Understanding the complications of pseudomonas aeruginosa biofilm exudates on wound healing

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**UNDERSTANDING THE COMPLICATIONS OF
PSEUDOMONAS AERUGINOSA BIOFILM EXUDATES
ON WOUND HEALING**

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Interdisciplinary Graduate School

Nanyang Institute for Health Technologies

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**Interdisciplinary Graduate School
Nanyang Institute for Health Technologies**

A thesis submitted to the Nanyang Technological University in
partial fulfilment of the requirement for the degree of

Doctor of Philosophy

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

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Commercial research, here I come!

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LIST OF ABBREVIATIONS

2-AA	2-aminoacetophenone
3T3	3-day transfer, inoculum 3×10^5 cells
3-Oxo-C ₁₂ -HSL	N-(3-oxo-dodecanoyl)-l-homoserine lactone
α -SMA	Alpha smooth muscle actin
AF	Alexa Fluor
AHL	Acyl homoserine lactones
ANOVA	Analysis of variance
BafA1	Bafilomycin A1
BCM	Biofilm conditioned media
BRC	Biological resource centre
C ₄ -HSL	N-butanoyl homoserine lactone
c-di-GMP	Cyclic diguanylate
c-Jun	Transcription factor AP-1
c-Rel	V-rel avian reticuloendotheliosis viral oncogene homolog
CD	Cluster of differentiation
CFU	Colony forming unit
Cox-2	Cyclooxygenase-2
Cx	Connexin
Da/ kDa	SI Unit: Dalton/kilo dalton
DAMP	Damage associated molecular patterns
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's medium
DNA	Deoxyribonucleic acid
eDNA	Extracellular deoxyribonucleic acid
EF	Elongation factor
eIF4H	Eukaryotic initiation factor 4H
ELISA	Enzyme-linked immunosorbent assay
EPS	Extracellular polymeric substances
ERK	Extracellular-signal-regulated kinase
Exl	Exolysin
Exo	Exotoxin
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
GFP	Green fluorescent protein
H&E	Hematoxylin and eosin
HI	Heat inactivation
HMGB1	High mobility group box 1
IACUC	Institutional Animal Care and Use Committee
I κ B- α /NF- κ B inhibitor α / NF κ BI	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
I κ K- α	I κ B kinase alpha
IL	Interleukin
IPAF	ICE-protease activating factor
JNK	c-Jun N-terminal kinase
K (e.g. K10)	Keratin (e.g. Keratin 10)
LasB	Elastase
LasR	LasR protein
LCWB	Lubbock chronic wound biofilm model
MAPK	Mitogen-activated protein kinase
MG132	Carbobenzoxymethyl-Leu-Leu-leucinal
MPO	Myeloperoxidase
mRNA	Messenger ribonucleic acid
MSCs	Mesenchymal stromal cells
MTT	3-{4,5-dimethylthiazol-2-yl}-2,5-diphenyltetrazolium bromide

MyoD	Myoblast determination protein 1
NF- κ B	Nuclear factor kappa light chain enhancer of activated B-cells
NS	Non-significant
OD	Optical density
p50/ NF- κ B1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
p52/ NF- κ B2	Nuclear factor kappa B subunit 2
p65/RelA/NF- κ B3	V-rel avian reticuloendotheliosis viral oncogene homolog A
PA	<i>Pseudomonas aeruginosa</i>
PAMP	Pathogen associated molecular patterns
PAO1	<i>Pseudomonas aeruginosa</i> strain PAO1
PAO1 BCM	Biofilm conditioned media of <i>Pseudomonas aeruginosa</i> strain PAO1
PAO1-GFP	<i>Pseudomonas aeruginosa</i> strain PAO1 modified to carry a green fluorescent protein
PBS	Phosphate buffered saline
PE	Polyester
Pel	Pellicle polysaccharide
PFA	Paraformaldehyde
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinases
PK	Proteinase K
PMSF	Phenylmethylsulfonyl fluoride
PQS	2-heptyl-3-hydroxy-4-quinolone, <i>Pseudomonas</i> quinolone signal
Psl	Polysaccharide synthesis locus
RelB	V-rel avian reticuloendotheliosis viral oncogene homolog B
RhlA	3-(3-hydroxydecanoyloxy)decanoate synthase
ROS	Reactive oxygen species
RT-qPCR	Real time-quantitative polymerase chain reaction
SA	<i>Staphylococcus aureus</i>
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
STAT3	Signal Transducer and Activator Of Transcription 3
T3SS	Type 3 secretion system
TAK	Transforming growth factor beta-activated kinase
TEWL	Transepidermal water loss
TNF- α	Tumour necrosis factor-alpha
TSA	Tryptic soy agar
TSB	Tryptic soy broth
ZO	Zona occludens

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ABSTRACT

This thesis illustrates the effects of secreted factors of *Pseudomonas aeruginosa* biofilm exudates on different wound healing responses of fibroblasts and keratinocytes *in vitro* and in an *in vivo* murine wound healing model. A dose-dependent effect of *P. aeruginosa* PAO1 biofilm conditioned media (BCM) on cell migration was observed. At moderate to high concentrations of PAO1 BCM (30-100 %), migration was hampered, together with a loss of cell viability *in vitro*. PAO1 BCM at low doses (1-10 %) may be able to promote migration. Differences in responses after 50 % PAO1 BCM incubation between fibroblasts and keratinocytes were also observed, with keratinocytes being more susceptible to the cytotoxic secreted factors and experiencing less loss in cell adhesion compared to fibroblasts *in vitro*. Occludin levels were decreased in both cell types after 50 % PAO1 BCM challenge. Cx43, β -catenin and N-cadherin levels were reduced in fibroblasts. The *in vivo* model revealed that 100 % PAO1 BCM challenge did not result in significant changes in wound sizes and E-cadherin junctions from controls. Interestingly, re-epithelialisation was hampered, with a concomitant decrease in leukocyte infiltration at the wound edge. The downregulation of inflammatory responses by PAO1 BCM was correlated to a decrease in p65/p50 heterodimer and unchanged I κ B- α levels, showing that the NF- κ B pathway was not activated. Further investigations revealed that p65 may have been degraded by the proteasome. The dampened NF- κ B pathway was correlated with a decrease in JNK1 and c-Jun levels. The effects of PAO1 BCM on keratinocyte differentiation were also checked due to the close association of this pathway with the NF- κ B pathway. Early and late keratinocyte differentiation were impaired *in vitro* and *in vivo*. Identification of the secreted factors that caused these effects was attempted via biochemical tests. Preliminary data showed that the possible factors involved in cytotoxicity were heat

sensitive around 100 °C, non-proteinaceous and neither were they LasB, RhlA, LasR nor LPS. Future work should identify these factors. This study highlights the importance of secreted biofilm factors in complicating chronic wound closure and underlines the potential of targeting these factors for more effective therapies.

1. LITERATURE REVIEW

1.1 Chronic wounds

1.1.1 Chronic wounds: Importance, economic burden and societal costs

Chronic wounds are wounds that are unable to resolve after 3 months (Nunan et al., 2014). They generally fall under 3 broad sub-categories: diabetic foot ulcers, pressure ulcers and venous leg ulcers. In clinical settings, they are often identified as having necrotic tissues, a putrid smell and yellow discharge (Percival et al., 2012). Due to their ability to recur and frequent association with underlying co-morbidities, chronic wounds are difficult for clinicians to treat.

Chronic wound care is a global problem that affects 5.7 million people in the United States and ranges from US\$ 28.1 – 96.8 billion annually (Sen., 2019). In the local context, chronic wounds account for SGD\$ 3 million of government spending and almost SGD\$ 1 million in total indirect costs (Tan et al., 2016). Given such statistics, chronic wound care is an important healthcare issue to be tackled worldwide. In addition, people suffering from chronic wounds may undergo amputation of the lower extremities, causing impaired daily movement, shortened life expectancy and hence productivity, creating a vicious cycle in which the economy is affected (Trikkalinou et al., 2017). Taken together, chronic wounds represent a serious socioeconomic consequence in society.

1.1.2 Hallmarks of normal wound healing

Normal wound healing is characterised by 4 distinct overlapping stages, namely, haemostasis, inflammation, new tissue formation, and tissue remodelling (Gurtner et al., 2008; Stadelmann et al., 1998).

In haemostasis, a blood clot is formed by aggregation of platelets and thrombocytes in fibrin bundles to prevent excessive blood loss (Martin et al., 1997). It also acts as the first line of defence by functioning as a protective barrier to reduce pathogen entry (Shaw and Martin., 2009). The fibrin network helps in cell migration and stimulation of fibroblast proliferation (de Oliveria Gonzalez., 2016).

Thereafter inflammation occurs, where neutrophils, followed later by macrophages, are rapidly recruited to the wound site, culminating in the clearance of both cellular debris and bacteria (Grose and Werner, 2004). Some key signs of inflammation are oedema and erythema at the wound site which can last as long as 2 days (de Oliveria Gonzalez., 2016). Neutrophils are recruited from damaged blood vessels or through diapedesis from local capillaries, while macrophages are either tissue residents or recruited monocytes from the local microenvironment (de Oliveria Gonzalez., 2016). Recruitment is activated through complement activation, presence of bacterial products, platelet degranulation and chemokine signalling (Gurtner et al., 2008; Eming et al., 2007). In the clearance of cellular debris and pathogens, neutrophils and macrophages release reactive oxygen species (ROS), neutrophil extracellular traps (NETs), pro-inflammatory cytokines, chemokines and lysosomal enzymes (Medrado et al., 2003; Brinkmann et al., 2004). Macrophages are initially bactericidal and phagocytic but have also been found to have roles in re-epithelialisation, angiogenesis, granulation tissue and scar formation (Lucas et al., 2010; Lawrence and Natoli., 2011). The wound repair roles of macrophages occur when class-switching of the polarised macrophage stages occur from M1 (pro-inflammatory) to M2 (anti-inflammatory and pro-repair) phenotype (Crane et al., 2014; Daley et al., 2010). Macrophages release growth factors such as

vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF) for tissue repair (Singer and Clark., 1999).

Subsequently, new tissue formation takes place via the proliferation and migration of keratinocytes and fibroblasts, resulting in re-epithelialisation and granulation tissue formation (Gurtner et al., 2008). This stage helps to contract the wound and can last up to 14 days post-wounding (Li et al., 2007b). In re-epithelialisation, keratinocytes are activated by signals such as damage, exposure to serum and sensing of pathogens (Freedberg et al., 2001). Wound edge keratinocytes undergo epithelial-to-mesenchymal transition (EMT), thereby promoting migration and proliferation (Savagner et al., 2005; Nieto., 2013; Bleaken et al., 2016; Nunan et al., 2015). Fibroblasts proliferate, collagen is deposited, and chemotactic factors and interferon- β are produced by fibroblasts (Calin et al., 2010; Medrado et al., 2010). Angiogenesis takes place to supply more nutrients to the newly repaired areas (Eming et al., 2007; Calin et al., 2010). In angiogenesis, endothelial cells proliferate, associate in tubular structures and recruit perivascular cells (de Oliveria Gonzalez., 2016; Mendonça and Coutinho-Netto., 2009). Following granulation tissue formation, some fibroblasts transform into myofibroblasts, which help to contract the wound (Calin et al., 2010). Migratory wound-edge keratinocytes also secrete new basement membrane components (Margadant et al., 2009). Matrix metalloproteases are then activated to degrade the scab and remodel the extracellular matrix (ECM) of the granulation tissue (Sudbeck et al., 1997). In this stage, type III collagen synthesised by fibroblasts in the granulation tissue is predominant (de Oliveria Gonzalez., 2016).

Finally, tissue remodelling occurs, which consists of reorganisation, degradation and re-synthesis of the ECM, achieving maximum tensile strength (Shaw and Martin., 2016). Inflammation is also resolved in this stage (de Oliveria Gonzalez., 2016). Migration stops, keratinocytes start to reconstruct the basal membrane and differentiate to generate the newly stratified epidermis (Li et al., 2007b). Collagen type III is degraded, and type I collagen is synthesised (de Oliveria Gonzalez., 2016). As healing occurs, apoptosis of fibroblasts and macrophages takes place (de Oliveria Gonzalez., 2016). Eventually, the wound resolves and a scar is formed (de Oliveria Gonzalez., 2016).

Figure 1: Normal wound healing. Image by Erica Teo Mei Ling.

1.1.3 Hallmarks of chronic wound healing

Patients suffering from chronic wounds frequently have underlying health issues such as vascular impairment, compromised immunity, neuropathy and persistent inflammation (Kranke et al., 2012; Tomic-Canic et al., 2004). Characterised by bacterial colonisation, persistent inflammation and hyper-proliferative epidermis, chronic wounds culminate in poor wound resolution (Tomic-Canic et al., 2004). Colonisation of bacteria also develops into a more persistent, biofilm state at the wound site (Tomic-

Canic et al., 2004). Despite reduced accessibility from poor vascular flow, neutrophils and macrophages are constantly recruited to kill and phagocytose the bacteria, resulting in chronic inflammation (Eming et al., 2009). Keratinocytes are unable to migrate while dermal fibroblasts are unable to proliferate, conferring a hyper-proliferative epidermis at the wound edge, characteristic of chronic wounds (Usui et al., 2008; Cha et al., 2008). Matrix deposition does not occur, and instead, the extracellular matrix is degraded by proteases such as matrix metalloproteases and elastases (McCarty et al., 2012; Sutcliffe et al., 2017). When no proper wound resolution is observed for 3 months, the wound is characterised as a chronic wound (Nunan et al., 2014).

1.1.4 Paradoxical role of inflammation in wound healing

Inflammation is the second phase of the wound healing process and is activated immediately after injury. During inflammation, neutrophils are first recruited to the wound site within 24 h through induction via damage associated molecular patterns (DAMPs), pathogen associated molecular patterns (PAMPs), cytokines and chemokines produced by immune cells at the site of injury (Zhang and Mosser., 2008; Ross and Odland., 1968; Engelhardt et al., 1998). Neutrophils help by providing antimicrobial activities such as phagocytosis of pathogens and release of ROS (Koh and Di Pietro., 2011; Schauer et al., 2014). Together with the hypoxic wound environment, pathogens and the chemokine IL-8 released by neutrophils, monocytes and macrophages are recruited to the wound site (Sen., 2009; Mosser., 2003).

Circulating monocytes then mature into macrophages, which phagocytose cell debris, pathogens and spent neutrophils (Ross and Odland., 1968). Macrophages in the M1 phenotype are pro-inflammatory, are also involved in production of pro-inflammatory

cytokines such as TNF- α , IL-6, IL-1, and promote angiogenesis (Barrientos et al., 2008; Murray et al., 2011). Collagenases and elastases are also secreted by macrophages to digest injured tissue. Lymphocytes are then recruited into the wound site around 72 h after injury (Barbul et al., 1989b). Lymphokines such as EGF and basic fibroblasts growth factor (bFGF) are secreted and T lymphocytes are recruited to regulate collagenase (Barbul et al., 1989a).

Finally, macrophages switch from the M1(inflammatory) to M2 (healing) phenotype which stimulates wound resolution (Stein et al., 1992; Brancato and Albina., 2011; Murray et al., 2011). The M2 phenotype induces the release of macrophage-derived growth factors such as TGF- β , PDGF and macrophage-derived angiogenic factors (Polverini et al., 1977; Hunt et al., 1984; Goren et al., 2009). This results in the influx of endothelial cells, and migratory fibroblasts and keratinocytes into the wound bed that is important for wound resolution (Polverini et al., 1977; Hunt et al., 1984; Kovacs and Di Pietro., 1994).

Inflammation is important in wound closure, as suggested by Cowin et al. (1998) and Martin and Leibovich. (2005), where it affects the final wound resolution such as normal wound healing, scar formation and fibrosis. For example, the phagocytic ability of macrophages to ingest neutrophils is necessary for the switch from the pro-inflammatory (M1) phenotype to the reparative (M2) phenotype (Fadok et al., 1998). A failure in this phagocytic ability causes the pathogenicity of non-healing wounds (Khanna et al., 2010; Acosta et al., 2008). As aberrant inflammation is characteristic of chronic wounds, it highlights the importance of controlled inflammation in wound resolution.

Chronic wounds are caused by underlying conditions such as diabetes, vascular impairment and neuropathy; and these conditions elicit high levels of oxidative stress (Rieder et al., 2007). In chronic wounds, the dysregulation of inflammation causes it to be persistently above basal levels (Rieder et al., 2007). Extended exposure of the wound to ROS causes massive amounts of cellular destruction and oxidation of cellular components and premature cell senescence (Demidova-Rice et al., 2012; Volpe et al., 2018). This is mainly caused by excessive neutrophil infiltration causing overproduction of ROS (Demidova-Rice et al., 2012). Consequently, the wound is constantly stuck in an inflammatory state and as a result, the wound is unable to heal (Gary Sibbald et al., 2008).

Oxidative stress represses the function of neutrophils such as their chemotaxis (Gustke et al., 1998). Studies have shown that advanced glycation end products (AGEs) repress the function and migration of neutrophils by binding to neutrophil receptors, causing scattering of neutrophils in the wound area and impairing endothelial cell migration (Glowacka et al., 2002; Tian et al., 2016a; Tian et al., 2013). Neutrophils of diabetic patients are also highly susceptible to apoptosis, decreasing their functional capability and increasing their chances of clearance by macrophages, thus culminating in a higher infection risk in these patients (Tennenberg et al., 1999). Moreover, if apoptotic neutrophils are not cleared away by macrophages, their presence will destroy normal tissue as well, due to their ability to digest a wide variety of factors and their production of ROS (Dovi et al., 2003; Wilgus et al., 2003; Meszaros et al., 2000; Meszaros et al., 1999). Hence, if macrophages are impaired in phagocytosis, such as in diabetic conditions, wound resolution will be impaired (Khanna et al., 2010).

During refractory wound healing, macrophages experience an imbalance in the M1-M2 class switching phenotype in the early stages of wound healing, with a decrease in M1 markers and an increase in M2 markers even though it is supposed to be at the inflammatory M1 phenotype (Miao et al., 2012). On the other hand, Sindrilaru et al. (2011) have reported that macrophages are in a dominant pro-inflammatory M1 state which impairs wound healing. The study revealed that the macrophages enhanced TNF- α and hydroxyl radical release, and induced fibroblast senescence, leading to impaired wound healing (Sindrilaru et al., 2011). The accumulation of ROS also causes rampant TNF- α production from macrophages, which impairs re-epithelialisation (Dong et al., 2016).

1.1.5 The NF- κ B pathway

The NF- κ B family of transcription factors regulate a wide array of genes involved in inflammation (Oeckinghaus and Ghosh., 2009). The members of the NF- κ B pathway include NF- κ B1 (p50), NF- κ B2 (p52), v-rel avian reticuloendotheliosis viral oncogene homolog (p65), v-rel avian reticuloendotheliosis viral oncogene homolog B (RelB) and v-rel avian reticuloendotheliosis viral oncogene homolog (c-Rel), and they mediate the transcription of target genes by binding to DNA (Hayden and Ghosh., 2012). These proteins are usually localised in the cytoplasm in the transcriptionally inactivate state with the help of inhibitory proteins such as nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (I κ B) (Sun. 2011). The post-translational modification of NF- κ B inhibitors is the first step of activation of the NF- κ B pathway, and this involves 2 pathways, namely the canonical and the non-canonical pathway (Zhang et al., 2017; Hayden and Ghosh., 2008).

In the canonical pathway, NF- κ B heterodimers are regulated by inhibitors of the NF- κ B pathway, which prevent the translocation of these heterodimers into the nucleus (Pires et al., 2018). The canonical pathway is activated by pro-inflammatory cytokine receptors such as tumour necrosis factor receptor (TNFR), growth factor receptors, antigen receptors and IL-1 receptor (IL-1R), which cause a cascade of I κ B kinase (I κ K) signalling events (Dondelinger et al., 2016; Pires et al., 2018). The receptor binding recruits receptor-interacting serine/threonine-protein kinase 1 (RIP-1), TGF β -activated kinase 1 (TAK1), TNF receptor type 1-associated DEATH domain protein (TRADD), TNF receptor-associated factor 2 (TRAF2), TGF β -activated kinase 1 and MAP3K7-binding protein (TAB) and cellular inhibitor of apoptosis protein (cIAP) into a protein complex, together with the linear ubiquitin assembly complex (LUBAC) (Dondelinger et al., 2016). Next, I κ K is recruited, triggering the ubiquitination of the NF- κ B essential modulator (NEMO-also known as I κ K γ) and phosphorylates I κ B, causing I κ B to be ubiquitinated and proteasomally degraded (Ben-Neriah., 2002; Hayden and Ghosh., 2008). Consequently, NF- κ B heterodimers such as p65/p50 or c-Rel/p50 translocate to the nucleus, bind DNA and induce gene expression of target genes such as IL-6 and IL-8 (Ben-Neriah., 2002).

Figure 2: The canonical NF-κB pathway. Image by Erica Teo Mei Ling.

