using a Wilcoxon Signed Rank Test. Normally distributed data were analysed by One-way ANOVA and percentage data was transformed by $Y=Log_{10}Y$ before analysis. No significant data. Horizontal lines denote the mean value for each repeat.

and fixed. Treatment was left on throughout the infection period and data was collected by viable counts (Figure 5.9). A significant reduction in bacterial load was observed with those skin pieces that had been treated with 800 peptide. This trend however was not observed with the 800:Nanocin™ or the SCR:Nanocin™ nanoparticles, indicating that these formulations no longer exert an anti-adhesive effect in the TEskin model.

To test if this was due to a change in localisation of the treatment caused by the Nanocin™, TEskin was treated with the peptides or the peptide:Nanocin™ formulations for 24 hours, then fixed. Analysis by histology suggested that the peptide and the nanoparticle formulation are both confined to the surface in un-wounded skin (Figure 5.10). No obvious penetration into lower layers of the skin was observed. This is not entirely unexpected due to the strong barrier provided by an intact stratum corneum, and confirms previous data that suggests Nanocin™ penetrates intact skin through hair follicles rather than by direct diffusion through the stratum corneum (Good et al, unpublished data).

Peptides tagged with tetramethylrhodamine as shown in chapter 3 were also combined into nanoparticles with FitC tagged Nanocin™, to test for co-localisation on the skin. Nanoparticle formation could not be observed with these peptides directly as the emission of the TMR tag disrupted to sensors on the NanoSight LM10, and a more advanced version would be required to detect the particles and determine their fluorescent composition. The new tagged peptides however are still of an appropriate size and charge for nanoparticle formation, and should behave in the same way as the standard peptide:Nanocin™ formulations. In unwounded TEskin we can see that the Nanocin™ and peptides are co-localised, suggesting that the formulations hold together when applied to the skin. There are areas however, as highlighted in Figure 5.11 A2 and B2, where the two components are co-localised, but the signal is concentrated in slightly different regions, which could indicate that the nanoparticles are not all composed of the same ratios of peptide and Nanocin™, or that the Nanocin™ is changing delivering the peptides into different regions.

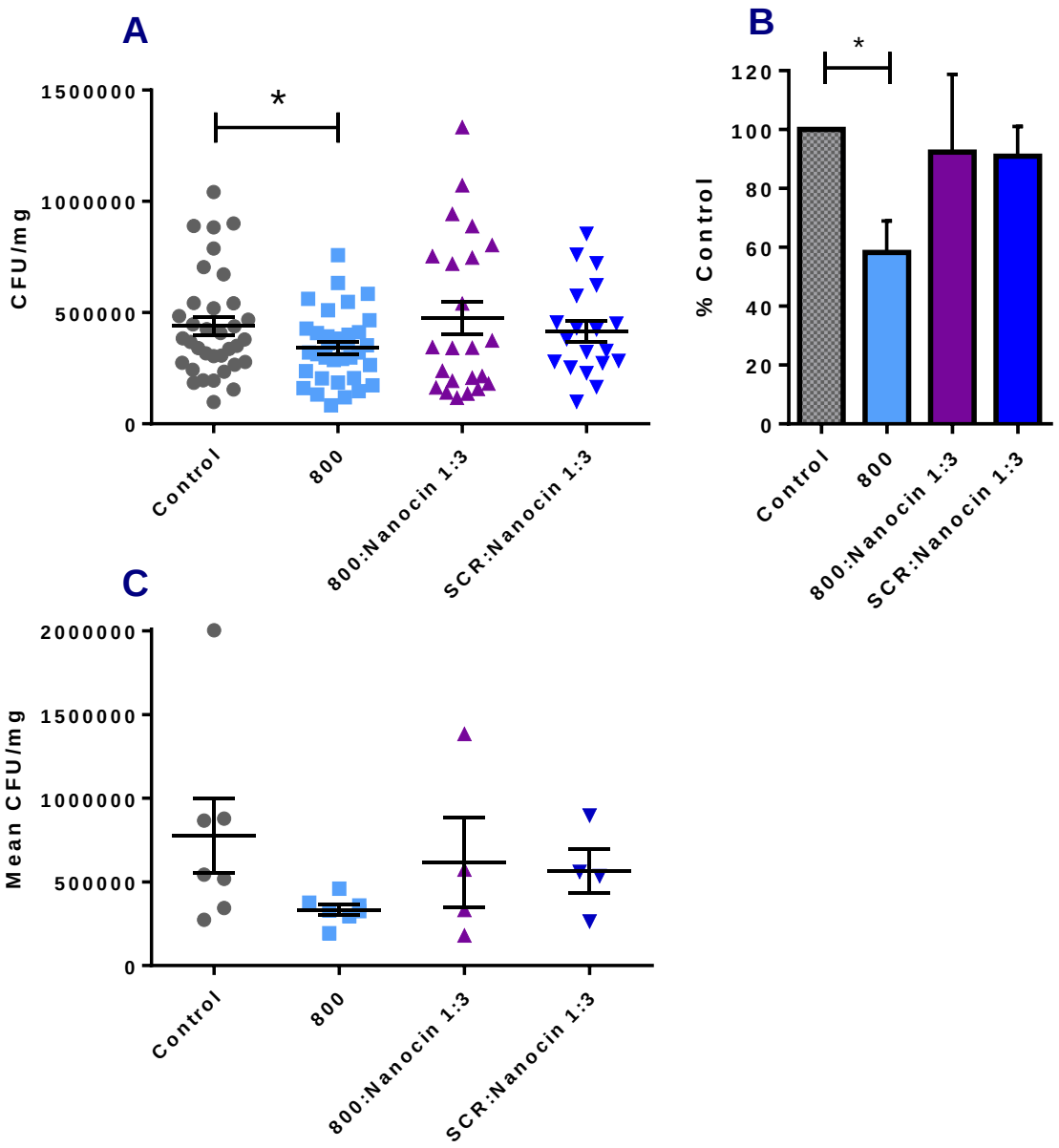


Figure 5.9: Nanocin™ Formulation Have a Lesser Effects on the Adherence of *Staphylococcus aureus* to TEskin: TEskin was burnt and treated continuously with 200nM 800 peptide or formulations. After 1 hour it was then infected with S235 strain *Staphylococcus aureus* for 5 hours, then the composite was washed and the bacteria harvested 24 hours after burning. A) Mean cfu count per mg of tissue B) Normalised as a percentage of the control C) Mean cfu/mg per. No skew is detected with skew analysis, data are parametrically distributed. Data for Control and 800 included from previous experiment as methods remain comparable. All data n=4, Control and 800 n=7. Percentage data were transformed by the function $Y=Log_{10}Y$ before all data were analysed by One-way ANOVA with Sidak's multiple comparisons. * $p \leq 0.05$.

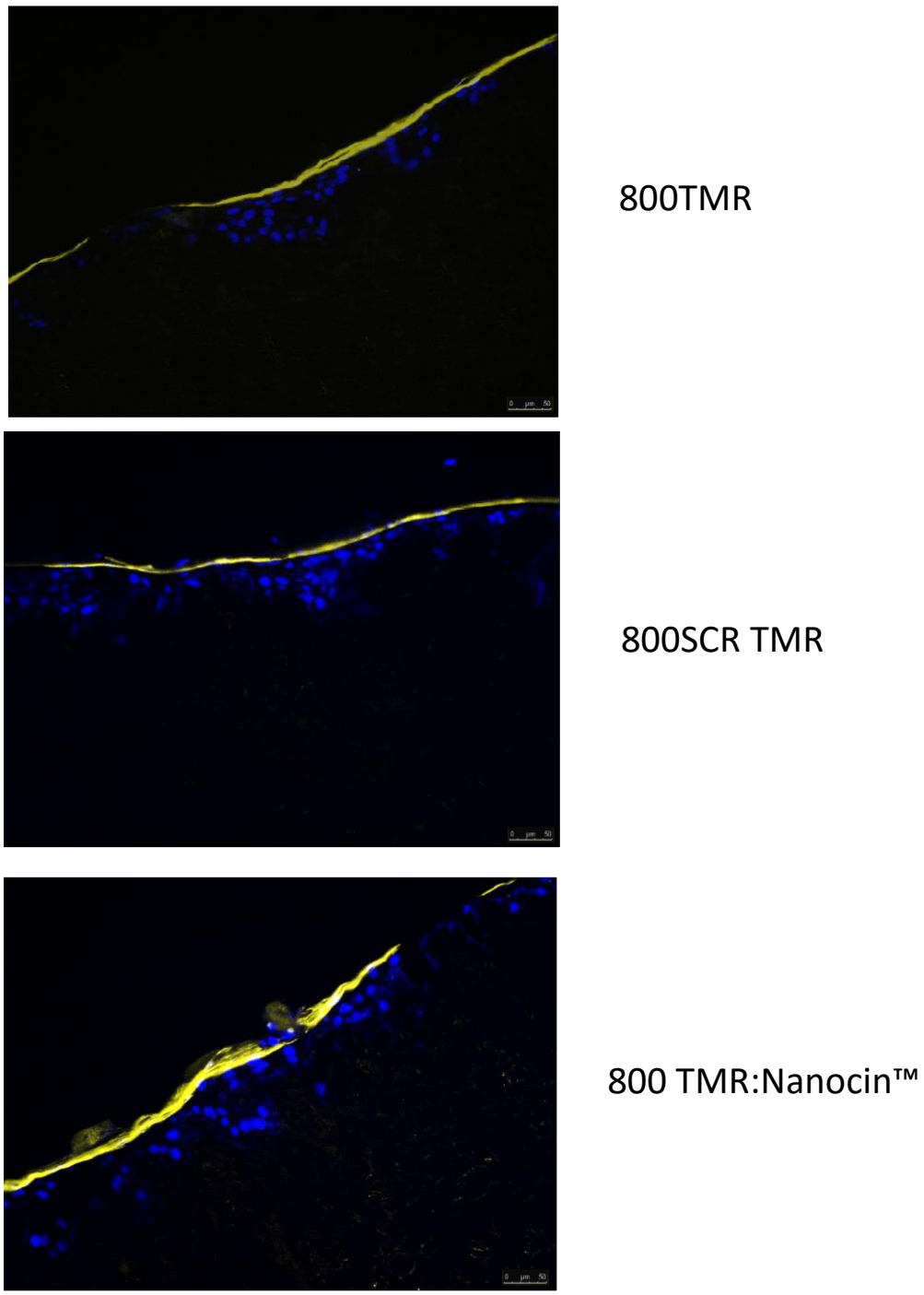


Figure 5.10: TMR tagged Peptides and Nanocin™ are Localised to the Surface of Unwounded TEskin: Peptides and formulations at 1µM in cell media were applied to the top of 14 days old TEskin in the centre of a 10mm diameter metal ring and left for 24 hours. TEskin was then washed once and fixed overnight in 4% paraformaldehyde. Sections were mounted in Vectashield with DAPI. Nuclei are shown in blue and peptides/Nanocin™ formulation in yellow. 20x magnification, scale bars 50µm.

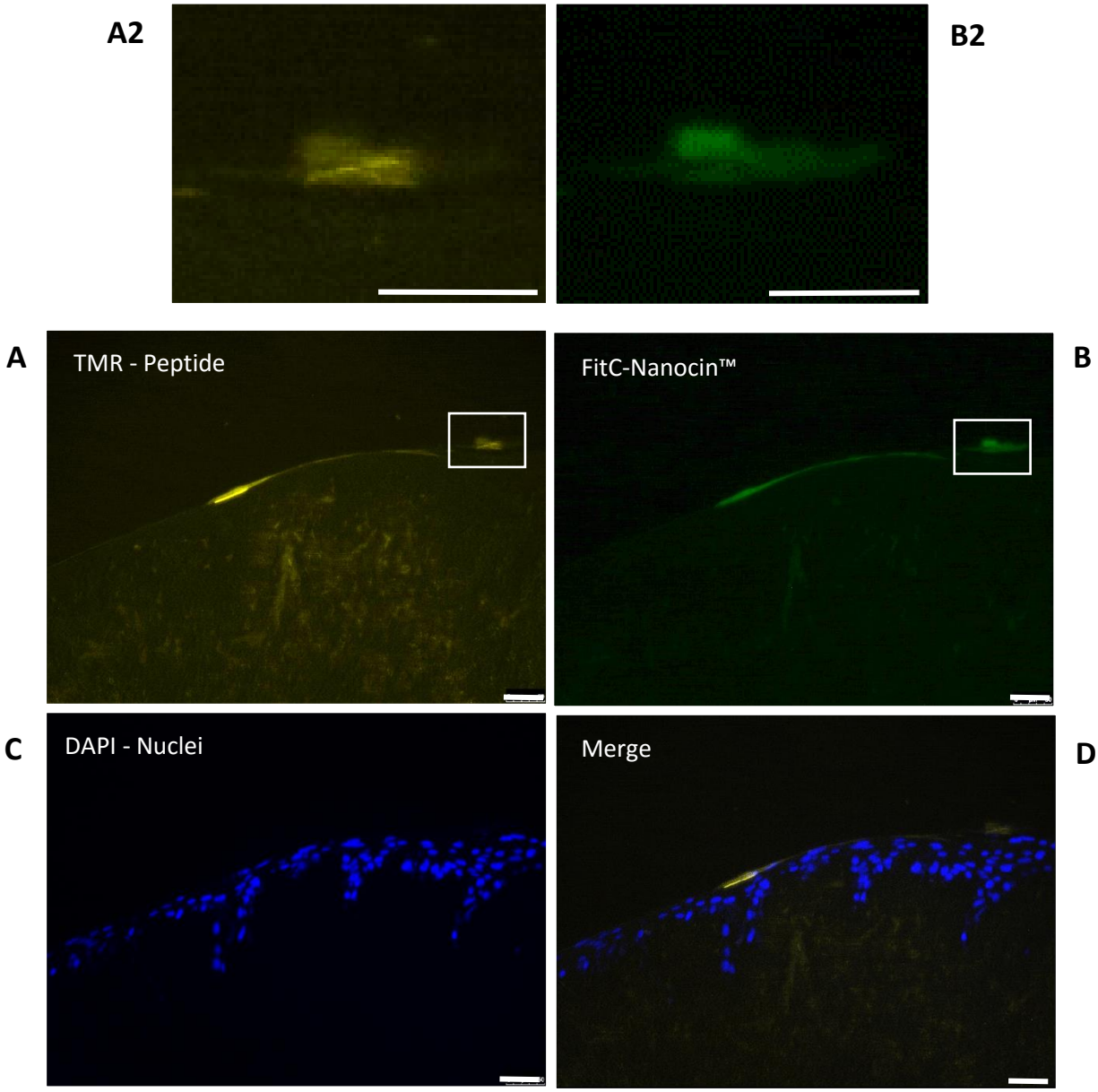


Figure 5.11: Peptide and Nanocin™ are Co-localised on TEskin: TMR labelled peptide 800 was combined with FitC labelled Nanocin™ and applied to TEskin for 24 hours as in figure 4.2.10. Sections were mounted in Vectashield with DAPI, nuclei are shown in blue and peptides/Nanocin™ formulation in yellow. 20x magnification, scale bars 50µm

5.3 Discussion

In this chapter we aimed to explore some avenues through which the anti-adhesive effects of the tetraspanin based peptide could be improved for therapeutic use. We showed that in a cell line model, the peptides can work synergistically with the antibiotic flucloxacillin, however this synergistic effect was not seen in a 3D model of human skin. We also showed that the packaging of peptide 800 into nanoparticles with Nanocin™ preserved the peptide's function, and improved the IC_{50} and retention time in a cell monolayer. This formulation however lost its anti-adhesive properties when applied to the TEskin model.

Combination therapy is commonly used for the treatment of wound infections. Therefore the ability of the peptides to work without interfering with other drugs, and possibly to aid their function is important. In an effort to test for this, we combined the peptides with a weak antibiotic recommended for methicillin sensitive *Staphylococcus aureus* treatment. Based on pharmacological data, bioavailability and recommended dose of flucloxacillin (Paton et al., 1982, Nauta and Mattie, 1975), we estimate that the final active concentration of antibiotic in tissues to be a maximum of 4µg/ml, varying depending on tissue location, individual size, rate of metabolism and many other factors. According to our data, this concentration of flucloxacillin is enough to kill approximately 95% of this clinical MRSA isolate in a cell line model, a figure which in our model is increased to 98% by pre-treatment with the peptides. With 800 peptide pre-treatment the proportion of remaining viable bacteria could potentially be halved.

In theory, as an anti-adhesive therapy, the peptides should leave non-adhered bacteria exposed on the wound surface, allowing for more effective antibiotic action and increased killing. This however does not appear to be the result in the TEskin model in which the synergistic effect observed in the cell line model is lost completely. This could be due to the peptide and antibiotic working against each other, although the mechanisms through which this could happen are unclear and an indication of this would be visible in the cell line model. Alternatively, how the antibiotics are administered in the more complex wound environment is a point for consideration; flucloxacillin is normally administrated orally as opposed to topically, and perhaps something in the wound effluent could be affecting its normal mechanism of action, or reducing the quantity of antibiotic present. This unknown factor could be upregulated in response to the peptide treatment, explaining the abolishing of

flucloxacillin activity in 800 treated TEskin. To assess this, flucloxacillin could be pre-incubated with wound effluent from treated and untreated TEskin, then tested against bacterial growth to assess any change in function. The presence of sub-inhibitory concentrations of antibiotics is known to alter, and often upregulate, the expression of adhesins by invading pathogens (Bisognano et al., 1997, Ohlsen et al., 1998), and so the infection dynamics over the course of a 24 hour infection and 3 hour flucloxacillin treatment are unpredictable. If the flucloxacillin is being degraded at all then it is possible that the anti-adhesive effects of the peptides are cancelled out by the pro-adhesive effects of low working concentrations of flucloxacillin.

It should be noted that this data is based only on 3 repeats due to limited materials and time, and the variance in the data could be causing a false result. The control figures in these experiments were also uncharacteristically small, so that despite the figures for skin treated with 800 peptide being very similar to those obtained in previous experiments, this is greater than the control figures. Therefore the assay should be repeated and the infection time, concentration of flucloxacillin and peptide, and methods of application should all be optimised in order to confirm a significant result before further experiments are carried out.

The TEskin model is also a very simple model of *in vivo* human skin. In a real wound, immune cells such as dendritic cells and macrophages, and pressure exerted by fluid movement contribute to a much more effective barrier against infection. These immune factors would constantly clear the non-adherent bacteria from the system, whilst flucloxacillin could diffuse constantly from basal skin layers into the wound and surrounding cells steadily to prevent infection. In this TEskin model those factors are missing, and the bacteria are confined in the system without clearing for large periods of time, with the flucloxacillin applied to the surface of the skin for that time.

In conclusion, these data do not tell us that the peptides are incompatible with all antibiotic treatments, and more testing should be carried out to elucidate any potential negative consequences of combination therapy. A range of antibiotics with varying modes of action, as well as multiple different strains of bacteria should be screened against.

As mentioned previously, one of the major pitfalls of anti-adhesive peptides is their inability to penetrate into tissues and their susceptibility to degradation (Krachler and Orth, 2013). In order to address these issues, peptide 800, which we have previously shown to have anti-

adhesive properties, and its scrambled peptide control, were combined with a nanotechnology based drug delivery platform developed by Tecrea™ known as Nanocin™. Due to IP restrictions not much information is available on the mechanism of action of this compound, and how and why it forms these 'nanoparticles', however the formation and presence of the nanoparticles is observable using a NanoSight.

We chose to combine the peptides with Nanocin™ for a number of reasons. Initially, we were interested to see if the peptides still retained their function when potentially delivered intracellularly, which was initially shown in the HaCaT cell line adherence assay. CD9, the peptide target, is seen expressed abundantly on the outer cell membrane, and therefore we hypothesised that combining the peptides with an agent known to deliver molecules inside of cells might abolish the function of the peptides. This however was not the case, and anti-adhesive effects were retained. We then hypothesised that perhaps intracellular delivery would cause some harm to the cells, as CD9 is known to also be expressed in low abundance on intracellular vesicles (Fernvik et al., 1995, Okochi et al., 1997, Ryu et al., 2000) and have intracellular functions, but no obvious toxicity was observed using an MTT test or migration assays. Based on these results it is possible to assume that the Nanocin™ is not delivering the peptides to the inside of the cells, or is delivering only a portion of the peptide, and this could be assessed using high resolution microscopy. These test however are limited, and more detailed assessment of CD9 dependent pathways should be performed.

From the perspective of pharmacological properties, the combination of Nanocin™ with 800 peptide has increased its applicability. At the concentrations used in these experiments, the Nanocin™ has no anti-microbial activity, as reflected by the lack of direct anti-adhesive effects in cell infection assay. Therefore, effects observed with the 800:Nanocin™ formulation are due to an increase in peptide efficacy, delivery or retention, as opposed to being an inherent property of the Nanocin™. In the two pharmacological characteristics tested for, IC_{50} and half-life ($t^1/_2$) of effect, a noticeable increase in the effectiveness of the peptide was seen using peptide nanoparticles. Further development, e.g. by incorporating this into a hydrogel or complexing with a polymer could increase the stability and retention of the peptide.

An interesting result obtained here was the lack of nanoparticle function in the TEskin model. We speculate that 800 peptide could become non-functioning when combined with the

Nanocin™ due to a change in the localisation of the peptide in the skin. In a wound setting, Nanocin™ is thought to deliver the peptide to deeper layers of the wound and into cells however data shown in chapter 3 suggested that after 24 hours, the majority of bacteria were either localised on top of the dermis, or were in the upper layers of the epidermis at the periphery of the wound, and therefore deeper penetration in this case would actively remove the peptide from where it would function, possibly leaving too low a concentration to prevent adherence. To try and determine if this was the case, some preliminary assays were undertaken to try and visualise peptide penetration into the skin. In intact skin both the peptide alone and the formulations appeared to be limited to the surface of the stratum corneum. On wounded skin the treatments may penetrate further, however in these skin pieces, the signal became to diffuse to detect with the microscopes available (data not shown) either due to simple diffusion, or the presence of proteinases produced by the wound. This microscopy however could be repeated with higher concentrations of peptide/nanoparticles or with a higher resolution, more sensitive microscope to determine the location of the treatment. Alternatively, the apparent lack of function of the nanoparticles on the TEskin could, as earlier, just be due to variance in the data, as the data for the 800:Nanocin™ formulation does appear slightly negatively skewed. As before, further repeats would be needed to confirm or deny this.

These data highlight the need for extensive testing of potential therapies before moving forward to animal and clinical trials. Based on data from previous chapters, changing variables such as the strain of *Staphylococcus aureus* or the location of the CD9 EC2 from which the peptide is derived could influence the outcome of each experiment. Additionally, a lot of potentially hazardous side effects would not be detected with the relatively simple assays performed here, and processes as complex and individualised as wound healing and infections, can never be modelled easily in cellular systems. Also, although the use of primary cells and models using primary cells are more representative models of the human reaction than cell line assays, using these cells also greatly increases the variance in data with donor bias, making conclusions difficult to draw.

Overall, we have explored some ways in which to practically apply the tetraspanin-based peptides developed in previous chapters, in progression towards the clinic. However we have also generated more questions to be addressed about both the usefulness of this TEskin

model as well as the necessary localisation and retention of therapies on the surface and top layers of the wound.

All work relating to Nanocin™ was carried out in collaboration with Blueberry Therapeutics, who possess an extension of the IP which covers the use of Nanocin™.

Chapter 6 - Discussion

Staphylococcus aureus is a major causative agent of skin and soft tissue infections (SSTIs) in the developed world. It is involved in a range of clinical conditions with different severities such as impetigo and necrotizing fasciitis, and estimates of its financial burden on the NHS exceed £1 billion per annum. Although the mortality rate associated with Staphylococcal SSTIs themselves is low, these infections allow entry of pathogens into the bloodstream to cause secondary infections and sepsis, which have much higher mortality rates.

The presence of antibiotic resistance in *Staphylococcus aureus* was first observed in the 1940s, and since then a large range of mechanisms have evolved, leading to a greater population of *S. aureus* with multiple resistance genes. Initially, MRSA was considered a nosocomial infection but many strains of community-acquired *Staphylococcus aureus* have now appeared, with no hospital visit required for an infection to set in. The demand for a wider range of treatments, including new antibiotics, is therefore becoming increasingly urgent.

For wound treatments specifically, there is a need for compounds with varying mechanisms of action to prevent the increase in resistance, and to treat SSTIs quickly, efficiently, and inexpensively. An increase in the speed in which these drugs are brought to the market is also needed, to keep up with the rate of microbial adaptation. For example, resistance to antimicrobial agents such as silver, a common component of wound dressings, is already beginning to be observed, although it has not yet been observed in pathogenic species (Woods et al., 2009).

One potential method is to target bacterial adherence, as reviewed in Cozens et al (Cozens and Read, 2012), preventing the initial attachment stages of bacterial infection. Resistance to these therapies should in theory be much slower to arise, as they do no kill the organisms directly and therefore do not apply as much selective pressure. Resistance may still appear however, by selection for bacteria with stronger or faster adhesins, but in combination with other therapies it could be sufficient to make highly resistant species more susceptible to killing.

The work undertaken here was motivated by previous work in Sheffield by Drs Luke Green and Daniel Cozens, which demonstrated that antibodies to tetraspanins, recombinant EC2

domains and tetraspanin-derived peptides could reduce the adherence of both Gram positive and Gram negative pathogens to host cells. The peptides are also currently being tested for their effects against *Salmonella enterica* adherence (Mr Fawwaz Ali), *Pseudomonas aeruginosa* adherence (Ms Jehan Alrahimi) and monocyte giant cell fusion (Mr Thomas Champion). These studies will hopefully help to further elucidate further the mechanism through which the peptides work, and to confirm or deny the theory that they function by disrupting receptor organization by disrupting tetraspanin enriched microdomain organization. An alternate theory is that the peptides and EC2s are causing the cross-linking and internalization of receptors associated with tetraspanins, and this could be investigated by assessing the amount of cell-surface tetraspanin throughout the peptide treatment.

Peptides as anti-microbial agents

The success of the peptides in preventing *S. aureus* adherence to host cells can be regarded as mixed. Different strains of *Staphylococcus aureus* showed different levels of response to the treatment, with the most virulent strains being most sensitive. Cell type also seemed to have an effect on the peptide efficacy, with keratinocytes exhibiting a large significant change in infection levels, as opposed to fibroblasts, which appeared unresponsive to treatment. We initially hypothesized that this could be due to incomplete saturation of cells with the peptides, which had previously been titrated for use on keratinocyte and epithelial cell lines, with an optimal dose of 50nM. Fibroblasts may for example, have a larger surface area than the other cell types so far tested. However, higher doses of peptides (up to 200nM) also failed to inhibit bacterial adhesion. When we compared the expression levels of tetraspanins between cell types however, we observed that CD9 expression in fibroblasts was very low compared to other cells, and a weak correlation between CD9 expression and peptide efficacy was observed. Although promising, this is still preliminary data and standardized studies in different cell types across different species should be completed before solid conclusions are drawn. Additionally, testing the peptides in a CD9 knockdown cell line would confirm that peptide function is dependent on CD9, and would help to elucidate whether the peptides act by interacting with CD9 or with its partner proteins.

Across all of the cell lines and bacteria tested, the maximum reduction in bacterial adherence by the peptides is around 60%, consistent with previous experiments using anti-tetraspanin

antibodies and recombinant fragments (Cozens, 2016). The reason for this is currently unknown, and requires further research into the mechanism by which the peptides act. As the number of bacteria per cell is unaffected by peptide treatment, this suggests that there is a subpopulation of the cells, around 40%, upon which the peptides are ineffective. The levels of tetraspanins expressed on the cell surface changes during the cell cycle, and this might have an effect on the efficacy of the peptides on cells in different places in the cell cycle (Green, 2010). This might also account for some of the reduced function of peptides on fibroblasts which are a highly proliferative cell type in culture. Alternatively, these bacteria may be attaching to cells via a CD9 independent mechanism, or may be capable of adhering despite the dispersal of receptors (Green et al., 2011).

This also leads to the question: as a therapeutic, is a 60% reduction in bacterial adherence good enough? Currently used systemic antibiotics function by killing or disrupting the bacteria's ability to grow or divide, and the efficacy of these treatments has been difficult to define *in vivo*. Even with infections of antibiotic-sensitive bacteria, a 100% clearance rate is not observed (O'Meara et al., 2000, Howell-Jones et al., 2005). There are multiple advantages to having these tetraspanin targeted peptides as a therapy. As mentioned previously, anti-adherence therapies put less selective pressure onto the pathogens and therefore resistance is less likely to develop. This is important in a new clinical drug, as the rate at which resistance is developing means standard drugs and antibiotics are less cost effective to develop and produce. Additionally, the tetraspanin based peptides have shown activity against a very broad range of both Gram-negative and Gram-positive pathogens at various target sites, and therefore could be very broadly applicable. Finally, as this therapy is host targeted it has strong potential for being combined with bacterially targeted drugs, allowing for a two pronged approach for the prevention and treatment of infection. This would be particularly useful in combination with antibiotics, which would initially kill a proportion of the bacteria, and later in the time-course, at sub-inhibitory concentrations, potentially reduce bacterial adhesion to host cells by altering bacterial surface properties (Krachler et al., 2011).

Many further experiments need to be conducted to determine the mechanism of action of the peptides. Some of these possible experiments, such as CD9 knockdown are mentioned above. Additionally, high-resolution microscopy such as 'STORM' microscopy would give us further information about how the TEM are organized and what effect peptide treatment

would have on this. Further studies could include testing the peptide efficacy on cells expressing chimeric or mutated versions of CD9, which would indicate how the peptides may be interacting with the cell. Furthermore, using different strains of *Staphylococcus aureus* with defined mutations in specific virulence factors and adhesins would define exactly which staphylococcal adherence mechanisms are affected by tetraspanin treatment.

Previous experiments have also been undertaken to show how effective the peptides are if applied at the same time as bacteria or after the bacteria. Although a reduction in peptide efficacy was observed, there were fewer adherent bacteria in the system after 30 minutes of peptide treatment. This has not been confirmed with *Staphylococcus aureus*.

Potential Toxicity

Tetraspanins are very diverse molecules with varying functions across the body. Interfering with CD9 could cause long ranging effects, and testing for these is also important. We have shown here that peptide treatment did not affect cell metabolism using an MTT test. We also showed that peptides 8001 and 810 had no effect on cell migration in a monolayer model, whilst 800 reduced migration in this model but had no effects on epidermal migration in the larger skin model. Additionally, peptide 800 treatment did not affect cytokine production from the TEskin model. Previous data has suggested the interfering with CD9 using blocking antibodies does not affect the uptake of bacteria by monocyte-derived macrophages, which would suggest the peptides should not affect initial phagocytic stages of innate immunity (Green, 2010). These tests are sufficient as preliminary data, however the toxicity of the therapy should be reviewed throughout all stages of development.