In the non-canonical pathway, IκB is absent and the RelB/p100 complex is inactive when localised in the cytoplasm (Sun., 2012). The pathway is activated when signalling results from the B cell-activating factor receptor (BAFFR), lymphotoxin β receptor (LTβR) and also the cluster of differentiation 40 ligand (CD40L) (Claudio et al., 2002; Coope et al., 2002; Kayagaki et al., 2002; Dejardin et al., 2002). This induces the activation of the NF-κB-inducing kinase (NIK), causing the phosphorylation of the homodimer IκK-α/ IκK-α (Senftleben et al., 2001; Xiao et al., 2001). Consequently, p100 becomes ubiquitinated and processed by the proteasome into p52, which culminates in the nuclear translocation of RelB/p52 into the nucleus and induction of gene expression (Senftleben et al., 2001).

1.1.6 Role of keratinocyte differentiation in wound healing

Keratinocyte differentiation is an important process in wound closure, as it is part of the re-epithelialisation process (Pastar et al., 2014). According to Pastar et al. (2014), re-epithelialisation defines the success of wound healing as a breach in the epidermal

barrier allows wounds to get infected by pathogens, which can result in the formation of chronic wounds. The impairment of epidermal barrier maintenance may also cause wounds to reoccur (Pastar et al., 2014).

Keratinocytes differentiation is a process by which cells mature from a proliferative state to a terminally differentiated state, where the skin forms a tough epidermal barrier to protect against foreign pathogens and prevent excessive transepidermal water loss (TEWL) (Wikaramanayake et al., 2014). In the epidermis, basal layer keratinocytes are proliferative and contain keratins 5 (K5) and 14 (K14) (Pastar et al., 2014). These keratinocytes then ascend through the epidermis from the spinous to the granular layer and finally the stratum corneum, in which they undergo differentiation during this process, where K1 and 10 are expressed in the suprabasal layers (Tomic-Canic., 1998). In this process, keratin filaments associate with cornified envelope proteins such as filaggrin and involucrin to form a cornified cell envelope (Wikaramanayake et al., 2014; de Guzman Strong, 2006). This is then followed by DNA degradation, nuclear and organelle destruction and substantial proteolytic activity, leading to cell death (Stojadinovic et al., 2008). This causes terminal differentiation, where the influx of intracellular calcium leads to transglutaminase activation and the formation of a cornified layer and subsequently desquamation of dead cells (Stojadinovic et al., 2008; Eckert, 1989) Finally, structural proteins such as involucrin, loricrin and filaggrin (late markers of differentiation) are covalently crosslinked by transglutaminase 1, together with the attachment of skin lipids in the stratum corneum (Eckert., 1989). Early markers of differentiation include K1 and 10, and late markers of differentiation include involucrin, transglutaminase 1 and filaggrin. Various signalling pathways that are essential for keratinocyte differentiation have been identified and include NF- κ B,

extracellular signal-regulated kinases (ERK), MAPK and the Notch signalling pathway (Blanpain and Fuchs., 2009; Rangarajan et al., 2001; Thelu et al., 1998). The major MAPK pathways involved are activated by multiple stimuli, such as the influx of calcium, and the levels of epidermal growth factor and tumor necrosis factor (Jost et al., 2001).

Apart from hampered migration, chronic wounds also have impaired keratinocyte differentiation. Keratinocytes at the non-healing edge of venous ulcers are not activated or differentiated, culminating in a callus-like formation at wound edges (Stojadinovic et al., 2008). Keratinocytes in chronic wounds have atypical cornified envelope assembly and are unable to complete the differentiation process (Stojadinovic et al., 2008). Stojadinovic et al. (2008) also have reported that early differentiation markers such as K1, 10 and small proline-rich proteins, and filaggrin, a late differentiation marker were downregulated, while late differentiation markers such as involucrin, loricrin and transglutaminase 1 and other small proline-rich proteins were expressed in ulcers compared to healthy skin. Taken together, these findings show that keratinocytes are unable to differentiate normally in chronic wounds, resulting in defective wound closure and barrier formation. Keratinocytes in diabetic foot ulcers are also shown to have reduced expression of K10, indicative of an impaired differentiation state. Other than the differentiation markers, pathways associated with keratinocyte differentiation have been reported to be dysregulated in chronic wounds. For example, I κ B- α (part of the NF- κ B pathway) expression was downregulated in venous ulcers, which is in line with findings of chronic inflammation (Stojadinovic et al., 2008). In the same paper, Notch 2 was also reported to be downregulated (Stojadinovic et al., 2008). Similarly, Takeda

et al. (1999) have found that keratinocytes in I κ B- α -deficient mice have impaired epidermal differentiation.

1.1.7 Role of fibroblasts in wound healing

Fibroblasts are involved in various steps of the wound healing process. In inflammation, fibroblasts are recruited by platelets via chemotaxis (Darby et al., 2014). During the proliferative phase, angiogenesis occurs and this contributes to fibroblast proliferation (and hence ECM deposition) and the formation of new blood vessels to supply nutrients to the wound site (Wietecha and DiPietro., 2013; Shaw and Martin., 2016). In the granulation tissue formation phase, fibroblasts are activated into myofibroblasts by expressing alpha smooth muscle actin (α -SMA) in stress fibers (Hinz and Gabbiani., 2003). Due to the presence of α -SMA, myofibroblasts are contractile in nature, which help with the contraction and maturation of the granulation tissue (Hinz and Gabbiani., 2003; Gabbiani et al., 1977). Lastly, in the tissue remodelling phase, myofibroblasts are important as they contribute to ECM remodelling. They have large focal adhesions for strong attachments to contract the ECM and transduce mechanical force in the tissue (Dugina et al., 2001; Goffin et al., 2006). Wound resolution also involves fibroblasts as cell number reduction is attributed to apoptosis of endothelial cells and myofibroblasts (Desmoulière et al., 1995). The continual presence of myofibroblasts is also a sign of fibrosis and scar formation, where many fibrotic diseases are caused by overactivity and overgrowth of myofibroblasts, leading to their association with cancer (Desmoulière et al., 2003; Desmoulière et al., 2004; Kalluri and Zeisberg., 2006).

1.2 Chronic wounds and bacterial biofilms

The presence of biofilms in wounds is well established, where biofilm-like structures of *Staphylococcus aureus* have been observed to be forming in the dermal and subcutaneous tissues in a mouse wound model (Akiyama et al., 1996). Establishment of biofilms was also detected in murine burns and porcine acute wounds (Rashid et al., 2000; Serralta et al., 2001). Bacterial biofilms exacerbate and complicate chronic wound healing (Schaber et al., 2007; James et al., 2008). Electron microscopy showed that 60 % of chronic wound biopsies contained biofilms, compared to 6 % in acute wounds, suggesting a possible growth preference of biofilms for chronic wounds (James et al., 2008). Meta-analysis of published data revealed that the prevalence of biofilms in 9 studies involving 185 human chronic wounds was 78 %, which support clinical assumptions that biofilms are ubiquitous in chronic wounds (Malone et al., 2017). Through pyrosequencing, chronic wound aetiologies such as venous leg ulcers, pressure ulcers and diabetic foot ulcers were revealed to have biofilm colonisation and infection (James et al., 2008; Wolcott et al., 2009; Smith et al., 2010; Percival et al., 2015). Additionally, apart from the major aetiologies, ulcerated burn wounds at 31 days post injury also had biofilm-like structures growing on them (Kennedy et al., 2010). This evidence reiterates that biofilm formation and development is characteristic of chronic wounds. This could be due to impaired host immunity and the presence of necrotic tissue that promote bacterial colonisation and biofilm development (Wolcott et al., 2008).

Biofilms are adherent, structured communities of bacteria enclosed in a matrix (Costerton et al., 1999). Some characteristics of biofilms include the ability to quorum sense and tolerance for antimicrobials, predation and the host immune system (Mah and O'Toole, 2001; Stewart and Costerton, 2001). They also have a different gene

expression and metabolic profile compared to their planktonic (single, free-floating bacterium) state (Waite et al., 2006). The biofilm development process consists of 4 stages: attachment, growth, maturation and dispersal (Percival et al., 2015). In the attachment stage, planktonic bacteria will start to attach reversibly, and subsequently, irreversibly onto surfaces (Ista et al., 2009). In cases where the bacterium is motile, such as *Pseudomonas aeruginosa*, the flagellum and type IV pili contribute to bacterial attachment (Mikkelsen et al., 2011). In the growth stage, planktonic bacteria proliferate to form microcolonies and secrete extracellular polymeric substances (EPS) to produce a surrounding matrix. The EPS consists of a variety of components such as polysaccharides, proteins, lipids and extracellular DNA (eDNA). It also has water channels to facilitate the exchange of nutrients and substances with the environment (Kaplan., 2010). The proliferation and matrix production culminate in the maturation of the biofilm and it becomes three-dimensional in structure. The matured biofilm is highly protective in nature due to the presence of EPS which acts as a diffusion barrier to antimicrobials and has gradients of metabolic activities (Lieleg et al., 2011). Finally, the biofilm disperses and this contributes to colonisation of new areas, disease transmission and pathogenic survival (Kaplan., 2010). Planktonic bacteria are dispersed from the biofilm via erosion, seeding or sloughing and the biofilm development cycle is repeated (Kaplan., 2010).

1.2.1 Biofilm and impaired wound healing

Biofilms are highly tolerant of antibiotics and debridement and have been reported to impair wound healing via a few mechanisms: inflammation, delayed re-epithelialisation and granulation tissue formation (Wolcott et al., 2010; Schierle et al., 2009; Gurjala et al., 2011; Nguyen et al., 2013). Studies by Schierle et al. (2009) and Seth et al. (2012)

showed that biofilms of bacteria such as *S. aureus*, *Staphylococcus epidermidis* and *Klebsiella pneumoniae* delayed wound re-epithelialisation. Apart from mono-species biofilms, polymicrobial biofilms, which are prevalent in non-healing chronic wounds, negatively affect wound healing as well. The combination of *P. aeruginosa* and methicillin-resistant *S. aureus* (MRSA) biofilms delayed re-epithelialisation of porcine wounds (Pastar et al., 2013). Moreover, polymicrobial biofilms transplanted onto the wounds of mice also resulted in infection (Dalton et al., 2011). The presence of biofilms is therefore highly associated with impaired wound healing.

1.2.2 Biofilm conditioned media

Recent research investigated the effects of bacterial secretory factors on wound healing using BCM as the bacterial secretome. Biofilm conditioned media are media in which biofilms have been grown and so they contain biofilm secretions. Most of the work focused on *S. aureus*, one of the most commonly isolated bacteria in chronic wounds (Gjødtsbol et al., 2006). Marano et al. (2015) reported that BCM from both *S. aureus* and *P. aeruginosa* negatively affect proliferation in human tympanic membrane derived keratinocytes, while also having toxic effects on human epidermal keratinocytes. Of the two BCM investigated in the article, only *S. aureus* BCM was found to inhibit migration (Marano et al., 2015).

Comparisons between soluble factors released by biofilm and planktonic states (termed as planktonic conditioned media, PCM) were also conducted. Upon proteomic and microarray analyses of the BCM and PCM generated by MRSA and clinical isolates of *S. aureus* respectively, an enrichment of genes was shown to be involved in apoptosis, inflammation, nitric oxide production, chemotaxis, signal transduction and reduced wound closure by inhibiting migration in both human fibroblasts and keratinocytes

(Secor et al., 2011; Kirker et al., 2012; Tankersley et al., 2014). However, the effects were more significant in BCM compared to PCM (Kirker et al., 2012; Tankersley et al., 2014). Additionally, sustained cytokine production at low levels was also induced in *S. aureus* BCM exposed keratinocytes through mitogen-activated protein kinase (MAPK)-independent mechanisms, compared to PCM where high levels of cytokine expression were detected (Secor et al., 2011). PCM induced higher expression of cytokines such as interleukin (IL)-6 and 8, growth factors like transforming growth factor beta (TGF- β) and epidermal growth factor (EGF), and matrix metalloproteinases (MMP) 1 and 3 compared to BCM, while BCM induced more tumour necrosis factor-alpha (TNF- α) expression (Kirker et al., 2012). As chronic wounds are usually characterised by persistent inflammation and delayed wound closure, these studies reinforce the possibility that BCM may contribute to the formation and persistence of chronic wounds (Tomic-Canic et al., 2004).

Apart from the context of wound healing, the effects of BCM have been investigated in other areas. BCM of clinical wound isolates of *S. aureus* reduced osteoblast viability with a concomitant increase in apoptosis (Sanchez et al., 2013a). It also inhibited osteogenic differentiation and promoted bone resorption (Sanchez et al., 2013a). Ward et al. (2015) have also demonstrated that exposure of mesenchymal stromal cells to *S. aureus* and *P. aeruginosa* BCM induces apoptosis, inhibits migration and differentiation of MSCs. Paracrine factors involved in inflammation and wound healing were contrastingly increased (Ward et al., 2015). Secreted factors from both BCM and PCM of dual-species bacteria involved in causing regenerative endodontics, *Streptococcus oralis* and *Actinomyces naeslundii*, promoted inflammation, while compromising cell viability, proliferation and mineralization capacity of apical papilla stem cells (Petridis

et al., 2018). Arima et al. (2018) have also showed that *Staphylococcus pseudintermedius* BCM induced inflammation via the Toll-like receptor (TLR) signalling pathway, and the severity increased with increased biofilm formation capacity. These studies suggest that the effects of bacterial biofilm secretome may be implicated in a plethora of host infections. Collectively, these studies reiterate that not all effects require direct bacteria-cell contact or interaction, and that the secretome may also be of clinical relevance.

1.2.3 Models of biofilm conditioned media generation

Although there are various studies using biofilm supernatants, the concept of BCM was introduced in 2009 by Kirker et al., to study the effects of biofilm secreted factors. Since then, various models of BCM generation have emerged, as listed in **Table 1**. Depending on the context of the study, some BCM are grown using bacteria growth media (e.g. tryptic soy broth (TSB), brain heart infusion (BHI) media), while others are grown in antibiotic-free cell culture media (e.g. DMEM, Roswell Park Memorial Institute (RPMI) media). Additionally, the timepoint in which media is being collected differs between studies and the researcher can also decide whether to pool the media together or to just use media from the final collection.

Table 1: Models of BCM generation

Model Name	Description	References
Co-culture	Biofilms were grown on tissue culture inserts (0.2 /0.4 µm) in static conditions and placed together in culture with cells grown as a monolayer. Mammalian cells are not in contact with the biofilms in this model. Fresh media was replenished for the insert-supported biofilms every 24 h for a total of 72 /96 h incubation. BCM was then obtained by incubating with cell culture medium (without Penicillin-Streptomycin (Pen-Strep)) and cell layer. Media was collected, fresh media replenished for the insert-supported biofilms every 24 h for a total of 72/96 h incubation and the media collections were pooled as BCM after pH adjustment to 7.4.	Developed by Kirker et al. (2009). Used by Tankersley et al. (2014), Secor et al. (2011, 2012), Sanchez et al. (2013a), Ward et al. (2015).
Static culture	Biofilms were cultured in cell culture media under static conditions on a surface (e.g. glass chamber slides, well plates, Constant Depth Film Fermenter) and the supernatant clarified by centrifuging to remove bacteria, and filter sterilised. Cells were then cultured in sterilised BCM.	Used by Arima et al. (2018), Petridis (2018) and Scherr (2015).
Shaking culture	Biofilms were cultured in media on a shaking incubator, and then collected, sterilised and applied on cells. Another example of biofilm formation using a shaking incubator is the Minimum Biofilm Eradication Concentration (MBEC™) high-throughput assay using the MBEC device which consists of a 96-peg-lid (top plate) that covers a ridged trough (bottom plate). The inoculated device is placed on a shaking platform which allows biofilms to be formed evenly on the pegs and incubated for the stipulated duration before media collection.	Used by Marano et al. (2015)
Calgary biofilm device	Biofilms were formed using the Calgary Biofilm Device for 4 h or 1 day at 150 rpm. BCM was also generated by co-culturing biofilms with cells for 4 h in serum-free DMEM and filtering the supernatant.	Used by Bowler et al. (2014) Bowler (2015-unpublished)

Apart from these BCM generation models, there are also other ways of growing biofilms *in vitro*. Other models of biofilm generation include the *in vitro* multi-species Lubbock chronic wound biofilm model (LCWB), collagen based models, surface-attached biofilm model (using polyester fleece), batch and flow cultures (Sun et al., 2008; Werthén et al., 2010; Kostenko et al., 2010; Thorn and Greenman., 2009; Thorn et al., 2009; Lipp et al., 2010; Schleheck et al., 2009). These methods could be used to generate biofilms for BCM, if modified to include supernatant collection.

1.3 *Pseudomonas aeruginosa* and chronic wounds

Pseudomonas aeruginosa is an opportunistic, Gram-negative, biofilm-forming pathogen commonly found in chronic wounds (Ge et al., 2002). This rod-shaped pathogen usually colonises and infects when natural defences are compromised and is responsible for causing infections such as cystic fibrosis, corneal infections, and burnt skin infections (Lyczak et al., 2000).

Amongst all the biofilm-forming bacteria detected in chronic wounds, *S. aureus*, *P. aeruginosa* and *Enterococcus faecalis* are most common (Gjødtsbol et al., 2006). Studies using 16S ribosomal DNA sequencing, fluorescence in situ hybridisation, denaturing gradient gel electrophoresis and standard culturing techniques have reported *P. aeruginosa*'s prevalence in chronic wounds, especially for diabetic foot ulcers and venous leg ulcers (Davies et al., 2004; Kirketerp-Møller et al., 2008). Gjødtsbol et al. (2006) have reported the presence of *P. aeruginosa* in 52.2 % of the chronic venous leg ulcers studied and Kirketerp-Møller et al. (2008) have observed *P. aeruginosa* microcolonies embedded in an alginate matrix, characteristic of the biofilm state. Through pyrosequencing, *Pseudomonas* was shown to be the third most prevalent bacterial population isolated from diabetic foot ulcers, and 23 out of 49 samples in decubitus ulcer samples had *P. aeruginosa* isolated (Dowd et al., 2008; Smith et al., 2010). These reiterate that *P. aeruginosa* is highly prevalent in chronic wounds.

Due to its ability to form biofilms and its intrinsic drug resistance capabilities, *P. aeruginosa* infections are known for being difficult to treat despite the development of drug cocktails (Moore and Flaws, 2011). *Pseudomonas aeruginosa* biofilms have been observed in chronic wounds, especially located in deeper tissues and in the wound bed (Fazli et al., 2009). It has been reported that third-degree scald burns have rampant *P.*

aeruginosa infection and their biofilms can form within 8 h post-infection (Rumbaugh et al., 1999; Schaber et al., 2007).

On this note, the presence of *P. aeruginosa* has been associated with poor prognosis for chronic wound healing. In a chronic non-diabetic wound model (created by the injection of *P. aeruginosa*-embedded alginate under a third-degree thermal lesion) established by Trøstrup et al. (2013), *P. aeruginosa* biofilms induced skin inflammatory response. Upon further investigation, Kim et al. (2015) have shown that the virulence of *P. aeruginosa* is induced by contact with the fascia layer of the skin, which is collagen-rich. The negative effects on the host defence system may stimulate a chronic inflammation state, thus contributing to impaired wound healing.

Using a diabetic wound model in mice, *P. aeruginosa* biofilm-challenged wounds had significantly hampered wound healing compared to the unchallenged (Zhao et al., 2010; Watters et al., 2013). Watters et al. (2013) then went on to show that insulin treatment for the diabetic mice did not improve bacterial clearance or wound closure. It was later reported that the inhibition in diabetic wound healing is primarily mediated by the type 3 secretion system (T3SS) of the bacterium (Goldufsky et al., 2015). Recently, there was an excessive activation of M1 phenotype and inadequate activation in *P. aeruginosa*-infected diabetic mice, contributing to the increase in pro-inflammatory cytokines as the switch is crucial in dampening inflammation for progression to the healing process (Chen et al., 2018).

Collectively, the prevalence of *P. aeruginosa* in chronic wounds, especially its biofilm state, is one of the major influences hampering wound closure and promoting inflammation.

1.3.1 Impact of *Pseudomonas aeruginosa* on directional migration

Collective migration, as defined by Friedl and Gilmour (2009), has a few hallmarks. Traction for migration is generated through functional intercellular junctions, cell polarity and cytoskeletal dynamics (Friedl and Gilmour., 2009). It also involves structural modification of the migration pathway such as clearing the path or through ECM modification (Friedl and Gilmour., 2009). In wound healing, directional, collective cell migration occurs when keratinocytes migrate as a monolayer across the granulation tissue, culminating in epidermal closure (Poujade et al., 2007; Zelenka and Arpitha., 2008). This is important for protecting the deeper tissues from further bacterial colonisation and other external forces (Nikolic et al., 2006).

Keratinocytes use intercellular junctions, such as integrins, to generate traction for movement with the help of the actin cytoskeleton (Grose et al., 2002; Moll et al., 1999; Vaezi et al., 2002). The link between integrins and the actin cytoskeleton is focal adhesions (Wozniak et al., 2004). Some of the focal adhesion proteins include the focal adhesion kinase, talin, vinculin and paxillin and together, they form the focal adhesion complex (Laukaitis et al., 2001). Regulated by the GTPases Rac, Cdc42 and Rho, focal adhesions are localised at the periphery of cells that are migratory and are also located centrally where they are associated with the ends of stress fibres (Ridley and Hall., 1992; Chrzanowska-Wodnicka and Burridge., 1996; Hotchin and Hall., 1995; Nobes and Hall., 1995). Rac activation allows the formation of focal adhesion complexes and then Rho activation follows to mature the focal adhesions and stabilise the cell through

lamellipodia protrusion during cell migration (Zaidel-Bar et al., 2003; Rottner et al., 1999; Kiosses et al., 2001; Ballestrem et al., 2001).

Cell protrusion is driven by lamellipodia, filopodia and lamella, which regulate cell migration. Both the lamellipodia and filopodia are made up of F-actin bundles at the leading edge of the cell (Bailly et al., 1999; Ichetovkin et al., 2002; Abercrombie et al., 1971; Mallavarapu and Mitchison., 1999). These structures generate leading edge protrusion when F-actin filament polymerisation outweighs the retrograde flow (Mallavarapu and Mitchison., 1999). The lamella is located behind the leading edge and contain stress fibers, polarity bundles and contraction arrays of F-actin for cell motility (Hotulainen and Lappalainen., 2006).

During directional cell migration, cell polarisation is achieved through a few mechanisms including the Rho GTPases, microtubule organising center (MTOC) and golgi apparatus. The localised activation of Rho family GTPases Cdc42 and Rac at the leading edge of the cells is important for the establishment and maintenance of polarity (Ridley et al., 2003). When a wound is created, this stimulus causes the secretion of molecules such as growth factors to stimulate wound repair (Gurtner et al., 2008). This causes the reorganisation of cytoskeleton and the repositioning of the MTOC and golgi apparatus such that they orientate themselves towards the leading edge of the wound or the direction of migration (Kupfer et al., 1982). This allows the golgi apparatus to have directed secretion towards the wound edge for epidermal wound repair (Caddy et al., 2010).

Bacterial pathogens have also been known to utilise cytoskeletal dynamics to facilitate host entry, having negative implications on host processes such as cell migration and polarisation (Colonne et al., 2016). The actin cytoskeleton is controlled by the Rho signalling pathway, which is often hijacked by pathogens for their own benefit (Popoff, 2014). Stationary phase supernatants of *P. aeruginosa* have been shown to reduce migration rates through altered actin cytoskeleton and inhibiting epithelial integrity restoration (de Bentzmann et al., 2000). Lipopolysaccharide from *P. aeruginosa* has been shown to inhibit keratinocyte migration in scratch wound assays (Loryman and Mansbridge, 2008). *Pseudomonas aeruginosa* toxins such as exotoxin T (ExoT) has been shown to inhibit cell migration through their GTPase-activating protein (GAP) domain that affects Rho family GTPases involved in migration (Garrity-Ryan et al., 2000; Kazmierczak and Engel., 2002; Krall et al., 2000). ExoT was found to stimulate actin reorganisation and GTPase activity of Ras-related C3 botulinum toxin substrate (Rac1) and cell division control protein 42 homolog (Cdc42) (Krall et al., 2000). Similarly, ExoT was sufficient to stimulate the disassembly of actin stress fibers through the GAP activity of ExoT in a variety of cells, especially at the wound edges (Geiser et al., 2001; Kazmierczak and Engel., 2002; Garrity-Ryan et al., 2000; Garrity-Ryan et al., 2004). In HeLa cells, expression of ExoT leads to a global loss of GTP-bound Ras homolog family member A (RhoA), Cdc42 and Rac1 (Kazmierczak and Engel., 2002). As RhoA, Cdc42 and Rac1 are involved in cell migration, these show that *Pseudomonas aeruginosa* hijacks cell migratory pathways through the Rho GTPase family (Zaidel-Bar et al., 2003; Rottner et al., 1999; Kiosses et al., 2001; Ballestrem et al., 2001). *Pseudomonas aeruginosa* exoproducts were also shown to significantly impair directional migration in primary non-cystic fibrosis human airway epithelial cells (Ruffin et al., 2016). ExoS and Y of the type 3 secretion system (T3SS) of *P. aeruginosa*

is also capable of altering the actin cytoskeleton through its adenosine diphosphate(ADP)-ribosyltransferase activity that modifies Ras (Ganesan et al., 1998; Soong et al., 2008; Cowell et al., 2005; Baldwin and Barbieri., 2005). They also target Rac, Cdc42 and Rho through the GAP activity (Pederson et al., 1999; Goehring et al., 1999; Soong et al., 2008; Cowell et al., 2005; Baldwin and Barbieri., 2005). Apart from hampering migration, studies have also shown that bacteria are capable of promoting migration. A more recent study by Karlsson et al. (2012) demonstrated that *P. aeruginosa* N-(3-oxo-dodecanoyl)-l-homoserine lactone (3-oxo-C₁₂-HSL) at lower concentrations promote migration, while higher concentrations suppress migration in human epithelial cells. This was achieved by the binding and co-localisation of 3-oxo-C₁₂-HSL with the IQ-motif-containing GTPase-activating protein (IQGAP1) in Caco-2 cells (Karlsson et al., 2012). Their findings suggest that IQGAP1 is a possible integrator of Cdc42 and Rac1-dependent cell migration (Karlsson et al., 2012).

Seen in this light, mechanisms that support directional migration by gaining traction, inducing cell polarisation and protrusion are closely knitted and are crucial to the wound healing process. However, they are often manipulated by bacteria for their own purposes. Most of the studies that have been reported are studies on epithelial cells, probably because of the close association between *P. aeruginosa* and cystic fibrosis. Additionally, they tend to be focused on the T3SS proteases of the pathogen, despite having a wide variety of virulence factors. A greater understanding of the mechanisms behind how bacteria influence the host migratory machinery may generate therapeutic strategies to promote healing in chronic wounds.

1.3.2 Impact of *Pseudomonas aeruginosa* on cell viability

The engulfment of apoptotic neutrophils by phagocytes is needed for resolution of inflammation, as this dampens the immune response and is essential for tissue homeostasis (Ohata et al., 1994; Tidball and Pierre., 1996; Greenlee-Wacker et al., 2016). Despite the usefulness of apoptosis, a dysregulation in cell viability proved to be detrimental to the proper wound healing response (Arya et al., 2011; Bhan et al., 2013). In diabetic wounds, apoptosis is elevated throughout the healing process (Baynes and Thorpe., 1999; Cai et al., 2002; Arya et al., 2011; Bhan et al., 2013). The increase in reactive oxygen species and metabolic alternations caused by the hyperglycaemic environment leads to apoptosis (Baynes and Thorpe., 1999; Cai et al., 2002). Apart from the usual causes of chronic wounds such as underlying vascular and neurological problems, the cell viability balance was also reported to be a major cause of diabetic foot ulcers (Rai et al., 2005). Hence, due to the importance of apoptosis in proper wound resolution, cell viability is an important aspect of wound healing.

Bacterial pathogens are well-known to cause reduced cell viability in the host, through cell death pathways (Jorgensen et al., 2017; Ashida et al., 2011). Despite being an intrinsic response to microbial infection, cell death mechanisms are often manipulated by pathogens to enhance their survival and replication in the host (Lamkanfi and Dixit., 2010). Apoptosis is programmed cell death that is non-inflammatory and is morphologically characterised by DNA fragmentation, membrane blebbing, cell shrinkage and caspase activation (Strasser et al., 2000; Galluzzi et al., 2018). Due to the plethora of studies on *P. aeruginosa* and apoptosis, this is a non-exhaustive summary. During microbial infection, apoptosis occurs to clear infected host cells to prevent bacterial replication and propagation. For example, induction of apoptosis was

necessary in *Legionella pneumophila*, *P. aeruginosa*, and *Helicobacter pylori* infected cells to control the spread of infection and tissue damage (Nogueira et al., 2009; Grassme et al., 2000; Jones et al., 2002). *Pseudomonas aeruginosa* has been shown to elicit apoptosis through virulence factors such as ExoT and ExoS and necrotic responses through ExoU (Kaminski et al., 2018; Wood et al., 2015; Sato et al., 2015). This has been shown through a study by Wood et al. (2015) whereby ExoT induces a focal adhesion protein (Crk) to transform into a cytotoxin, that induces anoikis by dysregulating focal adhesion kinase localisation to focal adhesion sites and interfering with integrin signalling. Most of the T3SS components induce apoptosis through their GAP domain (Kaminski et al., 2018; Wood et al., 2015b). Apart from the T3SS proteins, quorum sensing autoinducers such as 3-oxo-C12-HSL are also capable of inducing apoptosis (Tateda et al., 2003; Li et al., 2004; Schwarzer et al., 2012; Jacobi et al., 2009). Despite these studies, Roy et al. (2013) have shown that *P. aeruginosa* also elicits a non-apoptotic toxicity towards murine macrophages, highlighting that variability in experimental setups and differences in cell types could cause differences between results.

Necrosis is cell death that is induced by reactive oxygen species or stress signals such as adenosine triphosphate (ATP) depletion upon bacterial infection (Galluzzi et al., 2018). It is characterised by cell swelling and membrane rupture (Duprez et al., 2009). Exposure of cells to *Clostridium septicum* alpha-toxin formed pores in the cell membrane, leading to clostridial myonecrosis, which constitutes necrotic cell death (Kennedy et al., 2009). Kennedy et al. (2009) also reported an activation of calpains and cathepsins, increased reactive oxygen species (ROS) production and secretion of high mobility group box 1 (HMGB1) into the extracellular space.

Pyroptosis is an inflammatory programmed cell death induced by caspase-1 activation that is mediated by the inflammasome (Davis et al., 2011; Bergsbaken et al., 2007). It is characterised by rupturing of the cell membrane, DNA fragmentation and the production of pro-inflammatory cytokines such as TNF- α and IL-1 β (Schroder and Tschopp, 2010). Infection by T3SS (e.g. ExoU) and flagella components of *P. aeruginosa* induce the activation of the inflammasome by activating caspase 1 through the Nod-like receptor IPAF (Miao et al., 2008; Franchi et al., 2007; Sutterwala et al., 2007). Pyroptosis of neutrophils is also prevalent in *P. aeruginosa* lung infections (Ryu et al., 2017). Interestingly, flagellar motility determines the magnitude of pyroptosis induced by *P. aeruginosa*, as the absence of motility reduced inflammasome activation (Patankar et al., 2013). Despite being able to eradicate pathogenic bacteria, the inflammasome has also been utilised by pathogens to circumvent cell death and inflammation (Fink and Cookson, 2007). Recently, pyroptosis caused by T3SS of *P. aeruginosa* was also shown to be protective towards the pathogen through helping the pathogen to escape pore-induced intracellular traps immunity (Eren et al., 2019). Rampant, uncontrolled pyroptosis caused by *P. aeruginosa* infections can also lead to immunopathologies such as pneumonia (Cohen and Prince., 2013; Faure et al., 2014).

Based on the literature review, *P. aeruginosa* seems to elicit cell death in the host mainly through apoptosis and pyroptosis. Due to the presence of various cell death pathways apart from apoptosis and pyroptosis, they should also be checked for their involvement in *P. aeruginosa*-related infections (Galluzzi et al., 2018). Better insights on how pathogens usurp the cell death pathways would provide basis for new therapeutic approaches to eradicate bacterial infections.

1.3.3 Connexin modulation by *Pseudomonas aeruginosa* and its toxins

Connexins (Cx) are part of the gap junction family of proteins that are required for direct cell-cell communication (Kumar and Gilula., 1996; Wei et al., 2004). They function as intercellular channels for the exchange of small molecules (less than 1 kDa) such as secondary metabolites and ions (Alexander et al., 2003). Connexins are dynamically regulated in acute and diabetic wound healing and failure to regulate Cx levels can result in perturbed healing (Becker et al., 2012; Chanson et al., 2018; Wang et al., 2007). In normal wound healing, Cx43 is downregulated at the wound edges in the first 24 h postwounding (Coutinho et al., 2003; Brandner et al., 2004; Mori et al., 2006). This decrease in Cx43 expression has been shown to facilitate wound closure and is needed for proper wound healing. Additionally, antisense Cx43 has been used in *in vitro* and *in vivo* models and results showed a decrease in inflammation, improved granulation tissue deposition, and faster re-epithelialisation with lesser scarring (Qiu et al., 2003; Mori et al., 2006). Compared to normal wounds, Sutcliffe et al. (2015) reported the upregulation of Cx26, 30 and 43 in hyperthickened epidermis in the 3 major categories of chronic wounds, namely venous leg ulcers, pressure ulcers and diabetic foot ulcers. Wang et al. (2007) also showed the negative involvement of Cx43 in diabetic wound healing where Cx43 misexpression was rescued by Cx43 antisense, and healing was returned to normal. These studies show that misexpression of Cx is implicated in the failure of chronic wounds to heal.

Connexin modulation by bacterial pathogens and their toxins is dynamic, as both the downregulation and upregulation of connexin expression has been observed in a plethora of disease conditions (Ceelen et al., 2011; Castellano and Eugenin., 2014; Eugenin., 2014). Not only is the modulation dynamic, the regulation of connexins by

bacterial pathogens is highly complex. Studies correlating *P. aeruginosa* and gap junction communication are not that well established compared to other pathogens such as *H. pylori* and *S. aureus* (Ceelen et al., 2011; Castellano and Eugenin., 2014; Eugenin., 2014). In the context of the respiratory system, *P. aeruginosa* lipopolysaccharide (LPS) has been shown to downregulate Cx40 and Cx43 expression in both *in vitro* and *in vivo* settings (Yeh et al., 2005; Rignault et al., 2007). A lack of Cx40 in endothelial cells also caused an enhanced recruitment of neutrophils to the airways after intratracheal instillation of *P. aeruginosa* LPS (Chadjichristos et al., 2010). The authors suggested that Cx40-dependent regulation of cluster of differentiation 73 (CD73), an enzyme that hydrolyses extracellular adenine into adenosine, modulates leukocyte transmigration across the alveolar endothelium (Chadjichristos et al., 2010). In contrast, the same opportunistic pathogens have been shown to use or increase the intercellular communication to propagate infection in the host. In the respiratory system context as well, Sargedine et al. (2009) showed that intratracheal instillation of *P. aeruginosa* LPS resulted in upregulation of alveolar Cx43 to promote neutrophil recruitment to the airspace in the lungs. Losa et al. (2014) reported that induction of apoptosis by *P. aeruginosa* LPS in airway epithelial cells is caused by upregulated Cx43 expression through downregulating the c-Jun N-terminal kinase (JNK) pathway. Moreover, TLR2 signalling activated by *P. aeruginosa* triggered pro-inflammatory intracellular calcium ion waves that are transmitted and amplified through Cx43 channels (Martin and Prince., 2008). The study also showed that *P. aeruginosa* induced the tyrosine phosphorylation of Cx43 and its association with proto-oncogene tyrosine-protein kinase Src (c-Src), leading to a decrease in gap junction communication as an autoregulatory control (Martin and Prince., 2008). These studies show that given the same pathogen, both an up and downregulation of Cx expression can be observed under different circumstances.

These also show that various connexins can be regulated differentially in these systems as well. Collectively, these studies reinforce the multifaceted functions of gap junctions and emphasises the influence that pathogens can exert on gap junction intercellular communication.

1.3.4 Modulation of cell adhesion by *Pseudomonas aeruginosa*

Of all the focal adhesion molecules, integrins are the most important in regulating wound healing. Integrins are necessary for re-epithelialisation to occur as keratinocytes at the wound edge require integrins for epidermal migration into the wound bed (D'Alessio and Blasi, 2009). They are also important for providing the migration directionality to leading-edge keratinocytes (Choma et al., 2004). Cadherins are also important in re-epithelialisation. Adherens junctions are needed for cell-cell adhesion along the leading edge, but a downregulation of E-cadherin is also observed in migrating keratinocytes, resulting in the dissolution of these junctions so that wound repair can occur (Crawford and Dagnino, 2018). E-cadherin is also involved in Rac1 activation at the cell front, while P-cadherin is involved in Cdc42 activation at the rear in collective cell migration in re-epithelialisation (Cai et al., 2014; Plutoni et al., 2016). Mendoza-Naranjo et al. (2012) also showed that N-cadherin is upregulated in chronic wounds.

In the final stage of wound healing, granulation tissue formation, integrins also play a role in ECM deposition and removal of the fibrin provisional matrix (Eslami et al., 2009; Koivisto et al., 2012; Curnis et al., 2012). They are also important for the differentiation of fibroblasts to myofibroblasts through the activation of focal adhesion kinase (FAK) (Hinz, 2007; Thannickal et al., 2003). Integrins also play a role in angiogenesis, where they support endothelial and pericyte migration (Senger et al., 2011). They also act as

co-receptors for growth factor receptors and help in the assembly of the vascular basement membrane (Senger et al., 2011).

Tight junctions provide a barrier that protects the deeper tissues from pathogens and is the first line of defence (Bonazzi and Cossart., 2011). However, a plethora of bacterial pathogens have developed mechanisms to internalise into the host by targeting the host cell adhesion molecules, for colonisation, invasion and disruption of host barriers (Lu et al., 2014; Guttman and Finlay., 2009). This makes cell adhesion molecules one of the most exploited to gain access for bacterial host colonisation and infection (Lu et al., 2014; Guttman and Finlay., 2009; Golovkine et al., 2018). Due to the diverse range and numerous studies on *P. aeruginosa* and junctional breach, this is a non-exhaustive summary.

Focal adhesions appear to be the one of the leading targets by which bacteria bind to and contact the host (Pizarro-Cerdá and Cossart., 2006). Some pathogens directly contact focal adhesions via bacterial adhesins, while others contact indirectly with the help of extracellular matrix proteins (Foster and Hook, 1998; Patti et al., 1994). However, in terms of *P. aeruginosa*, the literature is not as well established as that of other pathogens. Recent studies have revealed that the type IV pilus-associated protein (PilY1) binds integrin, probably to mediate host epithelial binding (Johnson et al., 2011). Moreover, in a variety of respiratory cells, fibronectin, vitronectin, $\alpha 5\beta 1$ and $\alpha v\beta 5$ integrins have been shown to facilitate bacterial adherence and mediate *P. aeruginosa* entry into the host (Leroy-Dudal et al., 2004; Roger et al., 1999). In a recent study, $\beta 1$ -integrin was found to accumulate on the membrane of upper-airway epithelial cells in

cystic fibrosis mice and patients, which promotes bacterial infection such as *P. aeruginosa* (Grassmé et al., 2017).

Tight junctions are located at the most apical surface of the epithelium and are widely used by pathogens for propagating infections (Hartsock and Nelson., 2008; Zihni et al., 2016; Golovkine et al., 2018). They act as intercellular barriers between epithelial cells that separate tissue spaces (Schneeberger and Lynch., 2004; Yamazaki et al., 2008). They also regulate the diffusion of ions and solutes between cells (Zihni et al., 2016; Golovkine et al., 2018). Adherens junctions are linked to the actin cytoskeleton through vinculins and catenins, and are involved in the formation and maintenance of cell junctions and induction of migration (Golovkine et al., 2018; Hartsock and Nelson., 2008). Pathogens have been reported to indirectly or directly target them for adherence to the host, to facilitate internalisation or to gain access to the deeper tissues through disrupting the integrity of the epithelium (Wu et al., 2003; Bonazzi et al., 2009). Currently, most of the *P. aeruginosa* virulence factors affecting tight and adherens junctions are either secretion system or quorum sensing related. For example, the GAP activities of the T3SS proteins ExoS, T and Y have been shown to alter zona occludens 1(ZO-1) and occludin localisation in human airway epithelial cells (Soong et al., 2008). LasB from the T2SS has been shown to decrease tight junction levels such as claudin 5 and the proteolysis of VE-cadherin and occludin (Beaufort et al., 2013). In human nasal epithelial cells, *P. aeruginosa* elastase also downregulated claudin 1 and 4, occludin and tricellulin, mediated by signalling pathways such as JNK and NF- κ B (Nomura et al., 2014). Vikström et al. (2009) reported that the quorum sensing autoinducer 3-Oxo-C₁₂-HSL leads to tyrosine hyperphosphorylation of occludin, ZO-1, E-cadherin and β -catenin, causing disassembly in junction complexes and increased paracellular

permeability. The pore-forming toxin exolysin (ExlA) of *P. aeruginosa* trigger E-cadherin and VE-cadherin cleavage in epithelial and endothelial cells respectively by promoting calcium influx and metalloprotease disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) activation (Reboud et al., 2017). Apart from downregulating and cleaving junctional proteins, the elastase of *P. aeruginosa* did not alter the expression of adherens junctions β -catenin and E-cadherin, and tight junctions ZO-1 and 2 in human nasal epithelial cells (Nomura et al., 2014). These studies highlight the complexity of *P. aeruginosa* in regulating junctional expression and proteins to propagate infections in the host.