Table 6.1 outlines other molecules to which 800 and 800SCR peptide have high levels of homology (>6 matched amino acid). Some of these are membrane proteins and therefore accessible to the peptides in the system. If the peptides were to interfere with the functions of these proteins, there could be additional unwanted side effects that we have not tested for here.

Table 6.1: Proteins with homology to tetraspanin peptide sequences.

Peptide	Homologous Protein	Function	Homology	Reference
800	Type IV collagen, α5	Structural, basement membranes and ECM	7/8	Pöschl, 2004 (Poschl et al., 2004)
	Neurobeachin	Trafficking of neurotransmitter receptors	6/6, 4/4.	Nair, 2013 (Nair et al., 2013)
800SCR	Nucleoporin	Component of the nuclear pore complex	9/10	Krull, 2004 (Krull et al., 2004)
	Autophagy related protein 2	Autophagosome formation	8/11	Mizushima,2011 (Mizushima et al., 2011)
	EGF receptor	Cell signaling, motility	6/6	Wells, 1999 (Wells, 1999)

Obtained from a NCBI protein basic local alignment search tool (Altschul et al., 1990).

Peptides as a Tool for Studying Tetraspanins

Studies with these peptides, combined with studies using chimaeras of CD9 and CD81 mentioned previously, have been useful for highlighting functional regions within the EC2 domain of CD9. The generation of tagged peptides was also a useful tool in studying how the peptides interact with cells, and further high resolution microscopy such as STORM, with varying peptide concentrations and conditions that disrupt TEMs such as cholesterol depletion. The peptides could also be used for a wide range of reasons not relating to their potential as a therapeutic. For example, to deduce the role of specific regions of the EC2 domain in multinucleated giant cell fusion (Fanaei, 2014).

TEskin as a Model

In this study, TEskin constructed of primary adult keratinocytes and fibroblasts on a de-epidermised acellular dermis (DED), was used as a complex model to test for peptide effects in a bigger system prior to any animal testing. This has advantages over a cell line model as it creates a 3D environment for bacteria to interact with, and it mimics the structure of normal human skin. It also has advantages over an animal model in ethical terms, as it cuts out animal suffering, although it could be debated that the quantity of foetal calf serum used to culture

the TEskin is enough to narrow this ethical divide. The TEskin, unlike animal trials, did allow for multiple repeats of multiple experiments at a relatively low cost, however the availability of skin biopsies to use as DED and a cell source was a limiting factor throughout the length of the PhD, and more repeats and therefore less varied data could have been obtained if the supply was more abundant and consistent.

Much of the variability in TEskin data arose from donor bias, also observed in primary cell models. Cells derived from different patients varied in morphology, growth rate and viability, and in specific characteristics such as strength of dermal:epidermal junction. When TEskin was constructed, fibroblasts and keratinocytes were taken from different donors in order to combat this, however differences in keratinocyte growth and division, for example, can have a big effect on cell number over a 2 week growth period.

The TEskin model also allowed for testing of the cytokine response. Testing cytokines in this model led to a greater variation in the data, due to donor bias, size of wound, and remaining quantity of viable cells. It also however gives a more realistic measurement of skin cytokine response, as research has shown that the expression of cytokines by fibroblasts can affect those secreted by keratinocytes and vice versa (Barrientos et al., 2008, Marionnet et al., 2006, Nedelec et al., 2007, Nowinski et al., 2004). This interplay is also important when looking for effects on cell migration that could affect wound healing. As seen in this study (Sections 3, 4 and 5), a result obtained in a cell line model can yield different results to a result in a model involving multiple cell types, and therefore testing on multiple cell systems is a crucial step in therapeutic development.

This said, a variety of different models of skin are available with varying levels of complexity. Those that use immortalized cell lines such as HaCaT cells or N/TERT immortalized keratinocytes are easier to set up and culture and therefore larger numbers of repeats can be undertaken, however there are genetic differences between these cell lines and primary cells which may lead to false results. Furthermore there are skin models which use collagen scaffolds rather than DED, which reduce the quantity of biopsy needed for making the skin, however cells in these models struggle to make a good epidermal:dermal junction membrane which is already present in the DED, and therefore the skin architecture is compromised (Blackwood et al., 2008, MacNeil, 2007). As an alternate, skin straight from biopsy could be

used providing it is transported rapidly from the site of surgery and kept perfectly sterile. This however would require an even larger area of skin, would display more donor bias, and there would be no cell expansion for preliminary cell experiments. Finally, some model systems available consist of just the epidermis, constructed of keratinocytes and differentiated into a 3D structure. Although good for surface penetration studies, these models do not contain the complex basal membrane structures, and due to only containing one cell type cannot give any information regarding keratinocyte and fibroblast signalling (CELLnTEC, 2015).

Another advancement in TEskin models is immunoskin, which contains a fibroblast seeded dermis and a keratinocyte seeded epidermis, both grown on acrylic acid scaffolds, and also has a layer of monocyte derived dendritic cells within an agarose-fibronectin gel in the centre (Chau et al., 2013). Having an immune cell layer represented in the model signifies a first step towards a fully comprehensive skin model which can model immune cell migration and other immune functions. Research into angiogenesis within these models and a range of other characteristics is also ongoing, and in coming years we may see a commercially available immune competent model for the testing of therapeutics.

Enhancing Drug Potential with Nanocin™

Nanocin™ (Tecrea) is a commercially available tool for the intracellular delivery of small molecules. Upon mixing with the small molecules, Nanocin™ spontaneously forms nanoparticles which are capable of penetrating into tissue such as skin and nails and delivering the molecules. The current model of how this works is not the Nanocin forming an encompassing sphere, but more of a mixed particle containing both Nanocin™ and the target molecule (Good, unpublished). It has intrinsic antimicrobial properties at concentrations greater than 50µg/ml. The concentration used in this study is 0.13µg/ml, approximately 400x smaller than the toxic value, and cell line studies showed no decrease in bacterial number when treated with this concentration of Nanocin™.

Our initial hypothesis was that intracellular delivery of tetraspanin peptides using Nanocin™ would reduce the activity of 800 peptide, which by its nature should act extracellularly at the cell membrane. Dose response and retention experiments however showed that the nanoparticles improved the action of the peptides. The particles also showed no toxicity by MTT test and epidermal migration assay. This was also not expected as CD9 is involved in

intracellular vesicle trafficking which the peptides could have interfered with (Fernvik et al., 1995). One possible explanation for this is that the peptides are not being delivered into the cell cytoplasm, and that the formulations generated here are not capable of passing into the cells. Delivery of the PHMB into target cells is thought to occur through an endocytic pathway involving the GTPase protein dynamin (Firdessa et al., 2015). The expression of dynamin-2 specifically is known to be dependent on CD9 in pancreatic cancer cells (Tang et al., 2015), and therefore tetraspanin treatment could be affecting the internalization mechanisms of the cell and preventing Nanocin™ localization. This could be confirmed by confocal microscopy studies using the tagged peptides and FitC labelled Nanocin™.

Conclusion and Future Directions

In this study we have explored the potential of tetraspanin-based peptides as a therapeutic agent in the treatment of *Staphylococcus aureus* skin and wound infections. This therapy conferred a 60% reduction in adherent bacteria to host cells, and reduced the bacterial load in a 3D tissue engineered model of human skin. The redundancy within the tetraspanin system also would suggest that the peptides should not have any toxic effects, which was confirmed with an MTT test and migration assays. Packaging into nanoparticles using Nanocin™ improved their retention and lowered their inhibitory dose, however this also seemed to abolish their effects in the TEskin model, possibly suggesting a change to peptide localization due to the Nanocin™. Combination therapy also showed synergistic effects between flucloxacillin and the peptides in a cell line model, however this also was not reproduced in the TEskin.

The additional work needed to bring these peptides closer to the clinic has been mentioned throughout these chapters, specifically experiments examining the peptides mode of action. At each stage of development and improvement they should be examined closely for adverse effects on the host cell.

Overall, I believe that the global problem of antibiotic resistance is now becoming an urgent cause for concern, and that it will be incredibly fortunate if the treatments we currently use are effective even for another 20 years. Now therefore is the ideal time to search out alternatives. These tetraspanin based peptides exhibit some potential in preventing infection, and any improvement to current treatment that they can provide should be welcomed.

Furthermore, I believe that novel approaches to a problem as wide and complex as antibiotic resistance, such as anti-adherence therapies, should be pursued fully before rejection.

Bibliography

ABSOLOM, D. R. 1988. The Role Of Bacterial Hydrophobicity In Infection - Bacterial Adhesion And Phagocytic Ingestion. *Canadian Journal of Microbiology,* 34**,** 287-298.

ACH, T., ZIEMER, M., DAWCZYNSKI, J., STROBEL, J., SAUER, G. & DEISSLER, H. 2010. Differential expression of tetraspanin CD9 in basal cell and squamous cell carcinomas of the skin and actinic keratosis. *Oncology Letters,* 1**,** 37-40.

ACUTE TRUST, CCG & ENGLAND, N. 2015. *MMRSA bacteraemia: quarterly counts by acute trust and CCG, and financial year counts and rates by acute trust and CCG, up to financial year 2014 to 2015* [Online]. gov.uk. Available: https://www.gov.uk/government/statistics/mrsa-bacteraemia-annual-data [Accessed 27/01/2016 2016].

AL-WAILI, N. S., SALOM, K., BUTLER, G. & AL GHAMDI, A. A. 2011. Honey and Microbial Infections: A Review Supporting the Use of Honey for Microbial Control. *Journal of Medicinal Food,* 14**,** 1079-1096.

ALANIS, A. J. 2005. Resistance to antibiotics: Are we in the post-antibiotic era? *Archives of Medical Research,* 36**,** 697-705.

ALBELDA, S. M. & BUCK, C. A. 1990. Integrins and other cell adhesion molecules. *Faseb Journal,* 4**,** 2868-2880.

ALTSCHUL, S. F., GISH, W., MILLER, W., MYERS, E. W. & LIPMAN, D. J. 1990. Basic Local Alignment Search Tool. *Journal of Molecular Biology,* 215**,** 403-410.

ANANTA, M., BROWN, R. A. & MUDERA, V. 2012. A Rapid Fabricated Living Dermal Equivalent for Skin Tissue Engineering: An In Vivo Evaluation in an Acute Wound Model. *Tissue Engineering Part A,* 18**,** 353-361.

ARAI, T. 1967. The mechanisms of transfer of R factors. *Keio J Med,* 16**,** 55-82.

ARRECUBIETA, C., MATSUNAGA, I., ASAI, T., NAKA, Y., DENG, M. C. & LOWY, F. D. 2008. Vaccination with clumping factor A and fibronectin binding protein A to prevent Staphylococcus aureus infection of an aortic patch in mice. *Journal of Infectious Diseases,* 198.

ASHARANI, P. V., MUN, G. L. K., HANDE, M. P. & VALIYAVEETTIL, S. 2009. Cytotoxicity and Genotoxicity of Silver Nanoparticles in Human Cells. *Acs Nano,* 3**,** 279-290.

BARREIRO, O., YANEZ-MO, M., SALA-VALDES, M., GUTIERREZ-LOPEZ, M. D., OVALLE, S., HIGGINBOTTOM, A., MONK, P. N., CABANAS, C. & SANCHEZ-MADRID, F. 2005. Endothelial tetraspanin microdomains regulate leukocyte firm adhesion during extravasation. *Blood,* 105**,** 2852-61.

BARREIRO, O., ZAMAI, M., YANEZ-MO, M., TEJERA, E., LOPEZ-ROMERO, P., MONK, P. N., GRATTON, E., CAIOLFA, V. R. & SANCHEZ-MADRID, F. 2008. Endothelial adhesion receptors are recruited to adherent leukocytes by inclusion in preformed tetraspanin nanoplatforms. *Journal of Cell Biology,* 183**,** 527-542.

BARRIENTOS, S., STOJADINOVIC, O., GOLINKO, M. S., BREM, H. & TOMIC-CANIC, M. 2008. Growth factors and cytokines in wound healing. *Wound Repair and Regeneration,* 16.

BEACHEY, E. H. 1981. Bacterial adherence - Adhesion-receptor interactions mediating the attachment of bacteria to mucosal surfaces. *Journal of Infectious Diseases,* 143.

BERDITCHEVSKI, F. 2001. Complexes of tetraspanins with integrins: more than meets the eye. *Journal of Cell Science,* 114**,** 4143-4151.

BERDITCHEVSKI, F. & ODINTSOVA, E. 2007. Tetraspanins as regulators of protein trafficking. *Traffic,* 8.

BERRIDGE, M. V., HERST, P. M. & TAN, A. S. 2005. Tetrazolium dyes as tools in cell biology: New insights into their cellular reduction. *Biotechnology Annual Review, Vol 11,* 11**,** 127-152.

BISOGNANO, C., VAUDAUX, P. E., LEW, D. P., NG, E. Y. W. & HOOPER, D. C. 1997. Increased expression of fibronectin-binding proteins by fluoroquinolone-resistant Staphylococcus aureus exposed to subinhibitory levels of ciprofloxacin. *Antimicrobial Agents and Chemotherapy,* 41**,** 906-913.

BJARNSHOLT, T., KIRKETERP-MOLLER, K., JENSEN, P. O., MADSEN, K. G., PHIPPS, R., KROGFELT, K., HOIBY, N. & GIVSKOV, M. 2008. Why chronic wounds will not heal: a novel hypothesis. *Wound Repair and Regeneration,* 16**,** 2-10.

BLACKWOOD, K. A., MCKEAN, R., CANTON, I., FREEMAN, C. O., FRANKLIN, K. L., COLE, D., BROOK, I., FARTHING, P., RIMMER, S., HAYCOCK, J. W., RYAN, A. J. & MACNEIL, S. 2008. Development of biodegradable electrospun scaffolds for dermal replacement. *Biomaterials,* 29**,** 3091-3104.

BLOT, S. I., VANDEWOUDE, K. H., HOSTE, E. A. & COLARDYN, F. A. 2002. Outcome and attributable mortality in critically ill patients with bacteremia involving methicillin-susceptible and methicillin-resistant Staphylococcus aureus. *Archives of Internal Medicine,* 162**,** 2229-2235.

BLUMBERG, H. M., RIMLAND, D., CARROLL, D. J., TERRY, P. & WACHSMUTH, I. K. 1991. Rapid development of a ciprofloxacin resistance in methicillin-susceptible and methicillin-resistant staphylococcus aureus. *Journal of Infectious Diseases,* 163**,** 1279-1285.

BOSMAN, F. T. & STAMENKOVIC, I. 2003. Functional structure and composition of the extracellular matrix. *Journal of Pathology,* 200**,** 423-428.

BOUCHEIX, C., BENOIT, P., FRACHET, P., BILLARD, M., WORTHINGTON, R. E., GAGNON, J. & UZAN, G. 1991. Molecular cloning of the CD9 antigen - a new family of cell-surface proteins. *Journal of Biological Chemistry,* 266**,** 117-122.

BOUCHEIX, C. & RUBINSTEIN, E. 2001. Tetraspanins. *Cellular and Molecular Life Sciences,* 58**,** 1189-1205.