Although focal adhesions, tight junctions and adherens junctions are targets of *P. aeruginosa*, so far there are no reports of the negative effects on desmosomes. Collectively, these studies show that pathogen-host cell-cell attachment and modulation are crucial in propagating infectious diseases. These studies also reiterate that the secretion and quorum sensing systems of *P. aeruginosa* enhance the invasiveness of the pathogen by breaking junctional barriers. Further studies on how focal adhesions are affected by *P. aeruginosa* are required as they are currently in their infancy. It is also currently unclear as to how secreted factors of *P. aeruginosa* biofilms play a role in junction breach, so more research is warranted to decipher mechanisms that are involved.

1.3.5 *Pseudomonas aeruginosa* and the immune system

Host inflammatory responses during an infection are usually caused by pathogenic invasion of microbes such as bacteria, with the concomitant influx of immune cells such as neutrophils, monocytes and macrophages (Zhao et al., 2013; Wolcott et al., 2008; Zhao et al., 2016b). Inflammatory responses are induced when toll-like receptors (TLRs)

and pattern recognition receptors (PRRs) recognise DAMPs, PAMPs, lipopolysaccharide and peptidoglycan (Lemaitre et al., 1996; Medzhitov and Janeway., 2002; Medzhitov et al., 1997; Ruco et al., 1978). Similar to other infectious pathogens, *P. aeruginosa* is able to elicit inflammatory responses through the bacterium itself, lipopolysaccharide (LPS) and even its supernatant. Roy et al. (2013) have shown that *P. aeruginosa* and its supernatant are able to induce inflammatory responses by causing a nuclear translocation of p65 and an upregulation of pro-inflammatory cytokines such as TNF- α and IL-6. A recent study has also described the ability of *P. aeruginosa* biofilms to induce phagocyte hyperinflammation *in vitro* (Ciszek-Lenda et al., 2019).

Pathogens have evolved to develop strategies to evade, inhibit or manipulate the immune response, so as to potentiate infection in the host (Reddick and Alto., 2014). Some strategies employed include the evasion of detection by antimicrobial peptides and modification of PAMPs, manipulation of the host secretome and lastly by targeting intracellular signalling pathways like MAPK and NF- κ B (Reddick and Alto., 2014). The immunomodulatory and evasive mechanisms of *P. aeruginosa* have been well established and reported in various reviews (Alhede et al., 2014; Cohen et al., 2015; Cigana et al., 2011). As such, herein is a non-exhaustive summary of the literature. Rhamnolipids of *P. aeruginosa* biofilms were shown to protect the quorum sensing bacterium by causing necrosis in polymorphonuclear leukocytes (Jensen et al., 2007). In the same study, supernatants of planktonic cultures induced hemolysis and lysis of these leukocytes (Jensen et al., 2007). This was further supported by a study by van Gennip et al. (2009) showing that *rhlA* mutants were deficient in rhamnolipid production, thereby removing protection against polymorphonuclear leukocytes. *Pseudomonas aeruginosa* evades complement activation by the immune system by binding plasma

regulators Factor H and Factor H-related protein-1 to the bacterial surface (Kunert et al., 2007). The authors reported that Factor H induces degradation of complement C3b to possibly facilitate tissue invasion of the pathogen (Kunert et al., 2007). The polysaccharide Psl found in biofilm matrices of *P. aeruginosa* is also involved in immune evasion by protecting mucoid strains from opsonisation and complement activation in human serum (Jones and Wozniak., 2017). This helps in the establishment of chronic pulmonary infections in mucoid *P. aeruginosa* strains (Jones and Wozniak., 2017). Although virulence factors help in immune evasion of this opportunistic pathogen, the loss of *P. aeruginosa* swimming motility (independent of TLR signalling and flagellar expression) has been reported to help evade phagocytosis (Amiel et al., 2010). Contrastingly, other reports showed that the loss of flagellum decreased non-opsonic phagocytosis, and that the flagellum is the main determinant of immune evasion in *P. aeruginosa* (Mahenthiralingam et al., 1994; Mahenthiralingam and Speert., 1995; Balloy et al., 2007). Apart from the usual T3SS virulence factors that are highly involved in pathogenesis of *P. aeruginosa*, a T1SS effector protein, 4-hydroxy-2-oxovalerate aldolase 2 (TesG), was recently identified to suppress neutrophil influx, dampen cytokine and chemokine secretions and also reduce macrophage viability through cytoskeletal rearrangements (Zhao et al., 2019). The study reported that RhoA mediates *P. aeruginosa*-induced inflammation and TesG can inhibit RhoGEF-induced activation of RhoGTPases to manipulate the host immune response (Zhao et al., 2019). A toll/interleukin 1 receptor (TIR) domain-containing protein (PumA) of *P. aeruginosa* inhibits NF- κ B pathway by interacting with ubiquitin-associated protein 1 (UBAP1) and the TLR adaptors Toll/Interleukin-1 receptor domain-containing adapter protein (TIRAP) and myeloid differentiation primary response 88 (MyD88). This results in

dampened cytokine and TLR receptor signalling, which is another way of immune evasion by the pathogen (Imbert et al., 2017).

1.3.6 Impact of bacteria on keratinocyte differentiation

The elucidation of the effects of bacteria on keratinocyte differentiation is not well explored, and currently there are no studies on *P. aeruginosa*. The acne-causing bacterium, *Propionibacterium acnes* induced filaggrin expression on differentiated keratinocytes and in the suprabasal epidermis of explants via cell wall and cytosolic proteins (Jarrousse et al., 2007). The authors suggested that *P. acnes* probably modulates the terminal phase of keratinocyte differentiation (Jarrousse et al., 2007). Ryoko et al. (2009) reported that phenols from gut bacteria adversely affect keratinocyte differentiation by a failure of K10 expression in females. The extracellular adherence protein of *S. aureus* promoted bacterial internalisation and heightened expression of keratinocyte differentiation markers K10 and involucrin, as a possible mechanism to hide from the host during skin infections (Bur et al., 2013).

Reports showing the indirect effects of bacteria on keratinocyte differentiation were also conducted, through the detection of β -defensins, a family of antimicrobial peptides usually expressed in epithelial cells (Zhao et al., 1996; Valore et al., 1998). Frye et al. (2001) reported that human- β -defensin-1 promotes keratinocyte differentiation, while Liu et al. (2002) stated that differentiation is able to regulate human- β -defensin-2 production as well. Due to the connection between β -defensins and the immune response, studies to show the effect of pathogenic bacteria on β -defensins are aplenty. Some have reported that exposure of undifferentiated human keratinocytes in culture to non-toxic small molecules of *S. epidermidis* conditioned media that were <10 kDa

increased human β -defensin-2 and 3 mRNA production (Lai et al., 2010). Taken together, bacteria are able to indirectly enhance keratinocyte differentiation through β -defensins. The effects of bacteria on keratinocyte differentiation is not well researched, studies should be conducted as the differentiation process is crucial for maintenance of skin integrity and proper re-epithelialisation to occur.

1.4 Virulence factors of *Pseudomonas aeruginosa*

1.4.1 Protein-based virulence factors

The secretory systems of *P. aeruginosa* are one of the main sources of virulence for the pathogen, and they mainly produce proteinaceous virulence factors. Gram-negative bacteria have 6 types of secretion systems characterised and *P. aeruginosa* possess 5 out of the 6 (Economou et al., 2006).

The type I secretion system (T1SS) secretes alkaline proteases AprA and AprX (unknown function) (Guzzo et al., 1991; Duong et al., 2001). AprA degrades host complement proteins, fibronectin and interferes with flagellin signalling by degrading free flagellin monomers, culminating in immune evasion (Laarman et al., 2012; Bardoel et al., 2011; Matsumoto, 2004). Another secreted protein of the T1SS, haem acquisition protein HasA (HasAp), binds haem from haemoglobin and is involved in haem internalisation and thus promoting pathogen survivability in early infection stages (Wandersman and Delepelaire, 2004).

The T2SS encodes multiple hydrolysing proteins that are secreted from the periplasm to the extracellular environment (Jyot et al., 2011). Elastases such as staphylolysin (LasA) and elastase (LasB) are involved in the degradation of host proteins and inhibition of

macrophage activity to prevent clearance of the pathogen by the host (Bastaert et al., 2018; Kuang et al., 2011; Mun et al., 2009). LasA is a serine protease for peptidoglycan stabilisation in *Staphylococcus* cell wall and enhances the proteolytic activity of LasB (Toder et al., 1994; Matsumoto., 2004). On the other hand, LasB and protease IV degrade immunoregulatory proteins such as opsonising lung surfactant proteins A and D, thus conferring protection to the pathogen (Toder et al., 1994; Matsumoto., 2004; Mariencheck et al., 2003; Engel et al., 1998; Malloy et al., 2005). Phospholipase H and other lipolytic enzymes help in digesting host surfactant lipids and phospholipids, to increase surface tension (Kipnis et al., 2006; Holm et al., 1991; Gellatly and Hancock., 2013). Finally, ExoA elicits apoptotic cell death by negatively affecting protein synthesis through inhibiting elongation factor 2 (EF2) (Schultz et al., 2000; Wolf and Elsasser-Beile., 2009; Du et al., 2010; Gellatly and Hancock., 2013; Chatterjee et al., 2016; Driscoll et al., 2007).

The T3SS is one of the major virulence strategies of *P. aeruginosa* and its expression is usually associated with infections and increased mortality (Sadikot et al., 2005; Hauser, 2009). This system directly injects toxins (such as high concentrations of cyclic adenosine monophosphate) into host cells and the needle-like appendage promotes pathogenic invasion and colonisation by the translocation of bacterial effector proteins to the host cells through epithelial breaches such as pore formations (Costa et al., 2015). There are 4 effector proteins of the T3SS, namely ExoS, ExoT, ExoY and ExoU, which are responsible for eliciting cell death, cytoskeletal dysregulation, DNA synthesis inhibition, vesicular trafficking inhibition, and delayed wound closure in the host (Hauser., 2009). The disruption of epithelial barriers caused by these effectors aid the penetration of *P. aeruginosa* into the host (Kipnis et al., 2006; Hauser., 2009).

Widely represented in gram-negative bacteria, the T5SS allows for secretion of large proteins for microbial adhesion and virulence through the bacterial envelope (Bleves et al., 2010). The T5SS also helps to eradicate other bacteria by a contact-dependent growth inhibition (CDI) mechanism (da Mata Madeira et al., 2016). Being the only characterised *P. aeruginosa* autotransporter (T5aSS), esterase EstA (EstA) is involved in rhamnolipid production and its mutants were found to be deficient in motility and biofilm formation (Wilhelm et al., 1999; Wilhelm et al., 2007). In the T5bSS system, the large extracellular protease (LepA) activates the NF- κ B pathway through cleavage of protease-activated receptors (PAR) receptors in clinical isolates and not PAO1 (Kida et al., 2008). The other secreted protein, the TpsA6/adhesive protein CupB5 (CupB5) protein is a haemagglutinin-like protein interwoven with the CupB fimbriae (Ruer et al., 2008).

T6SS is the most recently discovered secretion system in *P. aeruginosa* and its secreted factor, haem carrier protein (Hcp1), can be detected in the sputum of cystic fibrosis patients (Mougous et al., 2006; Mougous et al., 2007; Hsu et al., 2009). More research should be pursued in this secretion system for deeper understanding on how it contributes to the pathogenesis of *P. aeruginosa*.

1.4.2 Lipid-based virulence factors

The most well-known lipid-based virulence factor of *P. aeruginosa* is LPS, which is a glycolipid responsible for anchoring LPS to the outer membrane of the bacterium (King et al., 2009). Overall, it is responsible for inflammation, functional and structural integrity of biofilms, and antigenicity (King et al., 2009; Lau et al., 2009; Wozniak et

al., 2003). Binding of the lipid A component of LPS to TLR-4 and CD14 causes the activation of inflammatory responses such as the NF- κ B pathway, causing the secretion of pro-inflammatory cytokines and chemokines such as TNF- α and IL-6 (Teghanemt et al., 2005; Akira et al., 2006; Pier., 2007). Lipid A can be modified to alter the susceptibility of the pathogen to antimicrobials and change its inflammatory properties. A fully hexa-acylated lipid A elicits a heightened inflammatory response (Pier., 2007). Alterations of lipid A binding and aminoarabinose addition contribute to resistance against antimicrobials, and these changes are regulated and can be induced as a response to environmental changes (Ernst et al., 1999). Wild type *P. aeruginosa* strains have lipid A attached to an oligosaccharide core which can be substituted by O-antigen, which is responsible for conferring antigenicity (King et al., 2009). In many of the chronic isolates of *P. aeruginosa*, the B-band is not expressed, and some strains do not produce the O-antigen or substitute the lipid A complex with only one O-antigen (Hancock et al., 1983). This may be a result of selective pressure for the bacteria to immune evade (King et al., 2009).

1.4.3 Small molecule virulence factors

Apart from the broad building block categories like proteins and lipids, small molecules are also important virulence factors of *P. aeruginosa*. Autoinducers, which are small membrane-diffusible signalling molecules, are involved in quorum sensing, a mechanism used by many bacteria to sense and respond to varied external factors such as bacterial population density, metabolic factors and selective expression of genes (Deep et al., 2011; Favre-Bonté et al., 2007). These molecules are constantly produced by the pathogen and become cofactors of specific transcriptional regulators when a certain threshold is reached (quorum), causing activation of specific downstream genes

and regulation of a wide arsenal of pathogenic genes such as pyocyanin, elastases and alginate (Keren et al., 2013; Deep et al., 2011).

The opportunistic pathogen *P. aeruginosa* secretes 3 autoinducers, 2 of which are acyl homoserine lactones (AHL), while the other is called the *Pseudomonas* quinolone signal (PQS or 2-heptyl-3-hydroxy-4-quinolone) (Deep et al., 2011; Rybtke et al., 2015). 3-Oxo-C₁₂-HSL is encoded for by the Las system and induces inflammation through production of IL-8, cyclooxygenase-2 (COX2) and prostaglandin E2 in human lung fibroblasts and epithelial cells (Smith et al., 2002). The Las system regulates the production of a plethora of virulence factors including alkaline protease, exotoxin A and LasA and Las B elastases (Passador et al., 1993; Toder et al., 1991; Jones et al., 1993; Gambello et al., 1993). Despite being under the acyl homoserine lactone family, N-butanoyl homoserine lactone (C₄-HSL) is encoded for by the Rhl system and is involved in the production of rhamnolipids and repressing the T3SS (Bleves et al., 2005). Unlike AHLs, the quinolone signal, 2-heptyl-3-hydroxy-4-quinolone, is encoded for by the quinolone-based system (Deep et al., 2011; Heeb et al., 2011). When quorum is reached, PQS and pyocyanin production are induced (Diggle et al., 2003; Deziel et al., 2005; Cao et al., 2001). Due to the involvement of these autoinducer molecules in quorum sensing, inhibition has been touted as a potential therapeutic target for not only *P. aeruginosa*-related infections, but also biofilm infections in general (Favre-Bonté et al., 2007; Moghaddam et al., 2014).

Apart from autoinducers, *P. aeruginosa* also produces pigmented metabolites. Pyocyanin is one of the pigmented signals and it responsible for giving *P. aeruginosa* colonies their distinct colour (Lau et al., 2004). This blue-green pigment is responsible

for dysfunctional cilia and for eliciting host inflammation and oxidative stress, while protecting the pathogen from ROS destruction (Lau et al., 2004; Denning et al., 1998). Studies have shown that purified pyocyanin can induce apoptosis in neutrophils and inhibit macrophage phagocytosis *in vitro* (Lau et al., 2004; Bianchi et al., 2008). Together with rhamnolipids, the production of this pigment is partially regulated by oxidative stress and hence seems to protect the pathogen from ROS produced by phagocytes during infection (Lau et al., 2005; Vinckx et al., 2010).

Apart from pyocyanin, pyoverdine, another coloured pigment, is a siderophore which helps to sequester iron from the host (Jimenez et al., 2012). When iron-bound, this signalling molecule interacts with the cell receptor ferric pyoverdine outer membrane receptor (FpvA), upregulating exotoxin A, endoprotease and even pyoverdine itself as a positive feedback mechanism (Jimenez et al., 2012). These signals are linked to iron chelation, which is vital for the establishment of chronic infections and this process occurs when there is a lack of iron in the environment for the pathogen's metabolism (Chatterjee et al., 2016; Jimenez et al., 2012).

1.4.4 Miscellaneous virulence factors

Compared to the planktonic brethren, biofilms are organised, structured communities of bacteria encased in a biofilm matrix that is usually limited in oxygen and nutrients (Bjarnsholt et al., 2010; Mah & O'Toole, 2001). The biofilm matrix comprises of EPS, lipids, proteins and extracellular DNA (eDNA) that help to hold the bacterial cells together (Billings et al., 2013; Colvin et al., 2012; Wei and Ma., 2013; Coulon et al., 2010). This matrix protects the pathogen from external forces such as antibiotics and antimicrobials which attempt to usurp biofilms and colonise when desired, thereby

driving antibiotic resistance (Bjarnsholt et al., 2010; Mah & O'Toole, 2001, Billings et al., 2013; Colvin et al., 2012; Wei and Ma., 2013; Yang et al., 2011).

EPS are involved in protecting bacteria from antibiotics, antimicrobials and host immune responses; providing support for biofilm formation, and for the initial attachment and adhesion to surfaces for growth (Billings et al., 2013; Colvin et al., 2012; Colvin et al., 2011). There are at least 3 polysaccharides produced for the EPS that contribute to *P. aeruginosa* biofilm formation such as alginate, polysaccharide synthesis locus (Psl) polysaccharide and pellicle (Pel) polysaccharide. Apart from biofilm formation, these polysaccharides have roles in host immune protection, adverse environmental protection, bacterial and host attachment and structural support in conjunction with eDNA (Wei and Ma., 2013; Simpson et al., 1988; Pier et al., 2001; Colvin et al., 2012; Colvin et al., 2011; Franklin et al., 2011; Jennings et al., 2015; Ryder et al., 2007; Mishra et al., 2012).

Extracellular DNA functions as an intercellular connector, is capable of inducing biofilm formation, protecting *P. aeruginosa* from antimicrobials and stabilising LPS (Watanabe et al., 1998; Whitchurch et al., 2002; Mulcahy et al., 2008). Together with interactions with EPS, eDNA enhances bacterial adhesion and surface aggregation, thereby forming microcolonies (Das et al., 2010; Klein et al., 2010).

Additionally, the motility of *P. aeruginosa* helps it to spread its area of colonisation with ease and is also one of the reasons why it is usually found in the deeper areas of the tissues and near blood vessels (Høiby et al., 2011; Schaber et al., 2007; Soave et al., 1978; Plotkowski et al., 1994). This pathogen possesses a single polar flagellum that is

important for swimming motility, inflammation and host surface interactions (Köhler et al., 2000; Miao et al., 2007; Bucior et al., 2012). The type IV pili is one of the most important adhesins of *P. aeruginosa* and hence important for adherence to surfaces and the host, biofilm formation and immunoevasion (Driscoll et al., 2007; Gellatly and Hancock., 2013; Sriramulu et al., 2005). Apart from these functions, the type IV pili is important for twitching motility swarming motility (together with flagellum) (Köhler et al., 2000; Yeung et al., 2009). It also drives microcolony formation of *P. aeruginosa*, such as those observed in chronic cystic fibrosis patients, which results in host immune and antibiotics protection (Craig et al., 2004; Bjarnsholt et al., 2009; Sriramulu et al., 2005). In conclusion, this shows the wealth of pathogenic mechanisms that *P. aeruginosa* possess and given their contributions to virulence, the pathogen has indeed evolved elegant strategies for survival.

1.5 In vivo animal models

In vivo animal models of biofilm infections are performed in either normal or diabetic animals, with pathogenic material such as the inoculation of bacterial cultures or transplanted biofilms, which can be monomicrobial or polymicrobial (Dai et al., 2011; Sami et al., 2019). These models have been widely used to study biofilm pathogenicity, therapeutic effects of new drugs in resolving infections, and acute and chronic wound healing (Dai et al., 2011; Sami et al., 2019). Herein, the different types of wounding models that have been conducted with bacterial/biofilm inoculations are summarised as shown in **Table 2**.

Various conditions are in play when optimising infection models, as maintenance of the biofilm infection is challenging. Studies have either used pre-formed *in vitro* biofilms

that are transplanted onto the wounds of the animals, while others have used wound dressing materials (e.g. Opsite dressing, Tegaderm, gauze patches, cotton bandages) to maintain a moist environment after inoculation of planktonic bacterial cultures (Brackman and Coenye., 2016). It has also been argued that the planktonic bacterial culture inoculation method reflects the initial colonisation phase of chronic wounds, as the wound bed becomes responsible for transformation of the planktonic bacteria to the biofilm state (Schultz et al., 2004; Cierny and DiPasquale., 2006). Moreover, the length of infection has to be considered, whereby only a few models study the infection for longer periods of time (e.g. 14, 21 days, 60 days) (Thompson et al., 2014; Zhao et al., 2012; Roy et al., 2014). Currently, there are no *in vivo* models that have used bacterial exudates on animals.

Table 2: *In vivo* animal models of biofilm infection

Model Name	Description	References
Full thickness Excisional Wound Model	<p>Wounds are created through a plethora of methods in terms of the excision sizes and tools such as using biopsy punches, scissors or burn injury to remove superficial keratinocytes (Sami et al., 2019; Kopecki et al., 2017). In rodents and pigs, the wounds are created on the dorsal side of the animal, while rabbits have wounds created on the ventral side of the ears prior to bacterial inoculation (Singer and McClain., 2003; Dunn et al., 2013; Qian et al., 2017). This is the most common type of wound model for studying wound healing and multiple wounds can be created on the same animal (Sami et al., 2019). The splinted full-thickness excisional wound model is also commonly used to prevent wound contraction in rodents, to stimulate wound closure that is similar to that of humans (i.e. re-epithelialisation) (Wang et al., 2013b). The splint is placed through the panniculus carnosus on a full-thickness wound (Wang et al., 2013b).</p> <p>Advantages: The splinted full-thickness excisional wound model minimises contraction, which makes it more replicative of that observed in humans (Grada et al., 2018).</p> <p>Disadvantages: There is some difficulty in preserving the integrity and location of the splinting materials in the splinted full-thickness excisional wound model (Grada et al., 2018). This may cause detachment of the materials and relocation into the wound sites, affecting wound closure (Grada et al., 2018).</p>	Singer and McClain (2003), Dunn et al. (2013), Qian et al., (2017)

Murine Incisional Wound Model	<p>This model is the second most common wound models being used (Sami et al., 2019). In this model, a longitudinal incisional wound is created parallel to the midline on the dorsal side of the rodent (Ziv-Polat et al., 2010). This wound cuts through the epidermis, dermis and subcutaneous tissue till the muscle (Ziv-Polat et al., 2010; Park et al., 2014).</p> <p>Advantages: This model is suitable for studying biomechanics of wound and epidermal migration with high reproducibility (Seaton et al., 2015).</p> <p>Disadvantages: Difficult to evaluate healing outcome in incisional wound models as there is lesser scars/wound tissue for analysis (Grada et al., 2018).</p>	Akiyama et al. (2002)
Subcutaneous ulcer/air sac model	<p>Bacterial cultures are inoculated via subcutaneous injection into the animal, resulting in the formation of an ulcer under the skin (Kopecki et al., 2017). In the air sac model, a subcutaneous air sac is created prior to infection and bacteria is injected into the air sac (Watson et al., 2016).</p> <p>Advantages: Due to the variability in the ulcer's border and location, it makes it more clinically relevant (Watson et al., 2016). Generally, this model is simple, cheap and high-throughput (Kopecki et al., 2017).</p> <p>Disadvantages: Data obtained is difficult to interpret as the ulcer's border and location is variable depending on the injection site and movement of the liquid that was injected (Watson et al., 2016).</p>	Kopecki et al. (2017), Watson et al. (2016)
Diabetic wound model	<p>Diabetic wound models are popular to mimic chronic wound conditions such as diabetic foot ulcers (Sami et al., 2019; Michaels et al., 2007; Chatzigeorgiou et al., 2009). Diabetes is induced in animals through 2 different ways. Firstly, type I diabetes is induced through the injection of reagents (e.g. Streptozotocin (STZ), Alloxan) that destroy the beta cells of the islets of langerhans in the pancreas, thereby destroying insulin-producing cells (Michaels et al., 2007). Secondly, type II diabetes is induced through high fat diet (Wang and Liao., 2012). Apart from chemical/diet-induced diabetes, usage of transgenic mice such as the db/db, Akita and NONcNZ010 mice have been used to model chronic diabetic wound healing (Michaels et al., 2007). Full-thickness excisional wounds are then created on the animals.</p> <p>Advantages: This model is sufficient to study acute wound healing in diabetic animals (Sami et al., 2019). It is clinically relevant to diabetic ulcers and enables testing of multiple conditions/agents on a single animal (Grada et al., 2018).</p> <p>Disadvantages: Sami et al. (2019) believe that this model is unsuitable for studying chronic wound healing due to the different wound etiologies as long-term effects of diabetes have not be fully developed before usage of the model. Other disadvantages include the high mortality rate of the animals upon diabetes induction through chemicals, the high breeding cost (for transgenics) and variability in protocols, making comparisons of</p>	Zhao et al. (2010), Watters et al. (2013), Chen et al. (2018)

	data difficult (Nunan et al., 2014). It is also physically impossible to replicate the diabetic pathologies and variabilities (Grada et al., 2018).	
Ischemic wound model	<p>The ischemic wound model has different setups in different animals and is usually clinically relevant to ischemic ulcers (Nunan et al., 2014). In the rodent, this model involves the detachment of a skin flap on the back of the animal from the deep tissues up to its base and subsequently sutured back, with a silicone sheet under the flap (Trujillo et al., 2015; Stadler et al., 2004; Mcfarlane et al., 1965). In rabbits (known as the rabbit ear ulcer model), 2 out of the 3 main arteries at the base of the ear are sutured to disrupt blood circulation (Ahn and Mustoe., 1990). Subsequently, ulcers are created through biopsy punches to the cartilage to create a full-thickness wound lacking in vascular supply (Ahn and Mustoe., 1990). In pigs, biopsy punches were used to create full-thickness excisional wounds in the middle of the flap (Patil et al., 2017).</p> <p>Advantages: In general, this model is useful to study ischemic wound healing resulting from diabetes or pressure ulcers (Roy et al., 2009). This model is also useful for pharmacological testing (Grada et al., 2018).</p> <p>Disadvantages: Depending on the choice of animal for modelling, it can be expensive (in pigs) and lacking in replication of hypoxic wounds in humans (Grada et al., 2018). This model is also technically challenging (Seaton et al., 2015).</p>	Roy et al. (2009), Patil et al. (2017)
Tape stripping model (Partial thickness wound model)	<p>This model is one of the partial-thickness wound model that involves the removal of the stratum corneum using an adhesive bandage to investigate skin barrier function and re-epithelialisation process (Kugelberg et al., 2005). Due to the removal of the stratum corneum, the permeability of the skin is temporarily compromised, and trans-epidermal water loss is measured (Kugelberg et al., 2005).</p> <p>Advantages: This model is useful for studies which are more targeted at barrier function and skin penetration as the usage of biopsy samples in such studies could have a mixture of epidermal and dermal structures, causing a complication in result analysis (Olesen et al., 2019). Moreover, tape stripping is minimally invasive and allows for secondary/follow-up studies on the same area as the skin is still intact (Olesen et al., 2019).</p> <p>Disadvantages: Some disadvantages of this model include inconsistencies in model establishment due to direction, speed and pressure of tape application and removal, number of tapes used and adhesiveness of the tape (Wilhelm et al., 2017). Moreover, this method is only applicable for studies that are targeting the skin barrier and is unable to penetrate deeper levels of the skin (Olesen et al., 2019).</p>	Kugelberg et al. (2005)

The murine *in vivo* full thickness excisional wound model was chosen for this study due to its reproducibility and usage by other studies for easy comparisons (Zhao et al., 2010; Zhao et al., 2012). This model also allows for the easy and localised application of BCM on wounds on the back of mice compared to invasive methods such as the subcutaneous ulcer model where an injection has to be performed. The use of biopsy punches to create the wounds also makes the wound size reproducible. The nature in which the wounds heal in mice is different from humans. The skin of rodents adheres loosely, they have a thin epidermis and dense hair (Dorsett-Martin., 2004; Ito and Cotsarelis., 2008; Cusumano et al., 2014). Compared to humans, they also have an endogenous source of vitamin C that is important for healing (Moores., 2013; Wong et al., 2011; Dorsett-Martin., 2004). Rodents also heal through wound contraction as well as re-epithelialisation and granulation tissue formation (Seaton et al., 2015; Schierle et al., 2009). Despite these differences, they are widely used due to their availability, low cost and small sizes compared to pigs, (which model human skin the closest) (Trøstrup et al., 2016). They also have better genetic tractability compared to pigs and rabbits (Kopecki et al., 2017). Similar to that of the immortalised cell usage, experimental limitations have to be considered and conclusions drawn may not be directly applicable to humans.

1.6 Aims and objectives

Despite the success of debridement in the clinical setting, recent research demonstrated that debridement is insufficient as a standalone method for biofilm removal (Gordon et al., 2012). For example, Kim et al. (2018) reported that the pre-debridement and post-debridement colony forming unit (CFU) counts were similar. Another study showed that a residual amount of 10^2 CFU was detected after ultrasonic debridement (Hiebert and Robson., 2016). Moreover, debridement is limited by the amount of pain inflicted on

the patient and is also a double-edged sword, where it stimulates the remnants to reform into mature biofilms as fast as 15 mins, with firmly attached aggregates formed in a day. Due to the limited efficacy of debridement and the ability of biofilm exudates to penetrate the skin, research on PAO1 BCM is essential.

Recently, studies to determine the *in vitro* effects of soluble factors of biofilms have been conducted, but most focused on *S. aureus* (Kirker et al., 2012; Marano et al., 2015). Mass spectrometry identified proteinaceous factors of *S. aureus*, alpha-toxin and leukocidin AB that inhibit macrophage phagocytosis, while alpha hemolysin and alcohol dehydrogenase were found to delay wound healing (Marano et al., 2015; Scherr et al., 2015). Although *S. aureus* is one of the most frequently identified bacteria found in chronic wounds, research should also be extended to study the effects of soluble factors of *P. aeruginosa* biofilms as it has been reported to be one of the most frequently identified biofilm-forming bacteria in chronic wounds (Gjødtsbol et al., 2006). While many *P. aeruginosa* virulence factors have been identified and their pathways described, little is known about soluble factors of *P. aeruginosa* biofilms affecting chronic wounds. These factors, if identified, could also be potential chronic wound biomarkers. Filling these research gaps will allow for a more effective treatment of *P. aeruginosa*-infected chronic wounds. Taken together, this study deepens our understanding on the limited efficacy of wound debridement and underlines the potential of targeting these secreted factors to increase the effectiveness of treatment.

The aims and hypotheses of the thesis are as follows:

General aims: To understand how biofilm secreted factors of *P. aeruginosa* impact wound healing through the use of BCM.

Aim 1: To determine the effects of *P. aeruginosa* BCM on cell migration, adhesion and viability.

Hypothesis 1: *P. aeruginosa* BCM will negatively affect cell migration, adhesion and viability.

This is achieved through improving current methods of BCM generation and application through in the first experimental chapter (**Chapter 3**).

Aim 2: To determine how *P. aeruginosa* BCM affects inflammation and keratinocyte differentiation.

Hypothesis 2: PAO1 BCM will increase inflammation through the NF- κ B signalling pathway and elevate expression of keratinocyte differentiation markers.

This is achieved through the second experimental chapter (**Chapter 4**).

Aim 3: To identify the possible secreted biofilm factors in PAO1 BCM that are responsible for eliciting the different effects on wound healing.

Hypothesis 3: The factors involved may be proteinaceous or metabolites given the wide spectrum of virulence factors that *Pseudomonas aeruginosa* is reported to produce (Gellatly and Hancock., 2013; Bleves et al., 2010).

This is achieved through the last experimental chapter (**Chapter 5**).

2. EXPERIMENTAL METHODS

2.1 *In vitro* techniques

2.1.1 Bacterial strains and cell lines used

The bacterial strains and cell lines used in this thesis are listed as follows:

Table 3: List of bacterial strains and cell lines.

Bacteria Strain/Cell Line	Source	Type
HaCaT	Prof Artur Schmidtchen	Immortalised human keratinocyte
BJ-5ta	Assoc Prof Kimberly Kline (ATCC CRL-4001)	Immortalised human fibroblast
NIH-3T3	ATCC CRL-1658 TM	Immortalised mouse fibroblast
PAO1	Assoc Prof Scott Rice	Wild-type <i>Pseudomonas aeruginosa</i>
PAO1-GFP	Assoc Prof Kimberly Kline	Wild-type <i>Pseudomonas aeruginosa</i> with GFP tag
PAO1 lasB	Gift from Prof Artur Schmidtchen	<i>Pseudomonas aeruginosa</i> elastase B mutant
PAO1 lasR	Gift from Assoc Prof Yang Liang	<i>Pseudomonas aeruginosa</i> lasR gene mutant
PAO1 rhIA	Gift from Assoc Prof Kevin Pethe	<i>Pseudomonas aeruginosa</i> rhamnolipid A mutant
PA 15159	Gift from Prof Artur Schmidtchen	Clinical strain derived from patient with chronic leg ulcer and obtained from Dept of Bacteriology, Lund University Hospital, Sweden

2.1.2 Generation of PAO1 biofilm conditioned media (BCM)

The generation of PAO1 BCM comprises of 4 major stages, summarised in **figure 3**.

Stage 1: Preculture

A PAO1 bacterial colony was inoculated into 10 ml tryptic soy broth (TSB) (Sigma-Aldrich, USA) to generate a bacterial preculture. The preculture was subsequently incubated at 37°C, 140 rpm until it reached an optical density (OD₆₀₀) of 0.8 (approximately 6 h) at an absorbance of 600 nm in wavelength. OD₆₀₀ was measured using the Multiskan Go spectrophotometer (Thermoscientific, USA). The preculture was then diluted to OD₆₀₀ 0.01 and inoculated in a 500 ml conical flask of 100 ml TSB. The conical flask contained a piece of aquaria polyester filter wool which is of 8.5 cm in diameter, 56.7 cm² in area and a weight of 0.15 g (the exact surface area of the filter

wool is unknown). The filter wool provides mechanical support for biofilm attachment and growth.

Stage 2: TSB incubation

Spent TSB was replaced with fresh media at 18 h and incubated at 37 °C, 140 rpm for another 6 h. The bacterial culture was then discarded and biofilm formation on the filter wool was checked through biofilm matrix staining, a procedure stated in **2.1.4 Biofilm matrix staining**, and imaged using a Leica TCS SP8 (Leica, Germany). Colony forming units counts of the bacteria on the filter wool were also conducted. The CFU procedure is stated in the next subsection of 'Experimental Methods' under **2.1.3 CFU counting**.

Stage 3: Dulbecco's Modified Eagle's medium (DMEM) incubation

Together with the bacteria inoculum, the filter wool was incubated for another 24 h in 100 ml Dulbecco's Modified Eagle's medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, USA).

Stage 4: Collection, sterilisation and pH optimisation of BCM

Biofilm formation on the filter wool was checked through biofilm matrix staining and imaged using an upright confocal microscope (SP8, Leica, Germany) and CFU counts of the bacteria on the filter wool were conducted. Bacterial cultures were spun down at 4000 rpm for 30 mins using a Sorvall ST 16R Centrifuge (Thermoscientific, USA). Subsequently, the supernatant was sterilised-filtered using 0.2 µm syringe filters (Sartorius, Germany) and pH optimised to 7.4. This resultant solution is then termed as BCM.

Figure 3: Workflow for PAO1-GFP BCM generation. Adapted from Schleheck et al. (2009). The BCM generation is made up of 4 major stages: Stage 1- preculture, Stage 2- tryptic soy broth (TSB) incubation, Stage 3- Dulbecco's Modified Eagle's medium (DMEM) incubation and lastly, Stage 4- the collection, sterilisation and pH optimisation.

2.1.3 CFU counting

Bacterial cultures were serially diluted in 1 X phosphate buffered saline (PBS) (1st Base, Malaysia) to generate dilutions of 10^{-1} to 10^{-7} . For CFU counts of biofilms formed on filter wool, an area of 1 cm² filter wool was excised and sonicated in 1 X PBS and the sonicated solutions were serially diluted. Next, 5 µl of each culture (diluted and non-diluted) was plated on tryptic soy agar (TSA) (Sigma-Aldrich, USA) and grown overnight at 37 °C. Single colonies of PA were counted and then multiplied by the dilution factor to give the resultant CFU counts for each culture.

2.1.4 Biofilm matrix staining

FilmTracerTM SYPRO Ruby Biofilm Matrix Stain (Invitrogen, USA) was used to stain PAO1 biofilm matrix. 1 cm² of filter wool was dipped in 5 ml of PBS to wash away free-floating planktonic bacteria and then dipped in 5ml of the stain for 30 -45 mins, protected from light. Filter wool was then dipped in filter-sterilised distilled water to remove excess stain and mounted on a glass slide. The biofilm matrix was then imaged

using the Leica TCS SP8 (Leica, Germany) with the 20 X dry objective. The stain was excited using 488 nm wavelength laser and images were acquired at 8-bit greyscale at 1024 x 1024 pixels.

2.1.5 Crystal violet staining of filter wool

Filter wool was dipped into PBS to remove unattached cells, and then dipped into crystal violet (0.1 % w/v) for 1 h. It was then washed in PBS 3 times to remove excess stain, blotted on paper towel and air dried overnight (24 h). Filter wool was photographed when dry for qualitative analysis. For a quantitative analysis, crystal violet on filter wool was solubilised in 10 ml 90 % ethanol for 12 h at 180 rpm on a shaker. The amount of crystal violet in the ethanol fraction was quantified at 580 nm using a spectrophotometer, with solutions diluted if $A_{580} > 1.5$. 90 % ethanol was used as a blank control.

2.1.6 Maintenance of mammalian cell lines

All mammalian cells were cultured routinely at 80 % confluency using DMEM (Gibco, USA) containing 10 % FBS with 1 % penicillin and streptomycin (pen-strep) (Gibco, USA), except for BJ-5ta fibroblasts which additionally require hygromycin B at 0.01 mg/ml. Briefly, cells were detached with 0.25 % Trypsin+EDTA (Gibco, USA), spun down at 1200 rpm for 5 mins before seeding at 20 % confluency in cell culture-treated EasYFlasks™ T-75 flasks (Nunc™, USA).

2.1.7 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay

The MTT assay was conducted using the Cell Proliferation Kit 1 (MTT) (Roche, Switzerland). Cells were seeded at 180,000 cells/ml on respective flat-bottomed 96-well plates the day before the experiment. On the day of the experiment, the respective dilutions of conditioned media (dilutions made with DMEM) were then added to make a total of 100 µl in each well. Cell cultures were then incubated at 37 °C with 5 % CO₂ with the conditioned media for the respective treatment timings to monitor the effects. After the respective incubation periods, conditioned media were removed and 10 % MTT labelling reagent (diluted with 90% DMEM) was added to each well with a subsequent incubation of 24 h. Subsequently, 100 µl of solubilisation solution was added to each well and incubated for another 24 h. The spectrophotometric absorbances of the samples were then measured using Cytation™ 3 Cell Imaging Multi-Mode Reader (BioTek, USA) at a wavelength of 570 nm. Experimental and biological triplicates were conducted to ensure reliability and reproducibility of results.

2.1.8 Scratch wound assay

Cells were seeded at 210,000 cells/ml on flat-bottomed 24 well plates the day before the experiment. On the day of experiment, cells in each well were scratched using sterile 200 µl pipette tips to create a “wound”. Respective dilutions of conditioned media were then added to make a total of 500 µl in each well. Propidium iodide (PI) (Life Technologies, USA) was also added in the concentration of 1 µg/ml as an uptake of PI by the cells would signify membrane compromise. Time-lapse images of the “wounded” areas were acquired every 15 mins over a period of 24 h using integrated modulation contrast (IMC) microscopy on the Leica DMI6000 B Inverted Microscope (Leica, Germany). Experimental and biological triplicates were conducted to ensure

reliability and reproducibility of results. Accumulated changes in migrated area (in pixels) were then quantified using ImageJ, by quantifying the changes in area (in pixels) between the wound edge from the previous wound edge at the respective timings.

In the scratch assay that involved transient cell injury, cells were incubated with 50 % PAO1 BCM for 5 h and then replaced with fresh complete DMEM and observed for 24 h (for 20 X objective) and 2 days (for 10 X objective) instead. For the LPS scratch wound assay, respective dilutions of lipopolysaccharide were then added to make a total of 500 µl in each well instead.

In scratch assays conducted for immunocytochemistry of fixed cells, cells were seeded at 230,000 cells/ml (for keratinocytes) and 210,000 cells/ml (for fibroblasts) on coverslips placed in 24-well plates with DMEM and left to grow for a day. Cells were scratched using 200 µl pipette tips and treated with respective PAO1 BCM dilutions for 5 h.

2.1.9 Hanging drop assay

Cells were trypsinised and resuspended in respective BCM conditions to get a concentration of 10^5 cells/ml. The cell suspension was aliquoted into droplets of 30 µl per replicate on a 24 well plate. The plate lid was filled with sterile 1 X PBS to humidify the plate. The well plate was then inverted on the plate lid and placed in the incubator for 5 h. After the stipulated incubation timing, cell clusters were dispersed by pipetting them up and down 8 times using 200 µl pipette tips that have been cut. Cell clusters were then transferred to slides and imaged immediately using the 4 X objective on the inverted widefield microscope DMI6000 (Leica, Germany). Images were then analysed

in ImageJ to determine the size of aggregates formed. Experimental and biological triplicates were conducted to ensure reliability and reproducibility of results.