BOUKAMP, P., PETRUSSEVSKA, R. T., BREITKREUTZ, D., HORNUNG, J., MARKHAM, A. & FUSENIG, N. E. 1988. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *Journal of Cell Biology,* 106**,** 761-771.

BREUER, K., HAUSSLER, S., KAPP, A. & WERFEL, T. 2002. Staphylococcus aureus: colonizing features and influence of an antibacterial treatment in adults with atopic dermatitis. *British Journal of Dermatology,* 147**,** 55-61.

BURGER, O., OFEK, I., TABAK, M., WEISS, E. I., SHARON, N. & NEEMAN, I. 2000. A high molecular mass constituent of cranberry juice inhibits Helicobacter pylori adhesion to human gastric mucus. *Fems Immunology and Medical Microbiology,* 29**,** 295-301.

CELLNTEC. 2015. *3D keratinocyte Starter Kit* [Online]. CELLnTEC. Available: http://cellntec.com/wp-content/uploads/pdf/3D_Starter_Kit_HPEK.pdf [Accessed 3/2/2016 2016].

CHAMBERS, H. F. 1997. Methicillin resistance in staphylococci: Molecular and biochemical basis and clinical implications. *Clinical Microbiology Reviews,* 10**,** 781-&.

CHAMBERS, H. F. & DELEO, F. R. 2009. Waves of resistance: Staphylococcus aureus in the antibiotic era. *Nature Reviews Microbiology,* 7**,** 629-641.

CHARRIN, S., LE NAOUR, F., OUALID, M., BILLARD, M., FAURE, G., HANASH, S. M., BOUCHEIX, C. & RUBINSTEIN, E. 2001. The major CD9 and CD81 molecular partner - Identification and characterization of the complexes. *Journal of Biological Chemistry,* 276**,** 14329-14337.

CHARRIN, S., LE NAOUR, F., SILVIE, O., MILHIET, P.-E., BOUCHEIX, C. & RUBINSTEIN, E. 2009. Lateral organization of membrane proteins: tetraspanins spin their web. *Biochemical Journal,* 420**,** 133-154.

CHARRIN, S., MANIE, S., OUALID, M., BILLARD, M., BOUCHEIX, C. & RUBINSTEIN, E. 2002. Differential stability of tetraspanin/tetraspanin interactions: role of palmitoylation. *Febs Letters,* 516**,** 139-144.

CHAU, D. Y. S., JOHNSON, C., MACNEIL, S., HAYCOCK, J. W. & GHAEMMAGHAMI, A. M. 2013. The development of a 3D immunocompetent model of human skin. *Biofabrication,* 5.

CHEMANI, C., IMBERTY, A., DE BENTZMANN, S., PIERRE, M., WIMMEROVA, M., GUERY, B. P. & FAURE, K. 2009. Role of LecA and LecB Lectins in Pseudomonas aeruginosa-Induced Lung Injury and Effect of Carbohydrate Ligands. *Infection and Immunity,* 77.

CHO, S. H., STRICKLAND, I., BOGUNIEWICZ, M. & LEUNG, D. Y. M. 2001. Fibronectin and fibrinogen contribute to the enhanced binding of Staphylococcus aureus to atopic skin. *Journal of Allergy and Clinical Immunology,* 108**,** 269-274.

CLARK, R. A., CHONG, B., MIRCHANDANI, N., BRINSTER, N. K., YAMANAKA, K., DOWGIERT, R. K. & KUPPER, T. S. 2006. The vast majority of CLA(+) T cells are resident in normal skin. *Journal of Immunology,* 176**,** 4431-4439.

COOK, D. & RIDDEN, J. 2014. *Nanocin Presentations - Blueberry Therapeutics* [Online]. Available: http://www.bionow.co.uk/search.aspx?search=nanocin [Accessed 05/01/2016 2016].

CORNELISSEN, L. H., BRONNEBERG, D., GIBBS, S., BOUTEN, C. V. C. & OOMENS, C. W. J. 2010. Cytokine Release in Tissue-Engineered Epidermal Equivalents After Prolonged Mechanical Loading. *Epidermal Cells: Methods and Protocols, Second Edition,* 585**,** 335-344.

COZENS, D. & READ, R. C. 2012. Anti-adhesion methods as novel therapeutics for bacterial infections. *Expert review of anti-infective therapy,* 10.

COZENS, D. J. 2016. *The Role of Tetraspanins and their Peptide Fragments in Bacterial Adhesion to Human Cells* PhD, University of Sheffield.

CUSHNIE, T. P. T. & LAMB, A. J. 2005. Antimicrobial activity of flavonoids. *International Journal of Antimicrobial Agents,* 26**,** 343-356.

DAVIES, J. & DAVIES, D. 2010. Origins and Evolution of Antibiotic Resistance. *Microbiology and Molecular Biology Reviews,* 74**,** 417-+.

DE KONING, H. D., SIMON, A., ZEEUWEN, P. L. J. M. & SCHALKWIJK, J. 2012. Pattern recognition receptors in infectious skin diseases. *Microbes and Infection,* 14.

DE SAINT JEAN, M., BAUDOUIN, C., DI NOLFO, M., ROMAN, S., LOZATO, P., WARNET, J. M. & BRIGNOLE, F. 2004. Comparison of morphological and functional characteristics of primary-cultured human conjunctival epithelium and of Wong-Kilbourne derivative of Chang conjunctival cell line. *Experimental Eye Research,* 78**,** 257-274.

DE VRIES, F. P., COLE, R., DANKERT, J., FROSCH, M. & VAN PUTTEN, J. P. M. 1998. Neisseria meningitidis producing the Opc adhesin binds epithelial cell proteoglycan receptors. *Molecular Microbiology,* 27**,** 1203-1212.

DECHEND, R., MAASS, M., GIEFFERS, J., DIETZ, R., SCHEIDEREIT, C., LEUTZ, A. & GULBA, D. C. 1999. Chlamydia pneumoniae infection of vascular smooth muscle and endothelial cells activates NF-kappa B and induces tissue factor and PAI-1 expression - A potential link to accelerated arteriosclerosis. *Circulation,* 100**,** 1369-1373.

DESAI, P., PATLOLLA, R. R. & SINGH, M. 2010. Interaction of nanoparticles and cell-penetrating peptides with skin for transdermal drug delivery. *Molecular Membrane Biology,* 27**,** 247-259.

DICKSON, M. A., HAHN, W. C., INO, Y., RONFARD, V., WU, J. Y., WEINBERG, R. A., LOUIS, D. N., LI, F. P. & RHEINWALD, J. G. 2000. Human keratinocytes that express hTERT and also bypass a p16(INK4a)-enforced mechanism that limits life span become immortal yet retain normal growth and differentiation characteristics. *Molecular and Cellular Biology,* 20**,** 1436-1447.

DONNARUMMA, G., PAOLETTI, I., BUOMMINO, E., TUFANO, M. A. & BARONI, A. 2004. alpha-MSH reduces the internalization of Staphylococcus aureus and down-regulates HSP 70, integrins and cytokine expression in human keratinocyte cell lines. *Experimental Dermatology,* 13**,** 748-754.

DOWD, S. E., SUN, Y., SECOR, P. R., RHOADS, D. D., WOLCOTT, B. M., JAMES, G. A. & WOLCOTT, R. D. 2008. Survey of bacterial diversity in chronic wounds using Pyrosequencing, DGGE, and full ribosome shotgun sequencing. *Bmc Microbiology,* 8.

DZIEWANOWSKA, K., CARSON, A. R., PATTI, J. M., DEOBALD, C. F., BAYLES, K. W. & BOHACH, G. A. 2000. Staphylococcal fibronectin binding protein interacts with heat shock protein 60 and integrins: Role in internalization by epithelial cells. *Infection and Immunity,* 68**,** 6321-6328.

EGESTEN, A., FRICK, I.-M., MORGELIN, M., OLIN, A. I. & BJORCK, L. 2011. Binding of Albumin Promotes Bacterial Survival at the Epithelial Surface. *Journal of Biological Chemistry,* 286.

EL HAJ, C., MURILLO, O., RIBERA, A., VIVAS, M., GARCIA-SOMOZA, D., TUBAU, F., CABELLOS, C., CABO, J. & ARIZA, J. 2015. Daptomycin combinations as alternative therapies in experimental foreign-body infection caused by meticillin-susceptible Staphylococcus aureus. *International Journal of Antimicrobial Agents,* 46**,** 189-195.

ELRAKAIBY, M., DUTILH, B. E., RIZKALLAH, M. R., BOLEIJ, A., COLE, J. N. & AZIZ, R. K. 2014. Pharmacomicrobiomics: The Impact of Human Microbiome Variations on Systems Pharmacology and Personalized Therapeutics. *Omics-a Journal of Integrative Biology,* 18**,** 402-414.

FALANGA, V., ISAACS, C., PAQUETTE, D., DOWNING, G., KOUTTAB, N., BUTMARC, J., BADIAVAS, E. & HARDIN-YOUNG, J. 2002. Wounding of bioengineered skin: Cellular and molecular aspects after injury. *Journal of Investigative Dermatology,* 119**,** 653-660.

FANAEI, M. 2014. *Investigating the Role of Tetraspanins in Multinucleated Giant Cell Formation Using Recombinant Proteins.* PhD, University of Sheffield.

FERNVIK, E., HALLDEN, G., HED, J. & LUNDAHL, J. 1995. Intracellular and surface distribution of CD9 in human eosinophils. *Apmis,* 103**,** 699-706.

FIGDOR, C. G. & VAN SPRIEL, A. B. 2010. Fungal pattern-recognition receptors and tetraspanins: partners on antigen-presenting cells. *Trends in Immunology,* 31**,** 91-96.

FIRDESSA, R., GOOD, L., AMSTALDEN, M. C., CHINDERA, K., KAMARUZZAMAN, N. F., SCHULTHEIS, M., ROEGER, B., HECHT, N., OELSCHLAEGER, T. A., MEINEL, L., LUEHMANN, T. & MOLL, H. 2015. Pathogen- and Host-Directed Antileishmanial Effects Mediated by Polyhexanide (PHMB). *PLoS Neglected Tropical Diseases,* 9.

FOSTER, T. J. & HOOK, M. 1998. Surface protein adhesins of Staphylococcus aureus. *Trends in Microbiology,* 6**,** 484-488.

GARTLAN, K. H., WEE, J. L., DEMARIA, M. C., NASTOVSKA, R., CHANG, T. M., JONES, E. L., APOSTOLOPOULOS, V., PIETERSZ, G. A., HICKEY, M. J., VAN SPRIEL, A. B. & WRIGHT, M. D. 2013. Tetraspanin CD37 contributes to the initiation of cellular immunity by promoting dendritic cell migration. *European Journal of Immunology,* 43**,** 1208-1219.

GERVER, S., MIHALKOVA, M., ABERNETHY, J., BOU-ANTOUN, S., NSONWU, O., KAUSAR, S., WASTI, S., APRAKU, D., DAVIES, J. & HOPE, R. 2015. Annual Epidemiological Commentary: Mandatory MRSA, MSSA and E. coli bacteraemia and C. difficile infection data, 2014/15. Public Health England.

GINSBERG, M. H., LOFTUS, J. C. & PLOW, E. F. 1988. Cytoadhesins, integrins and platelets. *Thrombosis and Haemostasis,* 59**,** 1-6.

GIUSTIZIERI, M. L., MASCIA, F., FREZZOLINI, A., DE PITA, O., CHINNI, L. M., GIANNETTI, A., GIROLOMONI, G. & PASTORE, S. 2001. Keratinocytes from patients with atopic dermatitis and psoriasis show a distinct chemokine production profile in response to T cell-derived cytokines. *Journal of Allergy and Clinical Immunology,* 107**,** 871-877.

GLASER, R., HARDER, J., LANGE, H., BARTELS, J., CHRISTOPHERS, E. & SCHRODER, J. M. 2005. Antimicrobial psoriasin (S100A7) protects human skin from Escherichia coli infection. *Nature Immunology,* 6**,** 57-64.

GOOD, L. 2016/2017. The antimicrobial polymer PHMB enters cells and selectively condenses bacterial chromosomes

GOOD, L. & RIDDEN, J. *Composition useful for e.g. combating antibacterial-resistant bacterial infection of human or animal comprises bacterium entry-promoting agent e.g. polyhexamethylene biguanide, and agent that inhibits antibacterial resistance e.g. peptide*.

GOTTRUP, F., JORGENSEN, B. & KARLSMARK, T. 2009. News in wound healing and management. *Current opinion in supportive and palliative care,* 3.

GREEN, L. 2010. *The Role of Tetraspanins in Bacterial Pathogenesis.* PhD, University of Sheffield.

GREEN, L. R., MONK, P. N., PARTRIDGE, L. J., MORRIS, P., GORRINGE, A. R. & READ, R. C. 2011. Cooperative role for tetraspanins in adhesin-mediated attachment of bacterial species to human epithelial cells. *Infection and immunity,* 79**,** 2241-2249.

GREIF, R., AKCA, O., HORN, E. P., KURZ, A., SESSLER, D. I. & OUTCOMES RES, G. 2000. Supplemental perioperative oxygen to reduce the incidence of surgical-wound infection. *New England Journal of Medicine,* 342.

GRICE, E. A., KONG, H. H., CONLAN, S., DEMING, C. B., DAVIS, J., YOUNG, A. C., BOUFFARD, G. G., BLAKESLEY, R. W., MURRAY, P. R., GREEN, E. D., TURNER, M. L., SEGRE, J. A. & PROGRA, N. C. S. 2009. Topographical and Temporal Diversity of the Human Skin Microbiome. *Science,* 324**,** 1190-1192.

GRONE, A. 2002. Keratinocytes and cytokines. *Veterinary Immunology and Immunopathology,* 88**,** 1-12.

GROSSMAN, R. M., GOTTLIEB, A. B., SEGHAL, P. B., KRUEGER, J., MAY, L. T., KUPPER, T., MURPHY, D. P. & RAY, A. 1989. Increased IL-6 in psoriatic plaques. *Journal of Investigative Dermatology,* 92**,** 437-437.

GUENTHNER, S. H. & WENZEL, R. P. 1984. In vitro activities of teichomycin, fusidic acid, flucloxacillin, fosfomycin, and vancomycin against methicillin-resistant Staphylococcus aureus. *Antimicrobial Agents and Chemotherapy,* 26**,** 268-269.

GUERRERO, C. A. & MORENO, L. P. 2012. Rotavirus receptor proteins Hsc70 and integrin alpha v beta 3 are located in the lipid microdomains of animal intestinal cells. *Acta Virologica,* 56**,** 63-70.

HAAKE, A., SCOTT, G. & HOLBROOK, K. 2001. *The Biology of The Skin: Structure and function of the skin: overview of the epidermis and dermis,* UK and Europe, The Parthernon Publishing Group.

HAAPASALMI, K., ZHANG, K., TONNESEN, M., OLERUD, J., SHEPPARD, D., SALO, T., KRAMER, R., CLARK, R. A. F., UITTO, V. J. & LARJAVA, H. 1996. Keratinocytes in human wounds express alpha v beta 6 integrin. *Journal of Investigative Dermatology,* 106**,** 42-48.

HAN, G., NGUYEN, L. N., MACHERLA, C., CHI, Y., FRIEDMAN, J. M., NOSANCHUK, J. D. & MARTINEZ, L. R. 2012. Nitric Oxide-Releasing Nanoparticles Accelerate Wound Healing by Promoting Fibroblast Migration and Collagen Deposition. *American Journal of Pathology,* 180**,** 1465-1473.