2.1.10 Bioactivity test of BCM in plasters (Using MTT assay)

300 µl DMEM and BCM were absorbed by plasters and then spun down at 1200 rpm for 1 min to collect the plaster-absorbed media. The plaster-absorbed media were then added to cells and incubated for 8 and 24 h. After 24 h, media were removed and 10 % MTT labelling reagent (diluted with 90 % DMEM) was added to each well with a subsequent incubation of 24 h. Subsequently, 100 µl of solubilisation solution was added to each well and incubated for another 24 h. The spectrophotometrical absorbances of the samples were then measured using Cytation™ 3 Cell Imaging Multi-Mode Reader (BioTek, USA) at a wavelength of 570 nm. Experimental and biological triplicates were conducted to ensure reliability and reproducibility of results. For detailed procedures of the MTT assay, refer to **2.1.7 3-{4,5-dimethylthiazol-2-yl}-2,5-diphenyltetrazolium bromide (MTT) Assay.**

2.1.11 Cell supernatant collection

Cell supernatant for enzyme-linked immunosorbent assay (ELISA) was collected by seeding HaCaT keratinocytes (230,000 cells/ml) on 24 well plates and incubating cells with respective conditions (Control-DMEM and 50 % PAO1 BCM) for 5 h. The cell supernatant was collected into eppendorf tubes which were then centrifuged briefly and the liquid collected from spinning down was used for ELISA.

2.2 *In vivo* techniques

2.2.1 Mice

Male C57BL/6 mice were bred and housed in Biological Resource Centre (BRC) of the Lee Kong Chian School of Medicine (LKC SOM), Singapore. They were housed under specific pathogen-free conditions with a 12-h light-dark cycle, where standard rodent diet and water were supplied *ad libitum*. Breeding was approved by the Institutional Animal Care and Use Committee (IACUC) under the protocol #A18045. Animals were 8 to 11 weeks of age and had an average weight of 20 g (range, 18 g to 22 g) prior to the start of the experiment. All animal procedures were done in accordance with the approved animal protocols obtained from the IACUC of the BRC.

2.2.2 Mice wounding procedure and harvest for histology

The IACUC of the BRC Singapore has approved of this protocol, #A0367. The overall mouse wounding procedure and harvest is shown in **figure 4**. Mice were anaesthetised with isoflurane gas and oxygen. Their dorsum was shaved using electric trimmers and depilatory cream applied for full removal of back fur and had their skin surfaces wiped with ethanol before wounding. Six mm biopsy punches were used to create full thickness excisional wounds on the back of each mouse. After this, 350 µl of the respective test solutions were applied onto plasters (plasters from Watsons, Guardian and Hansaplast). Tegaderm™ (3M, USA) was subsequently used to cover the wound and hold the plaster in place. Buprenorphine at a concentration of 2 mg/kg (approximately 200 µl per animal) (LKC Biological Resource Centre, Singapore) was delivered to the mice subcutaneously after wounding. The mice were then housed individually and monitored for their health statuses daily till the day of harvest.

On the day of harvest, mice were sacrificed by euthanising them in a carbon dioxide chamber. Wounded skin tissues were collected and bisected before fixing in 4 % PFA. The entire wound was used for histology. Pictures during the course of the animal experiment were taken using an iPhone 8.

Figure 4: Outline of mouse wounding procedure and harvest. Mice were anaesthetised using isoflurane gas, and 6 mm wounds were created using biopsy punches. Plaster and Tegaderm were then applied over the wounds. During harvest, skin tissues were collected for histology.

2.2.3 Wound fluid collection

Full-thickness excisional wounds were created in mice as mentioned in **2.2.1 Mice** and **2.2.2 Mice wounding procedure and harvest for histology**, with slight modifications to the experimental protocols for wound fluid analysis. Filter paper was cut out using a hole puncher and then placed on the wounds. Wounds were then covered by the BCM-filled plasters until the day of harvest. During harvest, instead of harvesting the entire wound for histology, the filter papers and plasters were harvested and placed in eppendorf tubes. The tubes were then centrifuged briefly and the liquid collected from spinning down was used for ELISA.

2.3 Histological techniques

2.3.1 Skin tissue processing, embedding and sectioning

All skin tissues used for histology were fixed in 4 % PFA overnight. They were transferred into 70 % ethanol solution and processed using the Leica ASP6025 automated vacuum tissue processor according to the steps shown in **table 4** (Leica, Germany). Subsequently, the tissues were embedded in paraffin (Leica, Germany) using the Leica EG1150 Modular Tissue Embedding Center (Leica, Germany) and sectioned at 5 μ m with the Leica Biosystems RM2255 Fully Automated Microtome (Leica, Germany).

Table 4: Table of steps involved in tissue processing.

Reagent	Timing
70% ethanol	45 min
80% ethanol	45 min
95% ethanol	45 min
100% ethanol	45 min (X3)
Xylene	45 min (X3)
Paraffin	45 min (X3)

2.3.2 Haematoxylin and Eosin (H&E) staining

Haematoxylin and eosin staining of the paraffin-sectioned skin tissues were performed using the Leica Biosystems ST5010 Autostainer XL (Leica, Germany). The staining process summarised in **table 5**:

Table 5: Table of H&E process.

Reagent	Timing
Clearene	5 min (2 X)
100% ethanol	2 min (2 X)
95% ethanol	2 min (2 X)
70% ethanol	2 min
Distilled water	2 min
Hematoxylin	1 min
Distilled water	3 min
0.3% acid alcohol	3 s
Distilled water	2 min
Scott's tap water	5 s
Distilled water	2 min
70% ethanol	2 min
Eosin	30 s
70% ethanol	2 min
95% ethanol	2 min
100% ethanol	1 min (3 X)
Clearene	1 min (2 X)

Coverslips were then mounted onto Polysine™ Microscope Adhesion slides (Thermo Scientific™, USA) using limonene (Sigma-Aldrich, USA). Images of the skin tissue samples were acquired using the Axio Scan.Z1 (Zeiss, Germany).

2.3.3 Antigen retrieval and immunofluorescence staining

Sections were dewaxed (**Table 6**) and antigen retrieval was conducted by boiling tissues in sodium citrate buffer in an autoclave at 121 °C for 15 mins. To block tissues, 250 µl of 1 % bovine serum albumin (BSA) in 1 X PBS (Sigma, USA) was incubated for 1 h at room temperature. Tissues were incubated with 300 µl of primary antibodies diluted in blocking buffer (BSA) (**Table 7**) at 4 °C overnight. Negative controls were incubated with blocking buffer at 4°C overnight. Tissues were then washed with washing buffer (0.05 % Tween 20 in 1 X PBS, also known as PBST) and then incubated with the respective secondary antibodies diluted in blocking buffer for 1 h at room temperature. All washing steps were performed 3 X for 5 mins each using 300 µl of washing buffer. Subsequently, cells were washed with PBST, incubated with DAPI solution for 10 mins

and then washed again with PBS. The coverslips were mounted in Citifluor (Citifluor, UK). Images were acquired using a Leica TCS SP8 (Leica, Germany).

Table 6: Table of dewax process.

Reagent	Timing
Clearene	5 min (2 X)
100% ethanol	2 min (2 X)
95% ethanol	2 min (2 X)
70% ethanol	2 min
Distilled water	2 min

Table 7: List of antibodies used for immunofluorescence.

Antibody Name	Company/Country	Cat No.	Dilution
Mouse monoclonal anti-E-cadherin	Cell Signalling Technology	#14472	1:500
Rabbit monoclonal anti-NFκB p65 antibody	Cell Signalling Technology	#8242	1:1000
Mouse monoclonal anti-Keratin 10 antibody	Invitrogen	MA5-13705	1:500
Mouse monoclonal anti-Involucrin antibody	Invitrogen	MA5-11803	1:500
Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor® 555 conjugate	Invitrogen	A21428	1:500
Goat anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor® 488 conjugate	Invitrogen	A11001	1:500

2.3.4 Immunocytochemistry

Cells were then fixed in 250 µl of 4% paraformaldehyde (PFA) (Sigma, USA) in distilled water at room temperature for 15 min and washed with 1 X PBS. All washing steps are done 3 times for 5 mins each using 300 µl of the respective washing buffer. Samples were then permeabilised using 250 µl 0.1% Triton-X 100 for 10 mins at room temperature. To block cells, 250 µl of 1% bovine serum albumin (BSA) in 1 X PBS (Biowest, USA) was then incubated for 1 h at room temperature. Cells were immune-labelled with 300 µl of primary antibodies that were diluted in blocking buffer (BSA). Primary antibody incubation was performed at 4 °C overnight. The types of antibodies used are listed in **table 8**. Negative staining controls were incubated with blocking buffer at 4 °C overnight instead. Cells were then washed with washing buffer (0.05 % Tween 20 in 1 X PBS, also known as PBST) and then incubated with the respective secondary

antibodies diluted in blocking buffer for 1 h at room temperature. Subsequently, cells were washed with PBST, incubated with DAPI solution for 10 mins and then washed again with PBS. The coverslips were mounted in Citifluor (Citifluor, UK) onto Polysine™ Microscope Adhesion slides (Thermo Scientific™, USA) and images acquired using the Leica TCS SP8 (Leica, Germany). Experimental and biological triplicates were performed for reliability.

Table 8: List of antibodies used for immunocytochemistry.

Antibody Name	Company/Country	Cat No.	Dilution
Mouse monoclonal anti-Gm130 antibody	BD Transduction Laboratories™	610822	1:4000
Mouse monoclonal anti- α -tubulin antibody	Sigma-Aldrich	T6074	1:4000
Rabbit polyclonal anti- β -catenin antibody	Abcam	Ab6302	1:4000
Rabbit polyclonal anti-Cx43 antibody	Sigma-Aldrich	C6219	1:4000
Mouse monoclonal anti-N-cadherin antibody	Invitrogen	8C11	1:500
Rabbit monoclonal anti-Occludin	Invitrogen	6H10L9	1:1000
Mouse monoclonal anti-E-cadherin	Cell Signalling Technology	#14472	1:500
Rabbit anti-cleaved caspase-3 (D175) antibody	Cell Signalling	9661	1:400
Mouse anti-HMGB1 antibody	Invitrogen	GT348	1:400
Mouse anti-vinculin antibody	Sigma	V9131	1:400
Rabbit polyclonal anti-p-vinculin (Y1065) antibody	Invitrogen	44-1078G	1:500
Actin phalloidin	Invitrogen	A12380	1:50
Rabbit monoclonal anti-NF κ B p65 antibody	Cell Signalling Technology	#8242	1:1000
Mouse monoclonal anti-Keratin 10 antibody	Invitrogen	MA5-13705	1:500
Mouse monoclonal anti-Involucrin antibody	Invitrogen	MA5-11803	1:500
Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor® 555 conjugate	Invitrogen	A21428	1:500
Goat anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor® 488 conjugate	Invitrogen	A11001	1:500

2.4 Molecular and biochemistry techniques

2.4.1 Drug inhibition of proteasomal and lysosomal assay

Cells were seeded on glass coverslips at a density of 230,000 cell/ml and left to grow for 1 day before the introduction of drugs diluted in DMEM. Cells were induced with bafilomycin (lysosome inhibitor) (Sigma, USA) and MG132 (proteasome inhibitor) (Sigma, USA) respectively at concentrations and for timings stated in **table 9**. After induction, media was removed and 50 % PAO1 BCM added and incubated for 5 h. Cells

were then fixed and stained with p65, as per **2.3.4 Immunocytochemistry**. Finally, microscope slides containing the fixed cells were imaged using the Leica TCS SP8 (Leica, Germany) and the p65 signal intensity was then quantified using FIJI software.

Table 9: List of drugs, their respective concentrations and induction timings.

Drug Name	Concentration	Induction timing
Bafilomycin A1	25 nM	1 and 2 h
	50 nM	
	100 nM	
	500 nM	
	1 μ M	
	10 μ M	
MG132	0.5 μ M	30 min, 1 h and 2 h
	1 μ M	
	2 μ M	
	5 μ M	
	10 μ M	

2.4.2 RNA extraction

Cells were seeded at 230,000 cells/ml in 6-well plates and left to grow for 2 days before incubating with the respective conditions (Control-DMEM, and 50 % PAO1 BCM for 5 h). Media was removed and 1 ml TRIzol reagent (Thermofisher, USA) was used to lyse the cells. Next, 200 μ l chloroform was added and incubated for 3 mins. Samples were centrifuged for 15 mins at 12,000 g, at 4 °C. The aqueous phase containing the RNA was then transferred to a new eppendorf tube and 500 μ l isopropanol added and incubated for 10 mins. The tubes were then centrifuged for 10 mins at 12,000 g at 4 °C, generating a RNA precipitate that is a white pellet. The supernatant was discarded and the RNA pellet resuspended in 1 ml 75 % ethanol. The RNA pellet was vortexed briefly and centrifuged for 5 mins at 7500 g at 4 °C. The supernatant was discarded and the pellet air dried for 5–10 mins. The pellet was then resuspended in 30 μ l diethyl pyrocarbonate (DEPC)- treated water (Invitrogen, USA) and the RNA quality and amount measured through the nanodrop.

2.4.3 cDNA synthesis

DNase digestion was conducted by combining reagents as listed in **table 10** in an RNase-free eppendorf tube and incubated at 37 °C for 30 mins. 1 µl of 50 mM EDTA was then added and incubated at 65 °C for 10 mins.

Table 10: List of reagents required for DNase digestion and their respective amounts.

Reagent	Amount	Company/Country Origin
RNA	1 µg	-
10X reaction buffer with MgCl ₂	1 µl	Thermofisher, USA
DNase I, RNase-free	1 µl	Thermofisher, USA
DEPC-treated water	To 10 µl	Invitrogen, USA

Next, reverse transcription was conducted. Firstly, 2 µl of deoxynucleotide (dNTP) (Invitrogen, USA) and 1 µl of oligo d(T)₁₂₋₁₈ (Invitrogen, USA) were added and incubated at 65 °C for 5 mins. Reagents listed in **table 11** were then added and incubated at 42 °C for 1 h, 85 °C for 5 mins and then at 4 °C for short-term storage.

Table 11: List of reagents required for reverse transcription and their respective amounts.

Reagent	Amount	Company/Country Origin
M-MLV Reverse Transcriptase 5X Reaction Buffer	4 µl	Promega, USA
Ribolock	1 µl	Invitrogen, USA
M-MLV Reverse Transcriptase	1 µl	Promega, USA

2.4.4 Quantitative real time- polymerase chain reaction (qRT-PCR)

The converted cDNA was then used for qPCR. The reagents required, primer sequences and PCR steps are stated in **tables 12, 13 and 14**. Experimental and biological triplicates were conducted for reproducibility and reliability.

Table 12: List of reagents required for qPCR and their respective amounts.

Reagent	Amount	Company/Country Origin
EXPRESS SYBR GreenER qPCR SuperMix Universal	10 µl	Invitrogen, USA
10 µM forward primer	0.4 µl	IDT, Singapore
10 µM reverse primer	0.4 µl	IDT, Singapore
ROX Reference Dye (25 µM)	0.4 µl	Invitrogen, USA
Template DNA	100 ng (2 µl of 10X diluted stock)	Invitrogen, USA
DEPC-treated water	To 20 µl	Invitrogen, USA

Table 13: List of primers used in qRT-PCR and their respective sequences.

Primer Name	Forward Primer (5'-3')	Reverse Primer (5'-3')
p65	AGCGCATCCAGACCAACAACAACC	CCGCCGCAGCTGCATGGAGACAC
p50	AGCCCCCAATGCATCCAACCTT	CCACCGCCGAAACTATCCGAAAAA
IKB-α	CAGCAGACTCCACTCCACTT	GAGAGGGGTATTTCTCTCGAA
GAPDH	ACAACCTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

Table 14: List of qPCR stages and their respective timings and cycles.

Stage	Temperature (°C)	Time	Number of Cycles
Holding stage	95	10 min	-
Cycling stage	95	15 s	40
	60	1 min	
Melt curve stage	95	15 s	Step and hold
	60	1 min	
	95	15 s	

2.4.5 ELISA

ELISAs were carried out as per manufacturer's instructions (Biolegend, USA). The types of antibodies used are listed in the **table 15**. Briefly, capture antibodies were diluted in 1X coating buffer and 100 µl of this capture antibody solution was then added to wells of the Nunc MaxiSorp ELISA plates (Thermofisher, USA). Plates were then sealed and incubated overnight at 4 °C. Plates were washed 4 times with 200 µl 1 X PBST per well and the residual liquid blotted by tapping the plate firmly upside down and shaking the liquid out. All subsequent washes were conducted in the same manner. Plates were then blocked using 200 µl 1 X assay diluent per well, sealed and incubated at room temperature for 1 h on an orbital plate shaker. All subsequent plate incubations

were conducted in a similar manner. Subsequently, plates were washed and cell supernatants/wound fluid and standards were added, sealed and incubated for 2 h. After this, plates were washed and 100 μ l avidin-HRP solution added to each well, sealed and incubated for 30 mins. Plates were then washed for 1 min for 5 times, and 100 μ l 3,3',5,5'-Tetramethylbenzidine (TMB) substrate solution was added and incubated for 15-30 mins (depending on the capture antibody used). Lastly, 100 μ l stop solution (2N H₂SO₄) was then added to each well and the absorbance read using a spectrophotometer at A₄₅₀ nm.

Table 15: List of ELISA antibodies.

Capture antibody	Company/Country	Cat No.	Concentration/Dilution
Human monoclonal anti-TNF- α antibody	eBioscience	BMS2034MST	2 μ g/ml
Human monoclonal anti-IL-6 antibody	eBioscience	BMS213/2MST	2.5 μ g/ml
Human monoclonal anti-IL-8 antibody	eBioscience	BMS204/3MST	5 μ g/ml
Mouse TNF- α ELISA MAX Capture Antibody	Biolegend	430905	1X
Detection antibody			
Biotin conjugate anti-human TNF- α total polyclonal antibody	eBioscience	BMS2034MST	1:1000
Biotin conjugate anti-human IL-6 total monoclonal antibody	eBioscience	BMS213/2MST	1:1000
Biotin conjugate anti-human IL-8 total monoclonal antibody	eBioscience	BMS204/3MST	1:1000
Mouse TNF- α ELISA MAX detection antibody	Biolegend	430905	1X
Streptavidin-HRP	Invitrogen	All the kits	1:5000/1:10000
	Biolegend		

2.4.6 Western blot

Cells were seeded at 230,000 cells/ml in 6-well plates and incubated for 1 day before BCM induction. Cells were then treated with respective conditions (Control- complete DMEM only, and 50 % PAO1 BCM) for 5 h and lysed for 3 mins, using RIPA buffer (Thermofisher, USA) with 1 X proteinase inhibitor added (Thermofisher, USA), before collecting the suspension into an eppendorf tube. The eppendorf was then spun down at

12,000 g at 4 °C for 5 mins and 1 X loading dye (Biorad, USA) was added. The protein samples were heated at 95 °C for 15 mins, cooled using ice and loaded into wells of 10 % NuPAGE 10 % Bis-Tris Protein gels (Thermofisher, USA). The gels were run at 130 V for 1.5 h and the proteins transferred using the iBlot 2 Dry Blotting System (Thermofisher, USA) onto PVDF iBlot 2 Transfer Stacks (Thermofisher, USA). After transferring the proteins, the membranes were blocked in 5 % bovine serum albumin (BSA) (Sigma, USA) in Tris-buffered Saline (TBS) (Axilscientific, Singapore) for 1 h before incubating in the respective primary antibodies listed in **table 16** overnight at 4 °C on a shaker. After primary antibody incubation, membranes were washed 3 times in 1X TBSTween 20 (TBST) for 10 mins each time. All subsequent washes were conducted in a similar manner. Membranes were then incubated in the respective secondary antibodies listed in table 11 for 1 h at room temperature on a shaker. After which, membranes were washed. TBST was then discarded and the membranes incubated with SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermofisher, USA) for 5 mins before developing the membrane on Gel Doc™ XR+ Gel Documentation System using the system optimised settings (Biorad, USA). The intensities of the protein bands were then quantified using the Image Lab Software (Biorad, USA).

Table 16: List of antibodies used for Western blot.

Antibody Name	Company/Country	Cat No.	Dilution
Rabbit monoclonal anti-c-Jun	Cell Signalling Technology	60A8	1:1000
Rabbit monoclonal anti-SAPK/JNK	Cell Signalling Technology	9252	1:1000
Goat anti-rabbit superclonal HRP	Invitrogen	A27036	1:10000

2.4.7 Generation of treated media

To generate heat-inactivated media, media were heated at respective temperatures (37 °C, 50 °C and 100°C) for 1 h. They were cooled to room temperature before usage.

To generate proteinase-K digested media, media were incubated with 50 µg/ml proteinase K at 37 °C and 50 °C for 1 h. To generate trypsin digested media, media were incubated with 75 and 25 µg/ml trypsin at 37 °C for 1 h. For both enzyme-digested media, the enzymatic reactions were inhibited using 5 mM PMSF for 1 h.