HANSSON, C., HOBORN, J., MOLLER, A. & SWANBECK, G. 1995. The microbial-flora in venous leg ulcers without clinical signs of infection- repeated culture using a validated standardized microbiological technique. *Acta Dermato-Venereologica,* 75.

HARTMAN, B. J. & TOMASZ, A. 1984. Low-affinity penicillin binding protein associated with beta-lactam resistance in Staphylococcus aureus. *Journal of Bacteriology,* 158**,** 513-516.

HARTMANN, M., PAPAVLASSOPOULOS, H., CHANDRASEKARAN, V., GRABOSCH, C., BEIROTH, F., LINDHORST, T. K. & ROEHL, C. 2012. Inhibition of bacterial adhesion to live human cells: Activity and cytotoxicity of synthetic mannosides. *Febs Letters,* 586**,** 1459-1465.

HASSUNA, N. 2010. *The role of tetraspannins in the uptake of intracellular pathogens.* Ph.D., The University of Sheffield.

HASSUNA, N., MONK, P. N., MOSELEY, G. W. & PARTRIDGE, L. J. 2009. Strategies for Targeting Tetraspanin Proteins Potential Therapeutic Applications in Microbial Infections. *Biodrugs,* 23**,** 341-359.

HATO, T., IKEDA, K., YASUKAWA, M., WATANABE, A. & KOBAYASHI, Y. 1988. Exposure of platelet fibrinogen receptors by a monoclonal antibody to CD9 antigen. *Blood,* 72**,** 224-229.

HELD, M., MEDVED, F., STAHL, S., BOESCH, C., RAHMANIAN-SCHWARZ, A. & SCHALLER, H.-E. 2015. Improvement of Split Skin Graft Quality Using a Newly Developed Collagen Scaffold as an Underlayment in Full Thickness Wounds in a Rat Model. *Annals of Plastic Surgery,* 75**,** 508-512.

HELLE, F. & COCQUEREL, L. 2008. Entry of HCV into target cells. *Virologie,* 12**,** 105-116.

HEMLER, M. E. 2005. Tetraspanin functions and associated microdomains. *Nature Reviews Molecular Cell Biology,* 6.

HERBERT, S., ZIEBANDT, A.-K., OHLSEN, K., SCHAEFER, T., HECKER, M., ALBRECHT, D., NOVICK, R. & GOETZ, F. 2010. Repair of Global Regulators in Staphylococcus aureus 8325 and Comparative Analysis with Other Clinical Isolates. *Infection and Immunity,* 78**,** 2877-2889.

HIGGINBOTTOM, A., TAKAHASHI, Y., BOLLING, L., COONROD, S. A., WHITE, J. M., PARTRIDGE, L. J. & MONK, P. N. 2003. Structural requirements for the inhibitory action of the CD9 large

extracellular domain in sperm/oocyte binding and fusion. *Biochemical and Biophysical Research Communications,* 311**,** 208-214.
HIRSCHHAUSEN, N., SCHLESIER, T., SCHMIDT, M. A., GOTZ, F., PETERS, G. & HEILMANN, C. 2010. A novel staphylococcal internalization mechanism involves the major autolysin Atl and heat shock cognate protein Hsc70 as host cell receptor. *Cellular Microbiology,* 12**,** 1746-1764.
HOLFORD, N., MA, S. C. & PLOEGER, B. A. 2010. Clinical Trial Simulation: A Review. *Clinical Pharmacology & Therapeutics,* 88**,** 166-182.
HONG, I.-K., JEOUNG, D.-I., HA, K.-S., KIM, Y.-M. & LEE, H. 2012. Tetraspanin CD151 Stimulates Adhesion-dependent Activation of Ras, Rac, and Cdc42 by Facilitating Molecular Association between beta1 Integrins and Small GTPases. *The Journal of biological chemistry,* 287.
HOWELL-JONES, R. S., WILSON, M. J., HILL, K. E., HOWARD, A. J., PRICE, P. E. & THOMAS, D. W. 2005. A review of the microbiology, antibiotic usage and resistance in chronic skin wounds. *Journal of Antimicrobial Chemotherapy,* 55.
HUEBNER, N. O. & KRAMER, A. 2010. Review on the Efficacy, Safety and Clinical Applications of Polihexanide, a Modern Wound Antiseptic. *Skin Pharmacology and Physiology,* 23**,** 17-27.
INDUSTRY, T. A. O. T. B. P. 2016. *Developing new medicines* [Online]. Available: http://www.abpi.org.uk/industry-info/new-medicines/Pages/default.aspx [Accessed 3/2/2016 2016].
INUI, S., HIGASHIYAMA, S., HASHIMOTO, K., HIGASHIYAMA, M., YOSHIKAWA, K. & TANIGUCHI, N. 1997. Possible role of coexpression of CD9 with membrane-anchored heparin-binding ECF-like growth factor and amphiregulin in cultured human keratinocyte growth. *Journal of Cellular Physiology,* 171**,** 291-298.
JAKUBZICK, C., GAUTIER, E. L., GIBBINGS, S. L., SOJKA, D. K., SCHLITZER, A., JOHNSON, T. E., IVANOV, S., DUAN, Q. N., BALA, S., CONDON, T., VAN ROOIJEN, N., GRAINGER, J. R., BELKAID, Y., MA'AYAN, A., RICHES, D. W. H., YOKOYAMA, W. M., GINHOUX, F., HENSON, P. M. & RANDOLPH, G. J. 2013. Minimal Differentiation of Classical Monocytes as They Survey Steady-State Tissues and Transport Antigen to Lymph Nodes. *Immunity,* 39**,** 599-610.
JIANG, X., TENG, M., GUO, X., ZHANG, D., ZHANG, Q., ZHANG, J. & HUANG, Y. 2014. Switch from alpha v beta 5 to alpha v beta 6 integrin is required for CD9-regulated keratinocyte migration and MMP-9 activation. *Febs Letters,* 588**,** 4044-4052.
JIANG, X.-P., ZHANG, D.-X., TENG, M., ZHANG, Q., ZHANG, J.-P. & HUANG, Y.-S. 2013. Downregulation of CD9 in Keratinocyte Contributes to Cell Migration via Upregulation of Matrix Metalloproteinase-9. *Plos One,* 8.
JONES, E., WEE, J., NGUYEN, J., RAMOS, J. V., HO, P. K., VILLADANGOS, J., HAEMMERLING, G. J., HICKEY, M. & WRIGHT, M. D. 2012. Tetraspanin CD82 plays a vital role in antigen presentation and processing in dendritic cells, and influences migration in opposition to CD37. *Immunology,* 137**,** 283-283.
JONES, E. L., WEE, J. L., DEMARIA, M. C., BLAKELEY, J., HO, P. K., VEGA-RAMOS, J., VILLADANGOS, J. A., VAN SPRIEL, A. B., HICKEY, M. J., HAMMERLING, G. J. & WRIGHT, M. D. 2016. Dendritic Cell Migration and Antigen Presentation Are Coordinated by the Opposing Functions of the Tetraspanins CD82 and CD37. *Journal of Immunology,* 196**,** 978-987.
JONES, P. H., BISHOP, L. A. & WATT, F. M. 1996. Functional significance of CD9 association with beta(1) integrins in human epidermal keratinocytes. *Cell Adhesion and Communication,* 4**,** 297-305.
KELLY, C. G., YOUNSON, J. S., HIKMAT, B. Y., TODRYK, S. M., CZISCH, M., HARIS, P. I., FLINDALL, I. R., NEWBY, C., MALLET, A. I., MA, J. K. C. & LEHNER, T. 1999. A synthetic peptide adhesion epitope as a novel antimicrobial agent. *Nature Biotechnology,* 17**,** 42-47.
KENNEDY, A. D., OTTO, M., BRAUGHTON, K. R., WHITNEY, A. R., CHEN, L., MATHEMA, B., MEDIAVILLA, J. R., BYRNE, K. A., PARKINS, L. D., TENOVER, F. C., KREISWIRTH, B. N., MUSSER, J. M. & DELEO, F. R. 2008. Epidemic community-associated methicillin-resistant

Staphylococcus aureus: Recent clonal expansion and diversification. *Proceedings of the National Academy of Sciences of the United States of America,* 105**,** 1327-1332.
KESTING, M. R., STOECKELHUBER, M., HOELZLE, F., MUECKE, T., NEUMANN, K., WOERMANN, K., JACOBSEN, F., STEINSTRAESSER, L., WOLFF, K. D., LOEFFELBEIN, D. J. & ROHLEDER, N. H. 2010. Expression of antimicrobial peptides in cutaneous infections after skin surgery. *British Journal of Dermatology,* 163**,** 121-127.
KIANG, J. G., JIAO, W., CARY, L. H., MOG, S. R., ELLIOTT, T. B., PELLMAR, T. C. & LEDNEY, G. D. 2010. Wound Trauma Increases Radiation-Induced Mortality by Activation of iNOS Pathway and Elevation of Cytokine Concentrations and Bacterial Infection. *Radiation Research,* 173**,** 319-332.
KIM, M.-S., PINTO, S. M., GETNET, D., NIRUJOGI, R. S., MANDA, S. S., CHAERKADY, R., MADUGUNDU, A. K., KELKAR, D. S., ISSERLIN, R., JAIN, S., THOMAS, J. K., MUTHUSAMY, B., LEAL-ROJAS, P., KUMAR, P., SAHASRABUDDHE, N. A., BALAKRISHNAN, L., ADVANI, J., GEORGE, B., RENUSE, S., SELVAN, L. D. N., PATIL, A. H., NANJAPPA, V., RADHAKRISHNAN, A., PRASAD, S., SUBBANNAYYA, T., RAJU, R., KUMAR, M., SREENIVASAMURTHY, S. K., MARIMUTHU, A., SATHE, G. J., CHAVAN, S., DATTA, K. K., SUBBANNAYYA, Y., SAHU, A., YELAMANCHI, S. D., JAYARAM, S., RAJAGOPALAN, P., SHARMA, J., MURTHY, K. R., SYED, N., GOEL, R., KHAN, A. A., AHMAD, S., DEY, G., MUDGAL, K., CHATTERJEE, A., HUANG, T.-C., ZHONG, J., WU, X., SHAW, P. G., FREED, D., ZAHARI, M. S., MUKHERJEE, K. K., SHANKAR, S., MAHADEVAN, A., LAM, H., MITCHELL, C. J., SHANKAR, S. K., SATISHCHANDRA, P., SCHROEDER, J. T., SIRDESHMUKH, R., MAITRA, A., LEACH, S. D., DRAKE, C. G., HALUSHKA, M. K., PRASAD, T. S. K., HRUBAN, R. H., KERR, C. L., BADER, G. D., IACOBUZIO-DONAHUE, C. A., GOWDA, H. & PANDEY, A. 2014. A draft map of the human proteome. *Nature,* 509**,** 575-+.
KIRBY, W. M. M. 1944. Extraction of a highly potent penicillin inactivator from penicillin resistant staphylococci. *Science,* 99**,** 452-453.
KITADOKORO, K., BOLOGNESI, M., GRANDI, G., MARCO, P., GALLI, G., PETRACCA, R. & FABIANA, F. 2003. 1IV5: New Crystal Form of Human CD81 Large Extracellular Loop. *Protein Data Bank,* 5.0.
KITADOKORO, K., BORDO, D., GALLI, G., PETRACCA, R., FALUGI, F., ABRIGNANI, S., GRANDI, G. & BOLOGNESI, M. 2001. CD81 extracellular domain 3D structure: insight into the tetraspanin superfamily structural motifs. *Embo Journal,* 20**,** 12-18.
KLECHEVSKY, E., MORITA, R., LIU, M. C., CAO, Y. Y., COQUERY, S., THOMPSON-SNIPES, L., BRIERE, F., CHAUSSABEL, D., ZURAWSKI, G., PALUCKA, A. K., REITER, Y., BANCHEREAU, J. & UENO, H. 2008. Functional specializations of human epidermal langerhans cells and CD14(+) dermal dendritic cells. *Immunity,* 29**,** 497-510.
KNOBLOCH, J. K. M., HORSTKOTTE, M. A., ROHDE, H., KAULFERS, P. M. & MACK, D. 2002. Alcoholic ingredients in skin disinfectants increase biofilm expression of Staphylococcus epidermidis. *Journal of Antimicrobial Chemotherapy,* 49.
KOLLIPARA, R., DOWNING, C., LEE, M., GUIDRY, J., CURTIS, S. & TYRING, S. 2014. Current and emerging drugs for acute bacterial skin and skin structure infections: an update. *Expert Opinion on Emerging Drugs,* 19**,** 431-440.
KRACHLER, A. M., HAM, H. & ORTH, K. 2011. Outer membrane adhesion factor multivalent adhesion molecule 7 initiates host cell binding during infection by Gram-negative pathogens. *Proceedings of the National Academy of Sciences of the United States of America,* 108**,** 11614-11619.
KRACHLER, A. M. & ORTH, K. 2011. Functional Characterization of the Interaction between Bacterial Adhesin Multivalent Adhesion Molecule 7 (MAM7) Protein and Its Host Cell Ligands. *Journal of Biological Chemistry,* 286**,** 38939-38947.
KRACHLER, A. M. & ORTH, K. 2013. Targeting the bacteria-host interface: Strategies in anti-adhesion therapy. *Virulence,* 4**,** 284-294.

KRAFT, S., JOUVIN, M. H., KULKARNI, N., KISSING, S., MORGAN, E. S., DVORAK, A. M., SCHRODER, B., SAFTIG, P. & KINET, J. P. 2013. The Tetraspanin CD63 Is Required for Efficient IgE-Mediated Mast Cell Degranulation and Anaphylaxis. *Journal of Immunology,* 191**,** 2871-2878.

KRAMER, B., SCHULTE, D., KORNER, C., ZWANK, C., HARTMANN, A., MICHALK, M., SOHNE, J., LANGHANS, B., NISCHALKE, H. D., COENEN, M., MOHL, C., VOGT, A., HENNENBERG, M., SAUERBRUCH, T., SPENGLER, U. & NATTERMANN, J. 2009. Regulation of NK cell trafficking by CD81. *European Journal of Immunology,* 39**,** 3447-3458.

KRASOWSKA, A. & SIGLER, K. 2014. How microorganisms use hydrophobicity and what does this mean for human needs? *Frontiers in Cellular and Infection Microbiology,* 4.

KROPSHOFER, H., SPINDELDREHER, S., ROHN, T. A., PLATANIA, N., GRYGAR, C., DANIEL, N., WOLP, A., LANGEN, H., HOREJSI, V. & VOGT, A. B. 2002. Tetraspan microdomains distinct from lipid rafts enrich select peptide-MHC class II complexes. *Nature Immunology,* 3**,** 61-68.

KRULL, S., THYBERG, J., BJORKROTH, B., RACKWITZ, H. R. & CORDES, V. C. 2004. Nucleoporins as components of the nuclear pore complex core structure and Tpr as the architectural element of the nuclear basket. *Molecular Biology of the Cell,* 15**,** 4261-4277.

KWAKMAN, P. H. S., TE VELDE, A. A., DE BOER, L., SPEIJER, D., VANDENBROUCKE-GRAULS, C. M. J. E. & ZAAT, S. A. J. 2010. How honey kills bacteria. *Faseb Journal,* 24**,** 2576-2582.

LABRECQUE, J., BODET, C., CHANDAD, F. & GRENIER, D. 2006. Effects of a high-molecular-weight cranberry fraction on growth, biofilm formation and adherence of Porphyromonas gingivalis. *Journal of Antimicrobial Chemotherapy,* 58**,** 439-443.

LAFLAMME, S. E., THOMAS, L. A., YAMADA, S. S. & YAMADA, K. M. 1994. Single subunit chimeric integrins as mimics and inhibitors of endogenous integrin functions in receptor localization, cell spreading and migration, and matrix assembly. *Journal of Cell Biology,* 126**,** 1287-1298.

LARJAVA, H., LYONS, J. G., SALO, T., MAKELA, M., KOIVISTO, L., BIRKEDALHANSEN, H., AKIYAMA, S. K., YAMADA, K. M. & HEINO, J. 1993. Anti-integrin antibodies induce type-IV collagenase expression in keratinocytes. *Journal of Cellular Physiology,* 157**,** 190-200.

LAU, L. M., WEE, J. L., WRIGHT, M. D., MOSELEY, G. W., HOGARTH, P. M., ASHMAN, L. K. & JACKSON, D. E. 2004. The tetraspanin superfamily member CD151 regulates outside-in integrin alpha(IIb)beta(3) signaling and platelet function. *Blood,* 104**,** 2368-2375.

LE NAOUR, F., ANDRE, M., GRECO, C., BILLARD, M., SORDAT, B., EMILE, J. F., LANZA, F., BOUCHEIX, C. & RUBINSTEIN, E. 2006. Profiling of the tetraspanin web of human colon cancer cells. *Molecular & Cellular Proteomics,* 5**,** 845-857.

LE NAOUR, F., RUBINSTEIN, E., JASMIN, C., PRENANT, M. & BOUCHEIX, C. 2000. Severely reduced female fertility in CD9-deficient mice. *Science,* 287**,** 319-321.

LEE, W. R., TOBIAS, K. M., BEMIS, D. A. & ROHRBACH, B. W. 2004. In vitro efficacy of a polyhexamethylene biguanide-impregnated gauze dressing against bacteria found in veterinary patients. *Veterinary Surgery,* 33**,** 404-411.

LEUNG, C. F., WEBB, S. E. & MILLER, A. L. 1998. Calcium transients accompany ooplasmic segregation in zebrafish embryos. *Development Growth & Differentiation,* 40**,** 313-326.

LEVY, S. & SHOHAM, T. 2005. The tetraspanin web modulates immune-signalling complexes. *Nature Reviews Immunology,* 5**,** 136-148.

LEVY, S. B. & MARSHALL, B. 2004. Antibacterial resistance worldwide: causes, challenges and responses. *Nature Medicine,* 10**,** S122-S129.

LUCKETT, L. R. & GALLUCCI, R. M. 2007. Interleukin-6 (IL-6) modulates migration and matrix metalloproteinase function in dermal fibroblasts from IL-6KO mice. *British Journal of Dermatology,* 156**,** 1163-1171.

LUPO, A., COYNE, S. & BERENDONK, T. U. 2012. Origin and evolution of antibiotic resistance: the common mechanisms of emergence and spread in water bodies. *Frontiers in Microbiology,* 3.

MACNEIL, S. 2007. Progress and opportunities for tissue-engineered skin. *Nature,* 445**,** 874-880.

MACNEIL, S., SHEPHERD, J. & SMITH, L. 2011. Production of Tissue-Engineered Skin and Oral Mucosa for Clinical and Experimental Use. *3d Cell Culture: Methods and Protocols,* 695**,** 129-153.
MADISON, K. C. 2003. Barrier function of the skin: "La Raison d'Etre" of the epidermis. *Journal of Investigative Dermatology,* 121**,** 231-241.
MANEERUNG, T., TOKURA, S. & RUJIRAVANIT, R. 2008. Impregnation of silver nanoparticles into bacterial cellulose for antimicrobial wound dressing. *Carbohydrate Polymers,* 72**,** 43-51.
MANGIN, P. H., KLEITZ, L., BOUCHEIX, C., GACHET, C. & LANZA, F. 2009. CD9 negatively regulates integrin alpha(IIb)beta(3) activation and could thus prevent excessive platelet recruitment at sites of vascular injury. *Journal of Thrombosis and Haemostasis,* 7**,** 900-902.
MANTEGAZZA, A. R., BARRIO, M. M., MOUTEL, S., BOVER, L., WECK, M., BROSSART, P., TEILLAUD, J. L. & MORDOH, J. 2004. CD63 tetraspanin slows down cell migration and translocates to the endosomal-lysosomal-MIICs route after extracellular stimuli in human immature dendritic cells. *Blood,* 104**,** 1183-1190.
MARIONNET, C., PIERRARD, C., VIOUX-CHAGNOLEAU, C., SOK, J., ASSELINEAU, D. & BERNERD, F. 2006. Interactions between fibroblasts and keratinocytes in morphogenesis of dermal epidermal junction in a model of reconstructed skin. *Journal of Investigative Dermatology,* 126**,** 971-979.
MARTINEZ, J. J., MULVEY, M. A., SCHILLING, J. D., PINKNER, J. S. & HULTGREN, S. J. 2000. Type 1 pilus-mediated bacterial invasion of bladder epithelial cells. *Embo Journal,* 19**,** 2803-2812.
MATTHEWS, K. H., STEVENS, H. N. E., AUFFRET, A. D., HUMPHREY, M. J. & ECCLESTON, G. M. 2005. Lyophilised wafers as a drug delivery system for wound healing containing methylcellulose as a viscosity modifier. *International Journal of Pharmaceutics,* 289**,** 51-62.
MAVRIC, E., WITTMANN, S., BARTH, G. & HENLE, T. 2008. Identification and quantification of methylglyoxal as the dominant antibacterial constituent of Manuka (Leptospermum scoparium) honeys from New Zealand. *Molecular Nutrition & Food Research,* 52**,** 483-489.
MCDEVITT, D., FRANCOIS, P., VAUDAUX, P. & FOSTER, T. J. 1994. Molecular characterization of the clumping factor (fibrinogen receptor) of staphylococcus aureus. *Molecular Microbiology,* 11**,** 237-248.
MENON, G. K. 2002. New insights into skin structure: scratching the surface. *Advanced Drug Delivery Reviews,* 54**,** S3-S17.
MEYER-WENTRUP, F., FIGDOR, C. G., ANSEMS, M., BROSSART, P., WRIGHT, M. D., ADEMA, G. J. & VAN SPRIEL, A. B. 2007. Dectin-1 interaction with tetraspanin CD37 inhibits IL-6 production. *Journal of Immunology,* 178**,** 154-162.
MITTELBRUNN, M., YANEZ-MO, M., SANCHO, D., URSA, A. & SANCHEZ-MADRID, F. 2002. Cutting edge: Dynamic redistribution of tetraspanin CD81 at the central zone of the immune synapse in both T lymphocytes and APC. *Journal of Immunology,* 169**,** 6691-6695.
MIYADO, K., YAMADA, G., YAMADA, S., HASUWA, H., NAKAMURA, Y., RYU, F., SUZUKI, K., KOSAI, K., INOUE, K., OGURA, A., OKABE, M. & MEKADA, E. 2000. Requirement of CD9 on the egg plasma membrane for fertilization. *Science,* 287**,** 321-324.
MIZUSHIMA, N., YOSHIMORI, T. & OHSUMI, Y. 2011. The Role of Atg Proteins in Autophagosome Formation. *Annual Review of Cell and Developmental Biology, Vol 27,* 27**,** 107-132.
MODAK, S. M. & FOX, C. L. 1981. Sulfadiazine silver-resistant pseudomonas in burns - new topical agents. *Archives of Surgery,* 116**,** 854-857.
MOHAMADZADEH, M., MULLER, M., HULTSCH, T., ENK, A., SALOGA, J. & KNOP, J. 1994. Enhanced expression of IL-8 in normal human keratinocytes and human keratinocyte cell line HaCaT in vitro after stimulation with contact sensitizers, tolerogens and irritants. *Experimental dermatology,* 3**,** 298-303.
MORI, M., ALMEIDA, P. V., COLA, M., ANSELMI, G., MAKILA, E., CORREIA, A., SALONEN, J., HIRVONEN, J., CARAMELLA, C. & SANTOS, H. A. 2014. In vitro assessment of biopolymer-modified porous silicon microparticles for wound healing applications. *European Journal of Pharmaceutics and Biopharmaceutics,* 88**,** 635-642.

MOSSER, D. M. & EDWARDS, J. P. 2008. Exploring the full spectrum of macrophage activation. *Nature Reviews Immunology,* 8**,** 958-969.

MROUE, R. & BISSELL, M. J. 2013. Three-dimensional cultures of mouse mammary epithelial cells. *Methods in molecular biology (Clifton, N.J.),* 945**,** 221-50.

MUELLER, G., KOBURGER, T. & KRAMER, A. 2013. Interaction of polyhexamethylene biguanide hydrochloride (PHMB) with phosphatidylcholine containing o/w emulsion and consequences for microbicidal efficacy and cytotoxicity. *Chemico-Biological Interactions,* 201**,** 58-64.

MUNRO, G. H., EVANS, P., TODRYK, S., BUCKETT, P., KELLY, C. G. & LEHNER, T. 1993. A protein fragment of streptococcal cell-surface antigen I/II which prevents adhesion of Streptococcus mutans. *Infection and Immunity,* 61**,** 4590-4598.

MURAYAMA, Y., SHINOMURA, Y., ORITANI, K., MIYAGAWA, J.-I., YOSHIDA, H., NISHIDA, M., KATSUBE, F., SHIRAGA, M., MIYAZAKI, T., NAKAMOTO, T., TSUTSUI, S., TAMURA, S., HIGASHIYAMA, S., SHIMOMURA, I. & HAYASHI, N. 2008. The tetraspanin CD9 modulates epidermal growth factor receptor signaling in cancer cells. *Journal of Cellular Physiology,* 216**,** 135-143.

NAIK, S., BOULADOUX, N., WILHELM, C., MOLLOY, M. J., SALCEDO, R., KASTENMULLER, W., DEMING, C., QUINONES, M., KOO, L., CONLAN, S., SPENCER, S., HALL, J. A., DZUTSEV, A., KONG, H., CAMPBELL, D. J., TRINCHIERI, G., SEGRE, J. A. & BELKAID, Y. 2012. Compartmentalized Control of Skin Immunity by Resident Commensals. *Science,* 337.

NAIR, R., LAUKS, J., JUNG, S., COOKE, N. E., DE WIT, H., BROSE, N., KILIMANN, M. W., VERHAGE, M. & RHEE, J. 2013. Neurobeachin regulates neurotransmitter receptor trafficking to synapses. *Journal of Cell Biology,* 200**,** 61-80.

NAKAMURA, K., MITAMURA, T., TAKAHASHI, T., KOBAYASHI, T. & MEKADA, E. 2000. Importance of the major extracellular domain of CD9 and the epidermal growth factor (EGF)-like domain of heparin-binding EGF-like growth factor for up-regulation of binding and activity. *Journal of Biological Chemistry,* 275.

NAN, Y. H., BANG, J.-K., JACOB, B., PARK, I.-S. & SHIN, S. Y. 2012. Prokaryotic selectivity and LPS-neutralizing activity of short antimicrobial peptides designed from the human antimicrobial peptide LL-37. *Peptides,* 35**,** 239-247.

NAUTA, E. H. & MATTIE, H. 1975. Pharmacokinetics of flucloxacillin and cloxacillin in healthy subjects and patients on chronic intermittent hemodialysis. *British Journal of Clinical Pharmacology,* 2**,** 111-121.

NEDELEC, B., DE OLIVEIRA, A., SAINT-CYR, M. & GARREL, D. R. 2007. Differential effect of burn injury on fibroblasts from wounds and normal skin. *Plastic and Reconstructive Surgery,* 119**,** 2101-2109.

NESTLE, F. O., DI MEGLIO, P., QIN, J.-Z. & NICKOLOFF, B. J. 2009. Skin immune sentinels in health and disease. *Nature Reviews Immunology,* 9**,** 679-691.

NHS. 2011. *Staphylococcal infections* [Online]. http://www.nhs.uk/conditions/Staphylococcal-infections/Pages/Introduction.aspx: NHS. [Accessed 18/03 2013].

NOBBS, A. H., LAMONT, R. J. & JENKINSON, H. F. 2009. Streptococcus Adherence and Colonization. *Microbiology and Molecular Biology Reviews,* 73.

NOWINSKI, D., LYSHEDEN, A. S., GARDNER, H., RUBIN, K., GERDIN, B. & IVARSSON, M. 2004. Analysis of gene expression in fibroblasts in response to keratinocyte-derived factors in vitro: Potential implications for the wound healing process. *Journal of Investigative Dermatology,* 122**,** 216-221.

NUESSLEIN-VOLHARD, C., GILMOUR, D. T. & DAHM, R. 2002. Introduction: Zebrafish as a system to study development and organogenesis. *Zebrafish: A practical approach***,** 1-5.

O'DRISCOLL, N. H., LABOVITIADI, O., TIM CUSHNIE, T. P., MATTHEWS, K. H., MERCER, D. K. & LAMB, A. J. 2013. Production and evaluation of an antimicrobial Peptide-containing wafer formulation for topical application. *Current microbiology,* 66**,** 271-8.

O'MEARA, S., CULLUM, N., MAJID, M. & SHELDON, T. 2000. Systematic reviews of wound care management: (3) antimicrobial agents for chronic wounds; (4) diabetic foot ulceration. *Health technology assessment (Winchester, England),* 4.

OFEK, I., HASTY, D. L. & DOYLE, R. J. 2003. *Bacterial adhesion to animal cells and tissues,* Washington, ASM Press, American Society for Microbiology.

OHLSEN, K., ZIEBUHR, W., KOLLER, K. P., HELL, W., WICHELHAUS, T. A. & HACKER, J. 1998. Effects of subinhibitory concentrations of antibiotics on alpha-toxin (hla) gene expression of methicillin-sensitive and methichillin-resistant Staphylococcus aureus isolates. *Antimicrobial Agents and Chemotherapy,* 42**,** 2817-2823.

OKOCHI, H., KATO, M., NASHIRO, K., YOSHIE, O., MIYAZONO, K. & FURUE, M. 1997. Expression of tetra-spans transmembrane family (CD9, CD37, CD53, CD63, CD81 and CD82) in normal and neoplastic human keratinocytes: an association of CD9 with alpha 3 beta 1 integrin. *British Journal of Dermatology,* 137**,** 856-863.

OLIVEIRA, S. M., REIS, R. L. & MANO, J. F. 2015. Towards the design of 3D multiscale instructive tissue engineering constructs: Current approaches and trends. *Biotechnology Advances,* 33**,** 842-855.

ONG, P. Y., OHTAKE, T., BRANDT, C., STRICKLAND, I., BOGUNIEWICZ, M., GANZ, T., GALLO, R. L. & LEUNG, D. Y. M. 2002. Endogenous antimicrobial peptides and skin infections in atopic dermatitis. *New England Journal of Medicine,* 347**,** 1151-1160.

PALMER, J. 2016. *Recombinant Expression and Analysis of Tetraspanin Extracellular-2 Domains.* PhD, University of Sheffield.

PANCHOLI, V. & FISCHETTI, V. A. 1989. Identification of an endogenous membrane anchor-cleaving enzyme for group-A streptococcal M-protein - its imlication for the attachment of surface-proteins in gram-positive bacteria. *Journal of Experimental Medicine,* 170.