2.4.8 Chromogenic LAL endotoxin detection assay

Endotoxin testing was conducted using the ToxicSensor Chromogenic LAL Endotoxin Assay Kit as per manufacturer's instructions (Genscript, USA). Briefly, 100 µl limulus amoebocyte lysate (LAL) was added to 100 µl samples and incubated at 37 °C for 1 h. 500 µl stop solution was added, followed by 500 µl of colour stabilisers 2 and 3. Absorbance at 545 nm was recorded and DMEM was used as a control.

2.5 Microscopy and image analyses

Images were acquired using an upright confocal microscope TCS SP8 (Leica, Germany) with a 40 X 1.2 NA oil objective. DAPI, Alexa Fluor 488 and 555 fluorophores were excited sequentially using 405 nm, 488 nm and 532 nm wavelength lasers respectively. The images were acquired at 8-bit greyscale at 1024 x 1024 pixels with a Z-series frame average of 3. Quantification of fluorescence signal (if any) were run via a Macros on FIJI, or by using a standardised thresholding and then analysing the signals. Identical parameters and photomultiplier tube (PMT) settings were used for all images (of that particular stain) to allow for direct comparison of staining intensities.

2.5.1 Golgi reorientation measurements

Measurements for the angle of golgi repolarisation were conducted on the first row of cells facing the wound using ImageJ as shown in **figure 5** (Bisel et al., 2008; Bisel et al., 2013; Magdalena et al., 2003; Mendoza-Naranjo et al., 2012). Briefly, lines were

drawn from the centre of the nucleus to the wound edge and from the centre of the nucleus to the center of the golgi apparatus. The angle formed between these 2 lines was then measured. Golgi apparatuses that are located within a 120° sector towards the wound edge from the center of the nucleus were considered repolarised. 5 field of views were used per replicate per condition. An average of 150 cells were counted per condition.

Figure 5: Method of Golgi apparatus reorientation measurements. Image by Erica Teo Mei Ling.

2.5.2 Lamellipodia area measurements

Measurements for lamellipodia protrusion area were conducted on the first row of cells facing the wound using ImageJ. Briefly, cell areas were traced and the area measured. 5 field of views were used per replicate per condition. An average of 150 cells were counted per condition.

2.6 Statistical analyses

All statistical comparisons were made using GraphPad Prism version 5.0 for Windows, (GraphPadTM Software, USA). Mann-Whitney tests were used to compared between 2 conditions, 1-way ANOVA with Dunnett's post hoc tests were used to compare within groups against a control, 1-way ANOVA with Tukey's post hoc tests were used to compare within groups against all conditions, 2-way ANOVA with Dunnett's post hoc

tests were used to compare between groups against a control and 2-way ANOVA with Tukey's post hoc tests were used to compare between groups against all conditions. The statistical significance of p values are summarised in **table 17**.

Table 17: List of statistical symbols and their respective significance.

Symbol	Statistical significance
NS	$P > 0.05$
*	$P \leq 0.05$
**	$P \leq 0.01$
***	$P \leq 0.001$

2.7 List of reagents used

Table 18: List of reagents.

Reagent	Company/Country
Phosphate buffered saline (PBS)	Axil Scientific, Singapore
(DMEM)	Thermofisher, USA
FBS	Hyclone, USA
TBS	Axil Scientific, Singapore
Bafilomycin A1	Sigma, USA
MG132	Sigma, USA

3. UNDERSTANDING THE EFFECTS OF PAO1 BIOFILM CONDITIONED MEDIA ON CELL MIGRATION, VIABILITY AND ADHESION

3.1 Introduction

Research on the secreted factors of biofilms and planktonic bacteria has been conducted recently, with most of the studies focusing on *S. aureus* (Secor et al., 2011; Kirker et al., 2012; Tankersley et al., 2014). Recently, studies on the effects of *Pseudomonas aeruginosa* conditioned media have been conducted (Marano et al., 2015). This study reported that BCM from *P. aeruginosa* negatively affects proliferation in human tympanic membrane derived keratinocytes, while also having toxic effects on human epidermal keratinocytes. Although this study has shown that the conditioned media is toxic, markers of the different processes were not checked for and so the cell death pathway is currently unknown. Moreover, the effects of the conditioned media on cell migration, which is a key factor in the wound healing process for proper wound closure, was not studied. Another study by Ward et al. (2015) demonstrated that exposure of mesenchymal stromal cells (MSC) to *P. aeruginosa* BCM induces inflammation, apoptosis, inhibits migration and differentiation of MSCs. However, how *P. aeruginosa* BCM affects keratinocytes and fibroblasts in wound healing were not pursued. Given the limited results of these two studies, there is a need for investigating the effects of *P. aeruginosa* BCM on other wound healing aspects such as cell adhesion and migration, which have been shown to be compromised in *P. aeruginosa* infections (Loryman and Mansbridge, 2008; Golovkine et al., 2018; Gellatly and Hancock., 2013). Moreover, the use of BCM in an *in vivo* wound healing model has not been explored. This would give the current *in vitro* studies more clinical relevance. In conclusion, this chapter fills the research gaps in the field regarding the effects of *P. aeruginosa* BCM on cell adhesion,

migration and cell death pathway, establishes an *in vivo* wound healing model with the application of BCM to determine its effects.

This chapter aims to characterise the *in vitro* and *in vivo* effects of PAO1 BCM on wound healing responses, namely cell migration, viability and adhesion. It is hypothesised that PAO1 BCM will have a negative effect on cell migration, adhesion and viability.

The strain PAO1 was chosen in this study as it is of moderate virulence and it is a reference strain, which allows for comparison with other studies and establishment of respective mutants if necessary in the project (Lee et al., 2006). Being a clinical strain, PAO1 was isolated from an infected, burn wound patient from Melbourne, Australia and is resistant to a broad spectrum of antimicrobial substances (Hare et al., 2012; Stover et al., 2000). Analysis of *in vitro* effects were through the use of immortalised human fibroblasts (BJ-5ta) and keratinocytes (HaCaT), the two main cell types in the skin. Immunocytochemistry, live cell imaging, viability and adhesion assays were conducted to determine the possible effects that PAO1-BCM could exert. Analysis of *in vivo* effects were through the use of a full-thickness murine wound excisional model. The effects of PAO1 BCM were observed over a span of 1 and 3 days post-wounding. Immunohistochemical staining of the tissue samples were carried out to quantify potential gene expression changes.

3.2 Results

3.2.1 Optimisation of PAO1 BCM generation

As shown in the literature review, there are various methods of growing BCM. Filter paper and polyester (PE) fibres were explored as surfaces for biofilm attachment. Bacteria grown on filter paper were easily sloughed off and not as thick and evenly coated as that of fibres of PE filters (**Figure 6**). Most of the bacteria accumulated at the sides of the filter paper, while those on the filter paper appeared as specks of planktonic bacteria (**Figure 6**). Thinner PE filters (1 layer) were observed to have dense biofilm attachment while thicker layers (3 layers) had sparse biofilm attachment (**Figure 6**).

Figure 6: Formation of biofilm-like structures (Green: PAO1-GFP) were the most on 1-layered PE fibres. Biofilm-like structures (Green: PAO1-GFP) formed on filter paper and on 1 and 3-layered PE fibres were imaged using the Leica TCS SPE (Leica, Germany) with the 20 X dry objective. White dotted lines indicate the edge of the filter paper. Green specks are planktonic PAO1-GFP. Scale bar: 50 μ m.

Various biofilm growth timings (24 and 48 h), and static and shaking cultures were also explored. Shaking conditions (140 rpm, adapted from Schleheck et al., 2009) resulted in a more even distribution of biofilm growth on the fibres in 24 h (**Figure 7A**). In static conditions, the biofilm-like structures were only concentrated only at specific areas in 24 h, but the growths were thicker than that of shaking conditions only after 48 h (**Figure 7A**). Incubation with DMEM beyond 24 h did not really increase biofilm formation (**Figure 7B**).

Figure 7: Biofilm-like structures (Green: PAO1-GFP) formed on PE fibres at (A) 24 and 48 h and (B) 1, 2 and 3 days in either static or shaking cultures. One-layered PE filters were placed in TSB and PAO1-GFP was grown on it for 24 h. TSB was then changed to DMEM and incubated for 1, 2 or 3 days. The biofilm-like structures were imaged using the Leica TCS SPE (Leica, Germany) with the 20 X dry objective. Scale bar: 100 µm.

To verify if these structures are biofilms, PE filters were incubated in FilmTracer™ SYPRO Ruby Biofilm Matrix Stain and imaged. This stain stains biofilm matrices (Invitrogen) and has been proven to stain the proteins within the biofilm matrices of *P. aeruginosa* PAO1, *Porphyromonas gingivalis* and *S. aureus* (Mohammed et al., 2013; Oniciuc et al., 2016; Ahire et al., 2016; Sanchez et al., 2013b). Staining at the areas that coincide with biofilm-like structure formation were observed (**Figure 8**).

PAO1 in SYPRO stain

Figure 8: Structures formed on PE filters are biofilms (green). FilmTracer™ SYPRO Ruby Biofilm Matrix Stain was used to stain PAO1 biofilm matrix that is shown in green. Scale bar: 100 μm .

These results show that PAO1 BCM was optimised for testing its effects on cellular processes such as migration and inflammation, which will be elaborated on in chapters 3 and 4. For the downstream experiments, DMEM was used as controls, as it was the medium in which BCM was grown in.

3.2.2 Different dosages of PAO1 BCM evoke contrasting effects on cell migration

As cell migration is an important aspect of wound closure, the effects of PAO1 BCM on migration of both fibroblasts and keratinocytes were checked. To observe changes in cell morphology and migration, time-lapse scratch wound experiments were conducted. In keratinocytes, accumulated change in migration (in pixels) was reduced in dosages of PAO1 BCM that were 30 % and above (**Figures 9 and 10**). Cell shrinkage was clearly seen as early as 5 h post 100% BCM incubation, together with the shrinkage of the leading edge of the scratch (until it disappears from field of view), suggesting a loss of cell adhesive properties (**Figure 9**). A 50 % dosage at 5 h caused the cell layer to shrink and retreat, compared to control (**Figure 9**). Uptake of PI, a marker of membrane integrity loss, was observed at 5 h post incubation in dosages at 10 % and beyond in

keratinocytes (**Figure 9**). The accumulated change in migration (in pixels) was reduced in dosages of PAO1 BCM that were 30 % and above in fibroblasts (**Figures 11 and 12**). Fibroblasts were observed to be spindle-like as early as 5 h post BCM challenge at 50 % dosage and cell rounding at 75 % dosage (**Figure 11**). On the other hand, low concentrations of PAO1 BCM were found to cause increased migratory capability in both fibroblasts and keratinocytes compared to controls, although the effects were statistically insignificant ($P > 0.05$) (**Figures 9-12**). This time-lapse experiment, together with preliminary experiments on fixing and permeabilising cells on cover slips (steps prior to immunostaining and imaging of stained markers) were used to determine the concentrations of PAO1 BCM and timings to use for subsequent experiments.

Figure 9: Keratinocytes incubated with PAO1 BCM at different dosages displayed contrasting effects. 10 X objective images of scratch wound assay with contrasting effects of PAO1 BCM on keratinocytes at different dosages (Control, 1 %, 3 %, 10 %, 30 %, 50 %, 75 %, 100 % PAO1 BCM) taken at various timepoints (0, 5, 10, 15, 24 h). For a positive control, normal cell culture media (DMEM) was used. Red signal: PI stain. Scale bar= 200 μ m. Figure is representative of 3 biological and technical replicates (N=3, n=3) for each condition.

Figure 10: Graph of accumulated change in keratinocyte migration (pixels) against treatment time (h). Time-lapse images of scratch wound assay after incubating with respective concentrations of PAO1 BCM on keratinocytes were taken and the accumulated change in migration (pixels) were measured. For a positive control, normal cell culture media (DMEM) was used. The plots for 75 % and 100 % BCM were cut off at 2 h post-incubation as cells were out of frame after those timings respectively. The remaining plots were cut off at 10 h as both the leading edges have met for certain conditions. Statistical comparisons between the various BCM concentrations against the control were conducted using 2-way ANOVA and Tukey post hoc tests. Statistical significance obtained for 30 % BCM was ** at 5 h and *** at 6 h and beyond. 50 % BCM was * at 3 h and *** at 4 h and beyond. 75 % and 100 % BCM were *** at 2 h and beyond. The rest that were unmentioned were NS. (P-value: NS $P > 0.05$, * $P \leq 0.05$, ** $P < 0.01$, *** $P < 0.001$). Data expressed as mean $\pm$ SD. Figure is representative of 3 biological and technical replicates (N=3, n=3) for each condition.

Figure 11: Fibroblasts incubated with PAO1 BCM at different dosages displayed contrasting effects. 10 X objective images of scratch wound assay with contrasting effects of PAO1 BCM on fibroblasts at different dosages (Control, 1 %, 3 %, 10 %, 30 %, 50 %, 75 %, 100 % PAO1 BCM) taken at various timepoints (0, 5, 10, 15, 24 h). For a positive control, normal cell culture media (DMEM) was used. Scale bar= 200 μ m. Figure is representative of 3 biological and technical replicates (N=3, n=3) for each condition.

Figure 12: Graph of accumulated change in fibroblast migration (pixels) against treatment time (h). Time-lapse images of scratch wound assay after incubating with respective concentrations of PAO1 BCM on fibroblasts were taken and the accumulated change in migration (pixels) were measured. For a positive control, normal cell culture media (DMEM) was used. The plots for 30 %, 50 %, 75 % and 100 % BCM were cut off at 17 h post-incubation as cells were out of frame after those timings respectively. The remaining plots were cut off at 10 h as both the leading edges have met for certain conditions. Statistical comparisons between the various BCM concentrations against the control were conducted using 2-way ANOVA and Tukey post hoc tests. Statistical significance obtained for 30 % BCM was * at 10 h. 50 % BCM was * at 6 h and *** at 7 h and beyond. 75 % BCM was * at 5 h and *** at 6 h and beyond. 100 % BCM was ** at 4 h and *** at 5 h and beyond. The rest that were unmentioned were NS. (P-value: NS $P > 0.05$, * $P \leq 0.05$, ** $P < 0.01$, *** $P < 0.001$). Data expressed as mean $\pm$ SD. Figure is representative of 3 biological and technical replicates (N=3, n=3) for each condition.

Since PAO1 BCM at high dose hampers migration while low doses seem to have slight improvements in migration rates, the effects of PAO1 BCM in both the high and low doses on various wound healing responses were checked. Although the increase in migration elicited by low doses of PAO1 BCM was statistically insignificant, it is still informative to investigate the reasons behind a consistently faster migration rate caused by low doses of PAO1 BCM compared to controls.

3.2.3 Exposure to 50 % PAO1 BCM hampers directional migration by disrupting cytoskeletal dynamics

3.2.3.1 Phosphorylated vinculin was reduced in fibroblasts but not keratinocytes

Compared to controls, total vinculin levels were unchanged ($P > 0.05$) in keratinocytes and fibroblasts when incubated with 50 % PAO1 BCM (**Figures 13 and 14A, B**).

Contrastingly, the phosphorylated state was only reduced ($P < 0.01$) in 50 % PAO1 BCM-challenged fibroblasts after 5 h incubation (**Figures 13 and 14A, B, C**). Apart from vinculin levels, vinculin size was also found to be larger in fibroblasts ($P \leq 0.05$) that were incubated with 50% PAO1 BCM after 5 h, while it was unchanged ($P > 0.05$) for keratinocytes (**Figures 13 and 14D**). The low dose PAO1 BCM-challenged cells displayed no significant differences ($P > 0.05$) in total and phosphorylated protein vinculin levels, and vinculin sizes (**Figures 13 and 14**).

Figure 13: Hampered migration in keratinocytes not attributed to phosphorylated vinculin (Y1065) expression. (A) Images of cells taken under 40 X oil objective (Blue: DAPI, Green: Total vinculin, Red: Phosphorylated vinculin (Y1065)). Monolayer of cells were scratched, incubated for 5 h at respective conditions (Control-DMEM, 3 % and 50 % PAO1 BCM) and stained for vinculin and phosphorylated vinculin (Y1065) respectively). Scale bar: 25 μ m. Figure is representative of 3 biological and technical replicates (N=3, n=3) for each condition.

(B) Graph of normalised total vinculin pixel:nuclei ratio. Statistical comparisons between the various BCM concentrations against the control were conducted using 1-way ANOVA and Dunnett's post hoc tests. (P-value: NS $P > 0.05$). Data expressed as mean $\pm$ SD. Figure is representative of 3 biological and technical replicates (N=3, n=3) for each condition.

(C) Graph of normalised phosphorylated-vinculin pixel:nuclei ratio. Statistical comparisons between the various BCM concentrations against the control were conducted using 1-way ANOVA and Dunnett's post hoc tests. (P-value: NS $P > 0.05$). Data expressed as mean $\pm$ SD. Figure is representative of 3 biological and technical replicates (N=3, n=3) for each condition.

(D) Graph of average vinculin sizes. Statistical comparisons between the various BCM concentrations against the control were conducted using 1-way ANOVA and Dunnett's post hoc tests. (P-value: NS $P > 0.05$). Data expressed as mean $\pm$ SD. Figure is representative of 3 biological and technical replicates (N=3, n=3) for each condition.

Figure 14: Hampered migration in fibroblasts could be affected by decreased phosphorylated vinculin (Y1065) expression and increased vinculin sizes. (A) Images of cells taken under 40 X oil objective (Blue: DAPI, Green: Total vinculin, Red: Phosphorylated vinculin (Y1065)). Monolayer of cells were scratched, incubated for 5 h at respective conditions (Control-DMEM, 3 % and 50 % PAO1 BCM) and stained for vinculin and phosphorylated vinculin (Y1065) respectively (Blue: DAPI, Green: Total vinculin, Red: Phosphorylated vinculin (Y1065)). Scale bar: 25 μ m. Figure is representative of 3 biological and technical replicates (N=3, n=3) for each condition.

(B) Graph of normalised total vinculin pixel:nuclei ratio. Statistical comparisons between the various BCM concentrations against the control were conducted using 1-way ANOVA and Dunnett's post hoc tests. (P-value: NS $P > 0.05$). Data expressed as mean $\pm$ SD. Figure is representative of 3 biological and technical replicates (N=3, n=3) for each condition.

(C) Graph of normalised phosphorylated-vinculin pixel:nuclei ratio. Statistical comparisons between the various BCM concentrations against the control were conducted using 1-way ANOVA and Dunnett's post hoc tests. (P-value: NS $P > 0.05$, ** $P < 0.01$). Data expressed as mean $\pm$ SD. Figure is representative of 3 biological and technical replicates (N=3, n=3) for each condition.

(D) Graph of average vinculin sizes. Statistical comparisons between the various BCM concentrations against the control were conducted using 1-way ANOVA and Dunnett's post hoc tests. (P-value: NS $P > 0.05$). Data expressed as mean $\pm$ SD. Figure is representative of 3 biological and technical replicates (N=3, n=3) for each condition.

3.2.3.2 50 % PAO1 BCM inhibits Golgi re-orientation towards the leading edge

Since vinculin expression and activation is linked to the activation of the Arp2/3 complex, which has downstream effects on cell polarity, the effects of PAO1 BCM on cell polarity were checked (Magdalena et al., 2003). During cell repolarisation, the microtubule-organising center (MTOC) and the Golgi apparatus are reorientated towards the leading edge, causing the cell to migrate in the direction of polarisation (Darido and Jane., 2013; Kupfer et al., 1982).

When incubated with a high dose of PAO1 BCM (50 %), Golgi re-positioning was inhibited in both keratinocytes ($P < 0.001$) and fibroblasts ($P \leq 0.05$) at the wound edge compared to their respective controls (**Figures 15 and 16**). There was also a significant reduction ($P < 0.001$) in percentage of re-positioned Golgi incubated with 50 % PAO1 BCM at 5 h compared to 3 h in keratinocytes (**Figure 15**). When incubated with a low dose of PAO1 BCM (3 %), there was no significant difference ($P > 0.05$) compared to controls (**Figures 15 and 16**).

Figure 15: Golgi re-orientation is inhibited in 50 % PAO1 BCM-incubated keratinocytes. (A) Images of cells taken under 40 X oil objective (Blue: DAPI, Green: Gm130-cis-golgi matrix protein). Monolayer of cells were scratched, incubated for 3 h and 5 h at respective conditions (Control-DMEM, 3 % and 50 % PAO1 BCM) and stained for Gm130. The Golgi apparatus is identified through Gm130 staining. The orientation of the Golgi with respect to the leading edge is measured as an angle and is considered as re-orientated if the angle falls within 120 °. White dotted lines indicate the *in vitro* wound edge that was made by the scratch and white arrowheads indicate the re-oriented Golgi. Scale bar: 25 μm. Figure is representative of 3 biological and technical replicates (N=3, n=3) for each condition. (B) Graph of percentage re-orientated Golgi. The percentage of cells with Golgi located in the 120 ° arc facing the wound were scored as positive (polarised). An average of 450 cells were evaluated per condition. Statistical comparisons between the various conditions were conducted using 1-way ANOVA and Tukey post hoc tests. (P-value: NS P > 0.05, *** P<0.001). Data expressed as mean ± SD. Figure is representative of 3 biological and technical replicates (N=3, n=3) for each condition.