PASPARAKIS, M., HAASE, I. & NESTLE, F. O. 2014. Mechanisms regulating skin immunity and inflammation. *Nature Reviews Immunology,* 14**,** 289-301.

PASTOR, D. M., PORITZ, L. S., OLSON, T. L., KLINE, C. L., HARRIS, L. R., III, KOLTUN, W. A., CHINCHILLI, V. M. & IRBY, R. B. 2010. Primary cell lines: false representation or model system? a comparison of four human colorectal tumors and their coordinately established cell lines. *International Journal of Clinical and Experimental Medicine,* 3**,** 69-83.

PATON, D. M., MANDER, P. & AVERY, R. J. 1982. Bioavailability and half-life of 2 preperations of flucloxacillin. *New Zealand Medical Journal,* 95**,** 766-768.

PEDDIBHOTLA, S. S. D., BRINKMANN, B. F., KUMMER, D., TUNCAY, H., NAKAYAMA, M., ADAMS, R. H., GERKE, V. & EBNET, K. 2013. Tetraspanin CD9 links junctional adhesion molecule-A to alpha v beta 3 integrin to mediate basic fibroblast growth factor-specific angiogenic signaling. *Molecular Biology of the Cell,* 24**,** 933-944.

PENAS, P. F., GARCIA-DIEZ, A., SANCHEZ-MADRID, F. & YANEZ-MO, M. 2000. Tetraspanins are localized at motility-related structures and involved in normal human keratinocyte wound healing migration. *Journal of Investigative Dermatology,* 114**,** 1126-1135.

PFEIFFER, A., BOTTCHER, A., ORSO, E., KAPINSKY, M., NAGY, P., BODNAR, A., SPREITZER, I., LIEBISCH, G., DROBNIK, W., GEMPEL, K., HORN, M., HOLMER, S., HARTUNG, T., MULTHOFF, G., SCHUTZ, G., SCHINDLER, H., ULMER, A. J., HEINE, H., STELTER, F., SCHUTT, C., ROTHE, G., SZOLLOSI, J., DAMJANOVICH, S. & SCHMITZ, G. 2001. Lipopolysaccharide and ceramide docking to CD14 provokes ligand-specific receptor clustering in rafts. *European Journal of Immunology,* 31**,** 3153-3164.

PILERI, P., UEMATSU, Y., CAMPAGNOLI, S., GALLI, G., FALUGI, F., PETRACCA, R., WEINER, A. J., HOUGHTON, M., ROSA, D., GRANDI, G. & ABRIGNANI, S. 1998. Binding of hepatitis C virus to CD81. *Science,* 282**,** 938-941.

POSCHL, E., SCHLOTZER-SCHREHARDT, U., BRACHVOGEL, B., SAITO, K., NINOMIYA, Y. & MAYER, U. 2004. Collagen IV is essential for basement membrane stability but dispensable for initiation of its assembly during early development. *Development,* 131**,** 1619-1628.

POSNETT, J., GOTTRUP, F., LUNDGREN, H. & SAAL, G. 2009. The resource impact of wounds on health-care providers in Europe. *Journal of wound care,* 18.

PRAJSNAR, T. K., CUNLIFFE, V. T., FOSTER, S. J. & RENSHAW, S. A. 2008. A novel vertebrate model of Staphylococcus aureus infection reveals phagocyte-dependent resistance of zebrafish to non-host specialized pathogens. *Cellular Microbiology,* 10**,** 2312-2325.

PRAJSNAR, T. K., HAMILTON, R., GARCIA-LARA, J., MCVICKER, G., WILLIAMS, A., BOOTS, M., FOSTER, S. J. & RENSHAW, S. A. 2012. A privileged intraphagocyte niche is responsible for disseminated infection of Staphylococcus aureus in a zebrafish model. *Cellular Microbiology,* 14**,** 1600-1619.

PRUITT, B. A. & LEVINE, N. S. 1984. Characteristics and uses of biological dressings and skin substitutes. *Archives of Surgery,* 119**,** 312-322.

RADU, F. A., BAUSE, M., KNABNER, P., LEE, G. W. & FRIESS, W. C. 2002. Modeling of drug release from collagen matrices. *Journal of Pharmaceutical Sciences,* 91**,** 964-972.

REICHELT, P., SCHWARZ, C. & DONZEAU, M. 2006. Single step protocol to purify recombinant proteins with low endotoxin contents. *Protein Expression and Purification,* 46**,** 483-488.

RIDDEN, J., DAVIES, M. & GOOD, L. *Topical composition, used to treat fungal infection e.g. fungal nail infection, dermatophytic and yeast infections, comprises polymer e.g. polyhexamethylene biguanide capable of forming nanoparticles, and antifungal agent e.g. nystatin*.

ROBERTS, M. C., SOGE, O. O. & NO, D. 2013. Comparison of multi-drug resistant environmental methicillin-resistant Staphylococcus aureus isolated from recreational beaches and high touch surfaces in built environments. *Frontiers in Microbiology,* 4.

ROMANSKA, H. M. & BERDITCHEVSKI, F. 2011. Tetraspanins in human epithelial malignancies. *Journal of Pathology,* 223**,** 4-14.

ROTH, R. R. & JAMES, W. D. 1988. Microbial ecology of the skin. *Annual Review of Microbiology,* 42**,** 441-464.

RUBINSTEIN, E., LENAOUR, F., BILLARD, M., PRENANT, M. & BOUCHEIX, C. 1994. CD9 antigen is an accessory subunit of the VLA integrin complexes. *European Journal of Immunology,* 24**,** 3005-3013.

RYU, F., TAKAHASHI, T., NAKAMURA, K., TAKAHASHI, Y., KOBAYASHI, T., SHIDA, S., KAMEYAMA, T. & MEKADA, E. 2000. Domain analysis of the tetraspanins: Studies of CD9/CD63 chimeric molecules on subcellular localization and upregulation activity for diphtheria toxin binding. *Cell Structure and Function,* 25**,** 317-327.

S, R. 2016. *Detailed Methods for Breeding Over Marbles* [Online]. ZFIN. Available: https://zfin.org/zf_info/zfbook/chapt2/2.6.html [Accessed 20/01/2016 2016].

SASAKI, T., KANO, R., SATO, H., NAKAMURA, Y., WATANABE, S. & HASEGAWA, A. 2003. Effects of staphylococci on cytokine production from human keratinocytes. *British Journal of Dermatology,* 148**,** 46-50.

SATO, H. & OKINAGA, K. 1987. Role of pili in the adherence of pseudomonas aeruginosa to mouse epidermal-cells. *Infection and Immunity,* 55.

SATO, M., SAWAMURA, D., INA, S., YAGUCHI, T., HANADA, K. & HASHIMOTO, I. 1999. In vivo introduction of the interleukin 6 gene into human keratinocytes: induction of epidermal proliferation by the fully spliced form of interleukin 6, but not by the alternatively spliced form. *Archives of Dermatological Research,* 291**,** 400-404.

SCHADE, L. & MARCHIONINI, A. 1928. On physical chemistry of the skins surface. *Archiv Fur Dermatologie Und Syphilis,* 154**,** 690-716.

SCHERBERICH, A., MOOG, S., HAAN-ARCHIPOFF, G., AZORSA, D. O., LANZA, F. & BERETZ, A. 1998. Tetraspanin CD9 is associated with very late-acting integrins in human vascular smooth muscle cells and modulates collagen matrix reorganization. *Arteriosclerosis Thrombosis and Vascular Biology,* 18**,** 1691-1697.

SCHMID-WENDTNER, M. H. & KORTING, H. C. 2006. The pH of the skin surface and its impact on the barrier function. *Skin Pharmacology and Physiology,* 19**,** 296-302.

SCHNEIDER, J. J., UNHOLZER, A., SCHALLER, M., SCHAFER-KORTING, M. & KORTING, H. C. 2005. Human defensins. *Journal of Molecular Medicine-Jmm,* 83**,** 587-595.

SCHUBERT, S., KEDDIG, N., HANEL, R. & KAMMANN, U. 2014. Microinjection into zebrafish embryos (Danio rerio) - a useful tool in aquatic toxicity testing? *Environmental Sciences Europe,* 26**,** 1-8.

SCHWACHA, M. G., THOBE, B. M., DANIEL, T. & HUBBARD, W. J. 2010. Impact of Thermal Injury on Wound Infiltration and the Dermal Inflammatory Response. *Journal of Surgical Research,* 158**,** 112-120.

SCHWARZ-LINEK, U., WERNER, J. M., PICKFORD, A. R., GURUSIDDAPPA, S., KIM, J. H., PILKA, E. S., BRIGGS, J. A. G., GOUGH, T. S., HOOK, M., CAMPBELL, I. D. & POTTS, J. R. 2003. Pathogenic bacteria attach to human fibronectin through a tandem beta-zipper. *Nature,* 423**,** 177-181.

SENESCHAL, J., CLARK, R. A., GEHAD, A., BAECHER-ALLAN, C. M. & KUPPER, T. S. 2012. Human Epidermal Langerhans Cells Maintain Immune Homeostasis in Skin by Activating Skin Resident Regulatory T Cells. *Immunity,* 36**,** 873-884.

SERRU, V., LE NAOUR, F., BILLARD, M., AZORSA, D. O., LANZA, F., BOUCHEIX, C. & RUBINSTEIN, E. 1999. Selective tetraspan-integrin complexes (CD81/alpha 4 beta 1, CD151/alpha 3 beta 1, CD151/alpha 6 beta 1) under conditions disrupting tetraspan interactions. *Biochemical Journal,* 340**,** 103-111.

SHENG, K. C., VAN SPRIEL, A. B., GARTLAN, K. H., SOFI, M., APOSTOLOPOULOS, V., ASHMAN, L. & WRIGHT, M. D. 2009. Tetraspanins CD37 and CD151 differentially regulate Ag presentation and T-cell co-stimulation by DC. *European Journal of Immunology,* 39**,** 50-55.

SHEPHERD, J., DOUGLAS, I., RIMMER, S., SWANSON, L. & MACNEIL, S. 2009. Development of Three-Dimensional Tissue-Engineered Models of Bacterial Infected Human Skin Wounds. *Tissue Engineering Part C-Methods,* 15**,** 475-484.

SHMUELY, H., OFEK, I., WEISE, E. I., RONES, Z. & HOURI-HADDAD, Y. 2012. Cranberry components for the therapy of infectious disease. *Current Opinion in Biotechnology,* 23**,** 148-152.

SHOHAM, T., RAJAPAKSA, R., BOUCHEIX, C., RUBINSTEIN, E., POE, J. C., TEDDER, T. F. & LEVY, S. 2003. The tetraspanin CD81 regulates the expression of CD19 during B cell development in a postendoplasmic reticulum compartment. *Journal of Immunology,* 171**,** 4062-4072.

SIERADZKI, K., ROBERTS, R. B., HABER, S. W. & TOMASZ, A. 1999. The development of vancomycin resistance in a patient with methicillin-resistant Staphylococcus aureus infection. *New England Journal of Medicine,* 340**,** 517-523.

SINCLAIR, R. D. & RYAN, T. J. 1994. Proteolytic enzymes in wound healing: the role of enzymatic debridement. *The Australasian journal of dermatology,* 35**,** 35-41.

SINHA, B., FRANCOIS, P., QUE, Y. A., HUSSAIN, M., HEILMANN, C., MOREILLON, P., LEW, D., KRAUSE, K. H., PETERS, G. & HERRMANN, M. 2000. Heterologously expressed Staphylococcus aureus fibronectin-binding proteins are sufficient for invasion of host cells. *Infection and Immunity,* 68**,** 6871-6878.

SPRENGER, A., WEBER, S., ZARAI, M., ENGELKE, R., NASCIMENTO, J. M., GRETZMEIER, C., HILPERT, M., BOERRIES, M., HAS, C., BUSCH, H., BRUCKNER-TUDERMAN, L. & DENGJEL, J. 2013. Consistency of the Proteome in Primary Human Keratinocytes With Respect to Gender, Age, and Skin Localization. *Molecular & Cellular Proteomics,* 12**,** 2509-2521.

ST JOHN, A. L., RATHORE, A. P. S., YAP, H., NG, M. L., METCALFE, D. D., VASUDEVAN, S. G. & ABRAHAM, S. N. 2011. Immune surveillance by mast cells during dengue infection promotes natural killer (NK) and NKT-cell recruitment and viral clearance. *Proceedings of the National Academy of Sciences of the United States of America,* 108**,** 9190-9195.

STEVENS, D. L., BISNO, A. L., CHAMBERS, H. F., DELLINGER, E. P., GOLDSTEIN, E. J. C., GORBACH, S. L., HIRSCHMANN, J. V., KAPLAN, S. L., MONTOYA, J. G. & WADE, J. C. 2014. Practice Guidelines for the Diagnosis and Management of Skin and Soft Tissue Infections: 2014 Update by the Infectious Diseases Society of America. *Clinical Infectious Diseases,* 59**,** E10-E52.

STEWART, P. S. & COSTERTON, J. W. 2001. Antibiotic resistance of bacteria in biofilms. *Lancet,* 358**,** 135-138.
SUGAWARA, T., GALLUCCI, R. M., SIMEONOVA, P. P. & LUSTER, M. I. 2001. Regulation and role of interleukin 6 in wounded human epithelial keratinocytes. *Cytokine,* 15**,** 328-336.
SUPP, D. M., WILSON-LANDY, K. & BOYCE, S. T. 2002. Human dermal microvascular endothelial cells form vascular analogs in cultured skin substitutes after grafting to athymic mice. *Faseb Journal,* 16.
SUTHERLAND, R., CROYDON, E. A. P. & ROLINSON, G. N. 1970. Flucloxacillin, a new isoxazolyl, penicillin, compared with oxacillin, cloxacillin and dicloxacillin. *British Medical Journal,* 4**,** 455-&.
SUZUKI, M., TACHIBANA, I., TAKEDA, Y., HE, P., MINAMI, S., IWASAKI, T., KIDA, H., GOYA, S., KIJIMA, T., YOSHIDA, M., KUMAGAI, T., OSAKI, T. & KAWASE, I. 2009. Tetraspanin CD9 Negatively Regulates Lipopolysaccharide-Induced Macrophage Activation and Lung Inflammation. *Journal of Immunology,* 182**,** 6485-6493.
SUZUKI, Y., TANIHARA, M., NISHIMURA, Y., SUZUKI, K., KAKIMARU, Y. & SHIMIZU, Y. 1998. A new drug delivery system with controlled release of antibiotic only in the presence of infection. *Journal of Biomedical Materials Research,* 42**,** 112-116.
TAJIMA, A., SEKI, K., SHINJI, H. & MASUDA, S. 2007. Inhibition of interleukin-8 production in human endothelial cells by Staphylococcus aureus supernatant. *Clinical and Experimental Immunology,* 147**,** 148-154.
TAKEDA, Y., KAZAROV, A. R., BUTTERFIELD, C. E., HOPKINS, B. D., BENJAMIN, L. E., KAIPAINEN, A. & HEMLER, M. E. 2007. Deletion of tetraspanin Cd151 results in decreased pathologic angiogenesis in vivo and in vitro. *Blood,* 109**,** 1524-1532.
TAKEDA, Y., TACHIBANA, I., MIYADO, K., KOBAYASHI, M., MIYAZAKI, T., FUNAKOSHI, T., KIMURA, H., YAMANE, H., SAITO, Y., GOTO, H., YONEDA, T., YOSHIDA, M., KUMAGAI, T., OSAKI, T., HAYASHI, S., KAWASE, I. & MEKADA, E. 2003. Tetraspanins CD9 and CD81 function to prevent the fusion of mononuclear phagocytes. *Journal of Cell Biology,* 161**,** 945-956.
TANG, M. C., YIN, G. J., WANG, F., LIU, H., ZHOU, S., NI, J. B., CHEN, C. Y., ZHOU, Y. Q. & ZHAO, Y. 2015. Downregulation of CD9 promotes pancreatic cancer growth and metastasis through upregulation of epidermal growth factor on the cell surface. *Oncology Reports,* 34**,** 350-358.
TECREA. 2016. *http://www.tecrea.co.uk/drug-formulation/nanocin/* [Online]. Available: http://www.tecrea.co.uk/drug-formulation/nanocin/ [Accessed 05/01/2016 2016].
TEKSTRA, J., BEEKHUIZEN, H., VAN DE GEVEL, J. S., VAN BENTEN, I. J., TUK, C. W. & BEELEN, R. H. J. 1999. Infection of human endothelial cells with Staphylococcus aureus induces the production of monocyte chemotactic protein-1 (MCP-1) and monocyte chemotaxis. *Clinical and Experimental Immunology,* 117**,** 489-495.
TODA, K. I., TUAN, T. L., BROWN, P. J. & GRINNELL, F. 1987. Fibronectin receptors of human keratinocytes and their expression during cell-culture. *Journal of Cell Biology,* 105**,** 3097-3104.
TOGNETTI, L., MARTINELLI, C., BERTI, S., HERCOGOVA, J., LOTTI, T., LEONCINI, F. & MORETTI, S. 2012. Bacterial skin and soft tissue infections: review of the epidemiology, microbiology, aetiopathogenesis and treatment. *Journal of the European Academy of Dermatology and Venereology,* 26.
TONG, S. Y. C., CHEN, L. F. & FOWLER, V. G., JR. 2012. Colonization, pathogenicity, host susceptibility, and therapeutics for Staphylococcus aureus: what is the clinical relevance? *Seminars in Immunopathology,* 34**,** 185-200.
TURKSEN, K., KUPPER, T., DEGENSTEIN, L., WILLIAMS, I. & FUCHS, E. 1992. Interleukin-6 - insights to its function in skin by overexpression in transgenic mice. *Proceedings of the National Academy of Sciences of the United States of America,* 89**,** 5068-5072.
UNTERNAEHRER, J. J., CHOW, A., PYPAERT, M., INABA, K. & MELLMAN, I. 2007. The tetraspanin CD9 mediates lateral association of MHC class II molecules on the dendritic cell surface.

Proceedings of the National Academy of Sciences of the United States of America, 104**,** 234-239.
VAN DRONGELEN, V., ALLOUL-RAMDHANI, M., DANSO, M. O., MIEREMET, A., MULDER, A., VAN SMEDEN, J., BOUWSTRA, J. A. & EL GHALBZOURI, A. 2013. Knock-down of filaggrin does not affect lipid organization and composition in stratum corneum of reconstructed human skin equivalents. *Experimental Dermatology,* 22**,** 807-812.
VAN DRONGELEN, V., DANSO, M. O., MULDER, A., MIEREMET, A., VAN SMEDEN, J., BOUWSTRA, J. A. & EL GHALBZOURI, A. 2014. Barrier Properties of an N/TERT-Based Human Skin Equivalent. *Tissue Engineering Part A,* 20**,** 3041-3049.
VENKATESAN, J., JAYAKUMAR, R., ANII, S., CHALISSERRY, E. P., PALLELA, R. & KIM, S.-K. 2015. Development of Alginate-Chitosan-Collagen Based Hydrogels for Tissue Engineering. *Journal of Biomaterials and Tissue Engineering,* 5**,** 458-464.
VOGT, A. B., SPINDELDREHER, S. & KROPSHOFER, H. 2002. Clustering of MHC-peptide complexes prior to their engagement in the immunological synapse: lipid raft and tetraspan microdomains. *Immunological Reviews,* 189**,** 136-151.
WANG, M., HERRMANN, C. J., SIMONOVIC, M., SZKLARCZYK, D. & VON MERING, C. 2015. Version 4.0 of PaxDb: Protein abundance data, integrated across model organisms, tissues, and cell-lines. *Proteomics,* 15**,** 3163-3168.
WELLS, A. 1999. EGF receptor. *International Journal of Biochemistry & Cell Biology,* 31**,** 637-643.
WERNER, S. & GROSE, R. 2003. Regulation of wound healing by growth factors and cytokines. *Physiological Reviews,* 83**,** 835-870.
WESTH, H., HOUGAARD, D. M., VUUST, J. & ROSDAHL, V. T. 1995. Erm genes in erythtomycin-resistant Staphylococcus aureus and coagulase negative staphylococci. *Apmis,* 103**,** 225-232.
WICKE, C., BACHINGER, A., COERPER, S., BECKERT, S., WITTE, M. B. & KOENIGSRAINER, A. 2009. Aging influences wound healing in patients with chronic lower extremity wounds treated in a specialized wound care center. *Wound Repair and Regeneration,* 17**,** 25-33.
WILHELM, M., SCHLEGL, J., HAHNE, H., GHOLAMI, A. M., LIEBERENZ, M., SAVITSKI, M. M., ZIEGLER, E., BUTZMANN, L., GESSULAT, S., MARX, H., MATHIESON, T., LEMEER, S., SCHNATBAUM, K., REIMER, U., WENSCHUH, H., MOLLENHAUER, M., SLOTTA-HUSPENINA, J., BOESE, J.-H., BANTSCHEFF, M., GERSTMAIR, A., FAERBER, F. & KUSTER, B. 2014. Mass-spectrometry-based draft of the human proteome. *Nature,* 509**,** 582-+.
WILKINSON, D. A., FITZGERALD, T. J. & JENNINGS, L. K. 1995. The newly discovered cell-adhesion molecule CD9 binds fibronectin and integrin alpha 2 beta 3. *Faseb Journal,* 9**,** A1500-A1500.
WILKINSON, L. J., WHITE, R. J. & CHIPMAN, J. K. 2011. Silver and nanoparticles of silver in wound dressings: a review of efficacy and safety. *Journal of Wound Care,* 20**,** 543-549.
WINSOR, T. & BURCH, G. E. 1944. Differential roles of layers of human epigastric skin on diffusion rate of water. *Archives of Internal Medicine,* 74**,** 428-436.
WOO, K. Y., COUTTS, P. M. & SIBBALD, R. G. 2012. Continuous Topical Oxygen for the Treatment of Chronic Wounds: A Pilot Study. *Advances in Skin & Wound Care,* 25.
WOODS, E. J., COCHRANE, C. A. & PERCIVAL, S. L. 2009. Prevalence of silver resistance genes in bacteria isolated from human and horse wounds. *Veterinary Microbiology,* 138**,** 325-329.
WRIGHT, M. D., GEARY, S. M., FITTER, S., MOSELEY, G. W., LAU, L. M., SHENG, K. C., APOSTOLOPOULOS, V., STANLEY, E. G., JACKSON, D. E. & ASHMAN, L. K. 2004. Characterization of mice lacking the tetraspanin superfamily member CD151. *Molecular and Cellular Biology,* 24**,** 5978-5988.
YAN, J., WU, B., HUANG, B., HUANG, S., JIANG, S. & LU, F. 2014. Dectin-1-CD37 association regulates IL-6 expression during Toxoplasma gondii infection. *Parasitology Research,* 113**,** 2851-2860.
YANEZ-MO, M., BARREIRO, O., GORDON-ALONSO, M., SALA-VALDES, M. & SANCHEZ-MADRID, F. 2009. Tetraspanin-enriched microdomains: a functional unit in cell plasma membranes. *Trends in Cell Biology,* 19**,** 434-446.

YEN, H. T., CHIANG, L. C., WEN, K. H., CHANG, S. F., TSAI, C. C., YU, C. L. & YU, H. S. 1996. Arsenic induces interleukin-8 expression in cultured keratinocytes. *Archives of Dermatological Research,* 288**,** 716-717.

ZANETTI, M., GENNARO, R., SCOCCHI, M. & SKERLAVAJ, B. 2000. Structure and biology of cathelicidins. *Biology and Pathology of Innate Immunity Mechanisms,* 479**,** 203-218.

ZHANG, J., DONG, J., GU, H., YU, S., ZHANG, X., GOU, Y., XU, W., BURD, A., HUANG, L., MIYADO, K., HUANG, Y. & CHAN, H. C. 2012. CD9 Is Critical for Cutaneous Wound Healing through JNK Signaling. *Journal of Investigative Dermatology,* 132**,** 226-236.

ZHOU, G., MO, W. J., SEBBEL, P., MIN, G. W., NEUBERT, T. A., GLOCKSHUBER, R., WU, X. R., SUN, T. T. & KONG, X. P. 2001. Uroplakin Ia is the urothelial receptor for uropathogenic Escherichia coli: evidence from in vitro FimH binding. *Journal of Cell Science,* 114.

ZHU, Y.-Z., LUO, Y., CAO, M.-M., LIU, Y., LIU, X.-Q., WANG, W., WU, D.-G., GUAN, M., XU, Q.-Q., REN, H., ZHAO, P. & QI, Z.-T. 2012. Significance of palmitoylation of CD81 on its association with tetraspanin-enriched microdomains and mediating hepatitis C virus cell entry. *Virology,* 429.

ZILBERMAN, M. & ELSNER, J. J. 2008. Antibiotic-eluting medical devices for various applications. *Journal of Controlled Release,* 130**,** 202-215.

ZUIDSCHERWOUDE, M., GOETTFERT, F., DUNLOCK, V. M. E., FIGDOR, C. G., VAN DEN BOGAART, G. & VAN SPRIEL, A. B. 2015. The tetraspanin web revisited by super-resolution microscopy. *Scientific Reports,* 5.

ZUMAQUERO, E., MUNOZ, P., COBO, M., LUCENA, G., PAVON, E. J., MARTIN, A., NAVARRO, P., GARCIA-PEREZ, A., ARIZA-VEGUILLAS, A., MALAVASI, F., SANCHO, J. & ZUBIAUR, M. 2010. Exosomes from human lymphoblastoid B cells express enzymatically active CD38 that is associated with signaling complexes containing CD81, Hsc-70 and Lyn. *Experimental Cell Research,* 316**,** 2692-2706.

Appendix

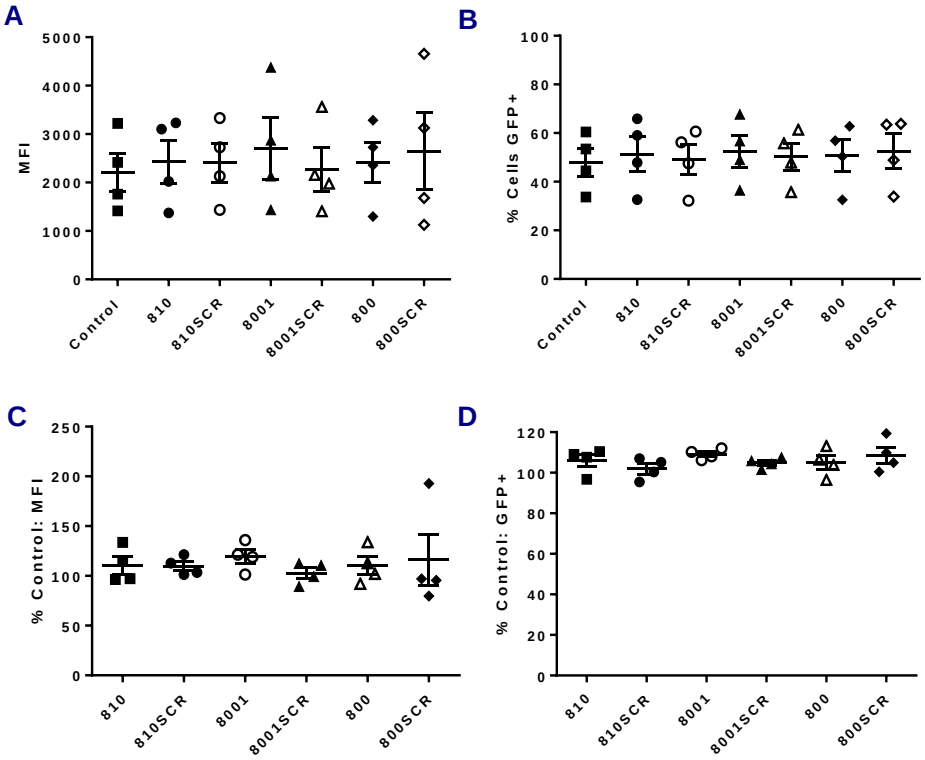
Quantification of Bacterial Adherence by Flow Cytometry:

Methods:

HaCaT cells were detached from flasks using cell dissociation solution (Sigma) and placed in a 96 well plate at a density of $1x10^5$ cells/well. They were then spun at 400rpm for 5 minutes, and washed using B/B/N buffer and spin cycles. Cells were peptide treated with 50nM peptides for 30 minutes. SH1000 expression gfp was then added to the cells at an MOI of 200 for 1 hour at 37°C with gentle agitation. Cells were washed 3 times to remove non-adherent bacteria and then fixed and re-suspended in B/B/N before quantification using the Attune Acoustic Focussing Flow Cytometer. Uninfected cells, bacteria alone, and cells infected with non-fluorescing SH100 were used for gating.

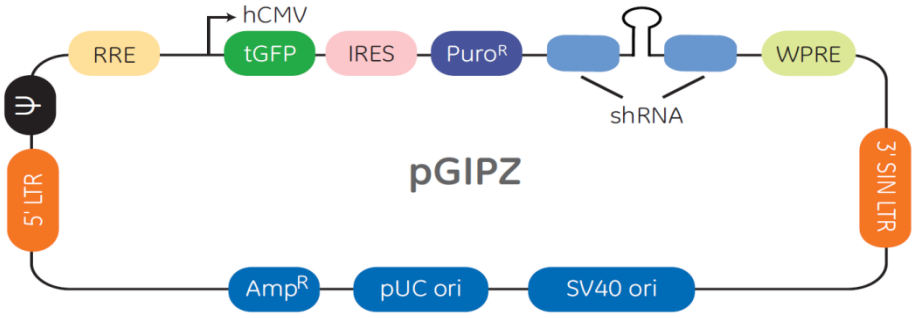
Results:

SH1000 bacteria were observed adhered to cells at an MOI of 200 No bacteria were observed at a lower MOI. No effect was of the peptide treatment was observed on Sh1000 adherence to HaCaT cells. This could be due to the increased MOI necessary to observe the fluorescence on the flow cytometer, or due to the mechanical stress of the experiment removing some of the adhered bacteria.



Supplementary Figure 1: Quantification of Bacterial Adherence by Flow Cytometry. (A) Median fluorescence of each condition. (B) Percentage of cells with gfp positive signal (C and D) Data normalised as a percentage of the no treatment control. Background fluorescence subtracted before plotting and analysis**.** % data transformed by $Y=\log_{10}(Y)$ before analysis by one way ANOVA. No significance. MFI is median fluorescence index.

N/TERT FLG knockdown:



Filaggrin knockdown in N/TERT cells was performed by Christabelle Goh. Briefly, the lentiviral plasmid pGIPz (depicted above, Dharmacon, GE Healthcare) containing silencing RNA for Filaggrin was transfected into N/TERT keratinicytes. Greatly reduced levels of pro-filaggrin were observed in cells transfected with this plasmid and selected with puromycin.