Figure 16: Golgi re-orientation is inhibited in 50 % PAO1 BCM-incubated fibroblasts. (A) Images of cells taken under 40 X oil objective (Blue: DAPI, Green: Gm130-cis-golgi matrix protein). Monolayer of cells were scratched, incubated for 3 h and 5 h at respective conditions (Control-DMEM, 3 % and 50 % PAO1 BCM) and stained for Gm130. The Golgi apparatus is identified through Gm130 staining. The orientation of the Golgi with respect to the leading edge is measured as an angle and is considered as re-orientated if the angle falls within 120 °. White dotted lines indicate the *in vitro* wound edge that was made by the scratch and white arrowheads indicate the re-oriented Golgi. Scale bar: 25 μm. Figure is representative of 3 biological and technical replicates (N=3, n=3) for each condition.

(B) Graph of percentage re-orientated Golgi. The percentage of cells with Golgi located in the 120 ° arc facing the wound were scored as positive (polarised). An average of 450 cells were evaluated per condition. Statistical comparisons between the various BCM concentrations against the controls were conducted using 1-way ANOVA and Dunnett's post hoc tests. (P-value: NS P > 0.05, * P ≤ 0.05). Data expressed as mean ± SD. Figure is representative of 3 biological and technical replicates (N=3, n=3) for each condition.

3.2.3.3 PAO1 BCM at 50 % dose impedes lamellipodia protrusions

To validate that PAO1 BCM hampers migration, keratinocytes and fibroblasts were stained with α -tubulin, a microtubule component involved in cellular processes such as mitosis, migration, intracellular transport and mechanical support (Alieva et al., 2010). Lamellipodia protrusions of cells drives cell migration as it provides the traction to move cells forward (DeMali et al., 2002; Krause and Gautreau., 2014).

In fibroblasts, the morphology of the α -tubulin filaments was orderly arranged in all conditions, no matter whether PAO1 BCM was incubated with the cells or not (**Figure 17**). However, the MTOC centres, which are depicted by the bright aggregation of α -tubulin filaments are only observed in control cells and cells incubated with 3 % PAO1 BCM after 5 h incubation (**Figure 17**). They were not observed in the 50 % PAO1 BCM condition (**Figure 17**). The measurements of lamellipodia sizes showed that 50 % PAO1 BCM causes a significant decrease in lamellipodia length compared to controls in both 3 (P<0.01) and 5 h (P<0.001) post-incubation (**Figure 17**). In comparison, 3 % PAO1 BCM caused no significant differences (P > 0.05) in lamellipodia sizes compared to controls in both 3- and 5 h post-incubation (**Figure 17**).

In keratinocytes, the morphology of the α -tubulin filaments seemed to be less well-organised in 50 % PAO1 BCM compared to 3 % PAO1 BCM and controls (**Figure 18**). The 50 % PAO1 BCM also caused a significant decrease (P<0.001) in lamellipodia sizes compared to controls in both 3 and 5 h post-incubation (**Figure 18**). In comparison, 3 % PAO1 BCM caused no significant differences (P > 0.05) in lamellipodia sizes compared to controls (**Figure 18**).

Figure 17: Lamellipodia protrusions are hampered in 50 % PAO1 BCM-incubated fibroblasts. (A) Images of cells taken under 40 X oil objective (Blue: DAPI, Green: α -tubulin). Monolayer of cells were scratched, incubated for 3 h and 5 h at respective conditions (Control-DMEM, 3 % and 50 % PAO1 BCM) and stained for α -tubulin. Arrows depict the lamellipodia and the varied lamellipodia length, while arrowheads depict the MTOC centers. Scale bar: 25 μ m. Figure is representative of 3 biological and technical replicates (N=3, n=3) for each condition.

(B) Graph of lamellipodia area (μ m²). Statistical comparisons between the various conditions were conducted using 1-way ANOVA and Tukey post hoc tests. (P-value: NS P > 0.05, ** P<0.01, *** P<0.001). Data expressed as mean $\pm$ SD. Figure is representative of 3 biological and technical replicates (N=3, n=3) for each condition.

Figure 18: Lamellipodia protrusions are hampered in 50 % PAO1 BCM-incubated keratinocytes. (A) Images of cells taken under 40 X oil objective (Blue: DAPI, Green: α -tubulin). Monolayer of cells were scratched, incubated for 3 h and 5 h at respective conditions (Control-DMEM, 3 % and 50 % PAO1 BCM) and stained for α -tubulin. Arrows depict the lamellipodia and the varied lamellipodia length. Scale bar: 25 μm . Figure is representative of 3 biological and technical replicates (N=3, n=3) for each condition. (B) Graph of lamellipodia area (μm^2). Statistical comparisons between the various conditions were conducted using 1-way ANOVA and Tukey post hoc tests. (P-value: NS P > 0.05, *** P<0.001). Data expressed as mean $\pm$ SD. Figure is representative of 3 biological and technical replicates (N=3, n=3) for each condition.

In summary, these results show that at high doses, PAO1 BCM hampers cell migration through preventing proper Golgi re-orientation towards the leading edge and impeding lamellipodia protrusion. Additionally, hampered migration in fibroblasts could be

affected by decreased phosphorylated vinculin (Y1065) expression and increased vinculin sizes.

3.2.4 Secreted factors of PAO1 biofilms contribute to cellular toxicity

To demonstrate that the detrimental effects of PAO1 BCM on fibroblasts and keratinocytes were not nutrient-related, PAO1 BCM was replaced with PBS as a control. The MTT assay conducted on fibroblasts demonstrated that presence of only 25 % DMEM was nutritionally sufficient for cells to survive for at least 8 h (**Figure 19**). However, the cell viability of PAO1 BCM-challenged cells was worse than their respective controls (**Figure 19**). This shows that the effects of PAO1 BCM on cells were mostly due to the biofilm secretions rather than nutrient deprivation of the cell culture media.

Figure 19: PAO1 BCM-treated cells experiences a lower cell viability compared to cells treated with PBS of the same concentrations. Different conditions were tested using the MTT assay for a period of 1, 4, 8 h respectively to show that PAO1 BCM effects were not due to nutrient deprivation of the culture media. For a positive control, the MTT reagent was diluted in DMEM and lysis buffer in a well containing cells, while the negative control was prepared the same way without cells. Treatment results were normalised to the positive control of 1. Statistical analyses using 2-way ANOVA and Tukey's post hoc tests were obtained by comparing treatments of the same concentrations after the respective treatment timings (e.g. 30% PBS + 70% DMEM VS 30% PAO1 BCM + 70% DMEM) (P-value: *** P<0.001, ** P<0.01, * P<0.05). Data has been expressed as mean $\pm$ SD. Figure is representative of 3 biological and technical replicates (N=3, n=3) for each condition.

3.2.5 Exposure to 50 % PAO1 BCM elicits apoptosis in keratinocytes and promotes fibroblasts' resistance to apoptosis and necrosis

3.2.5.1 Cell viability is attenuated by PAO1 BCM dose-dependently with time

To determine the effect of PAO1 BCM on cell viability of fibroblasts and keratinocytes, MTT assays were conducted. Doses of BCM at 10 % and above led to significant decrease in cell viability in keratinocytes (**Figure 20A**). Statistical significance increased dose- and time-dependently (**Figure 20A**). However, low doses of BCM (1 and 3 %) resulted in unchanged cell viability in keratinocytes, until at 24 h where 3 % BCM-challenged cells saw a decrease in viability (**Figure 20A**).

Similarly, in fibroblasts, cell viability decreased in a dose- and time-dependent manner (**Figure 20B**). In earlier timepoints of 1 and 4 h post-incubation, fibroblasts did not demonstrate significant differences ($P>0.05$) between the different conditions (**Figure 20B**). At 8 h post-incubation, doses of BCM at 30 % and beyond produced decreased cell viability which was even more significant at 24 h post-incubation with fibroblasts (**Figure 20B**).

Comparatively, keratinocytes experience a decrease in cell viability faster than that of fibroblasts (**Figure 20C**). As early as 1 h post-incubation, 50 % BCM-challenged keratinocytes displayed a significant loss of cell viability compared to fibroblasts ($P<0.001$) (**Figure 20C**). When incubated with lower doses of BCM, 3 % BCM-challenged keratinocytes showed a significant loss ($P<0.001$) of cell viability at 24 h post-incubation compared to fibroblasts (**Figure 20C**).

Figure 20 (A, B, C): Higher concentrations of PAO1 BCM had a significant negative effect on cell viability of (A) keratinocytes and (B) fibroblasts, but (C) keratinocytes were more negatively affected compared to fibroblasts when subjected to the same treatments. Log concentrations of PAO1-BCM were incubated for 1, 4, 8 and 24 h on the respective cell lines in an MTT assay. For a positive control, the MTT reagent was diluted in DMEM and lysis buffer in a well containing cells, while the negative control was prepared the same way without cells. Treatment results were normalised to the positive control of 1. Statistical analyses using 2-way analysis of variance (ANOVA) and Tukey's post hoc tests were performed amongst all conditions (P-value: *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, NS $P > 0.05$). Data has been expressed as mean $\pm$ standard deviation (SD). Figure is representative of 3 biological and technical replicates ($N=3$, $n=3$) for each condition.

(A)- Statistical significance for 1 and 3 % BCM against the control are NS unless labelled. Statistical significance for 10 % -100 % BCM against the control are *** unless labelled.

(B) Statistical significance for 1, 3 and 10 % BCM against the control are NS unless labelled. Statistical significance for 30 % BCM against the control at 8 h is *. Statistical significance obtained for 50 % BCM against the control was ** at 8 h and beyond. 75 and 100 % BCM against the control were ** at 8 h and *** at 24 h. The rest that were unmentioned were NS. (P-value: NS $P > 0.05$, * $P \leq 0.05$, ** $P < 0.01$, *** $P < 0.001$).

(C) Statistical analyses using 2-way analysis of variance (ANOVA) and Tukey's post hoc tests were performed amongst all conditions (P-value: NS $P > 0.05$, ** $P < 0.01$, *** $P < 0.001$).

3.2.5.2 PAO1 BCM induces cell death in keratinocytes via apoptosis

Since cell viability was compromised in keratinocytes and fibroblasts, the cell death pathways that were involved in eliciting reduced cell viability were determined. Two common cell death pathways were checked, namely apoptosis and necrosis. Apoptosis was checked through the presence of cleaved caspase 3, an executioner caspase whose

cleavage activates the apoptotic pathway (Riedl and Shi., 2004; McIlwain et al., 2013). Necrosis, a more advanced form of apoptosis, was checked through the marker high mobility group box 1 protein (HMGB1) (Raucci et al., 2007). HMGB1 is usually located in the nucleus of cells and is only localised in the cytoplasm and then secreted upon initiation of necrosis (Raucci et al., 2007).

Incubation of 50 % PAO1 BCM with keratinocytes caused cleaved caspase 3 to be detected (**Figure 21**). However, the presence of cleaved caspase 3 was not observed in fibroblasts (**Figure 21**). When stained for HMGB1, both keratinocytes and fibroblasts incubated with 50 % PAO1 BCM did not show any signs of HMGB1 cytoplasmic localisation nor presence in supernatant (**Figure 22**).

Figure 21: PAO1 BCM causes apoptosis in keratinocytes, but not fibroblasts. (A) Images of cells taken under 40 X oil objective (Blue: DAPI, Red: Cleaved caspase 3). 0.5 µM staurosporine incubated for 5 h was used as a positive control for apoptosis and the negative control was just cells incubated with DMEM. A 10 X objective image was included to depict the extent of apoptosis in a field of view. Scale bar: 25 µm. Figure is representative of 3 biological and technical replicates (N=3, n=3) for each condition.

Figure 22: PAO1 BCM does not cause necrosis. (A) Images of cells taken under 40 X oil objective (Blue: DAPI, Red: HMGB1). 0.5 µM staurosporine incubated for 7 h was used as a positive control for necrosis and the negative control was just cells incubated with DMEM. White arrows represent the cytoplasmic localisation of HMGB1 in cells (non-exhaustive of the number of cells having cytoplasmic HMGB1). Scale bar: 25 µm. Figure is representative of 3 biological and technical replicates (N=3, n=3) for each condition.

3.2.5.3 Transient exposure to PAO1 BCM causes irreversible damage to keratinocytes but not fibroblasts after 1 day post exposure

As PAO1 BCM was shown to elicit loss of cell viability in both keratinocytes and fibroblasts, we wanted to determine if this loss was reversible if it was just a transient exposure. This would mimic the wound environment where biofilms were debrided and then allowed to heal in an *in vitro* setting. To determine if the impact of wounding plays a role in causing more damage to the cells, experiments to compare both scratch and

non-scratch wounds *in vitro* were conducted. Cells were incubated for 5 h and then the media was replaced with fresh complete DMEM and left for 1 or 2 days. The amount of damage was determined qualitatively through the PI signal, but it was not quantified as cell lysis would cause of the leakage of the dye into the surrounding medium, causing it to be inaccurate.

Results showed that scratch wounds caused slight damage to the keratinocytes compared to the non-scratch areas, as seen by the presence of PI signal in control cells (**Figure 23A**). Upon challenge with 50 % PAO1 BCM, there were many rounded cells with PI signals detected in keratinocytes at 12 h post-wounding onwards compared to control cells (**Figure 23**). To determine if this was a short- or long-term phenomenon, the time-lapse was left to go for a span of 2 days post-media change. The scratch wound eventually closed around 1 day post-media change in controls and 2 days in 50 % PAO1 BCM-challenged keratinocytes (**Figure 24**).

In fibroblasts, challenge with 50 and 75 % PAO1 BCM caused rounding up of cells and cytoplasmic shrinkage to become spindle-like but no PI was taken up (**Figure 25**). Upon media change, fibroblasts incubated with BCM regained their lamellipodia sizes and proliferated similar to that of controls (**Figure 25**). When left for a span of 2 days, 50 % BCM-incubated cells migrated to close the wound after 1 day post-media change, similar to that of controls (**Figure 26**). However, cells that have been incubated with 75 % BCM did not close the wound even after 2 days post-media change (**Figure 26**).

Figure 23: Transient exposure to PAO1 BCM causes irreversible damage to keratinocytes after 24 h. (A, B) 20 X objective images of scratch and non-scratch wound areas taken at various timepoints. DMEM (A) or 50 % BCM (B) was incubated with cells for 5 h before switching to fresh DMEM and then incubated for another 24 h. For a positive control, normal cell culture media (DMEM) was used. Red signal: PI stain. Scale bar: 75 μ m. Figure is representative of 3 biological and technical replicates (N=3, n=3) for each condition.

Figure 24: Transient exposure to PAO1 BCM still closes scratch wound in keratinocytes after 2 days, albeit slower than controls.

(A, B) 10 X objective images of scratch and non-scratch wound areas taken at various timepoints. DMEM (A) or 50 % BCM (B) was incubated with cells for 5 h before switching to fresh DMEM and then incubated for another 2 days. For a positive control, normal cell culture media (DMEM) was used. Red signal: PI stain. Scale bar: 200 μ m. Figure is representative of 3 biological and technical replicates (N=3, n=3) for each condition.

Figure 25: Transient exposure to PAO1 BCM causes reversible damage to fibroblasts. (A, B, C) 20 X objective images of scratch and non-scratch wound areas taken at various timepoints. DMEM (A), 50 % BCM (B), or 75 % BCM (C) was incubated with cells for 5 h before switching to fresh DMEM and then incubated for another 24 h. For a positive control, normal cell culture media (DMEM) was used. Scale bar: 75 μ m. Figure is representative of 3 biological and technical replicates (N=3, n=3) for each condition.

Figure 26: Transient exposure to PAO1 BCM causes reversible damage to fibroblasts. (A, B, C) 10 X objective images of scratch and non-scratch wound areas taken at various timepoints. DMEM (A), 50 % BCM (B), or 75 % BCM (C) was incubated with cells for 5 h before switching to fresh DMEM and then incubated for another 2 days. For a positive control, normal cell culture media (DMEM) was used. Scale bar: 200 μ m. Figure is representative of 3 biological and technical replicates (N=3, n=3) for each condition.

3.2.6 Expression of gap junctions is dysregulated by PAO1 BCM

Connexins (Cx) have been reported to regulate cytoskeletal elements, correlated to wound healing status and propagate death signals through their channels (Mori et al., 2006; Cogliati et al., 2015 Udawatte et al., 2005; Cusato et al., 2006; Krysko et al., 2005; Mendoza-Naranjo et al., 2012; Matsuuchi and Naus., 2013). Results showed that the expression of Cx43 in keratinocytes challenged with 50 % BCM were not significantly

different ($P > 0.05$) from that of controls (**Figure 27**). However, in low dose incubated keratinocytes, Cx43 expression was significantly increased in both 3 ($P < 0.01$) and 5 ($P \leq 0.05$) h timepoints (**Figure 27**).

In fibroblasts, Cx43 expression was lower in 50 % PAO1 BCM-challenged cells at 3 h post-incubation, but it was not significant ($P \leq 0.05$) until it reached the 5th h (**Figure 28**). Moreover, in cells incubated with 3 % PAO1 BCM, Cx43 expression was unchanged ($P < 0.01$) in the 3rd h, but significantly increased by the 5th h (**Figure 28**). Additionally, most of the cytoplasmic localised Cx43 observed in control cells were lost in 50 % BCM-challenged cells at 5 h post-incubation (**Figure 28A**).

Figure 27: 3 % PAO1 BCM-challenged keratinocytes experience increased Cx43 levels. (A) Images of cells taken under 40 X oil objective (Blue: DAPI, Green: α -tubulin, Red: Cx43). Monolayer of cells were scratched, incubated for 3 h and 5 h at respective conditions (Control-DMEM, 3 % and 50 % PAO1 BCM) and stained for α -tubulin and Cx43. Scale bar: 25 μ m. Figure is representative of 3 biological and technical replicates (N=3, n=3) for each condition.

(B) Graph of Cx43 expression. Statistical comparisons between the various conditions were conducted using 1-way ANOVA and Tukey post hoc tests. (P-value: NS $P > 0.05$, * $P \leq 0.05$, ** $P < 0.01$, *** $P < 0.001$). Data expressed as mean $\pm$ SD. Figure is representative of 3 biological and technical replicates (N=3, n=3) for each condition.

Figure 28: 50 % PAO1 BCM-incubated fibroblasts experience reduced Cx43 levels at 5 h post-incubation. (A) Images of cells taken under 40 X oil objective (Blue: DAPI, Green: α -tubulin, Red: Cx43). Monolayer of cells were scratched, incubated for 3 h and 5 h at respective conditions (Control-DMEM, 3 % and 50 % PAO1 BCM) and stained for α -tubulin and Cx43. Scale bar: 25 μ m. Figure is representative of 3 biological and technical replicates (N=3, n=3) for each condition.

(B) Graph of Cx43 expression. Statistical comparisons between the various conditions were conducted using 1-way ANOVA and Tukey post hoc tests. (P-value: NS $P > 0.05$, * $P \leq 0.05$, ** $P < 0.01$, *** $P < 0.001$). Data expressed as mean $\pm$ SD. Figure is representative of 3 biological and technical replicates (N=3, n=3) for each condition.

Taken together, high doses of PAO1 BCM reduce cell viability in both keratinocytes and fibroblasts. This reduction in cell viability seems to be more irreversible in keratinocytes than fibroblasts. Lastly, gap junction levels (that control cellular communication) are upregulated and downregulated for keratinocytes and fibroblasts respectively, which could play a role in cell death signal propagation.

3.2.7 Exposure to secreted factors of PAO1 biofilms disrupts cell-cell adhesion by dysregulating junctional proteins

3.2.7.1 Cell-cell adhesion of fibroblasts is negatively affected by PAO1 BCM compared to keratinocytes

To determine if PAO1 BCM has an effect on the cell-cell adhesion of skin cells, a hanging drop assay was conducted to measure the strength of cell-cell adhesion between cells through the visualisation and quantification of cell clump sizes. In keratinocytes, the cell clump sizes of all conditions (Control, 3% PAO1 BCM and 50 % PAO1 BCM) were similar ($P > 0.05$) (**Figure 29**). From the visual observations and graph plotted for cell clump size quantification, it is notable, albeit insignificant, that the cell clumps of 3 % PAO1 BCM were slightly smaller than that of controls, as shown by the more compact visual observation and decreased cell clump size (**Figure 29**).

In fibroblasts, the cell clump sizes of control and 3 % PAO1 BCM-challenged cells were similar, but the gaps between the cells within the clump were greater than controls (**Figure 29**). Upon incubation with 50 % PAO1 BCM, the cell clump sizes of fibroblasts were observed to be significantly decreased ($P \leq 0.05$) (**Figure 29**). The cell clumps of keratinocytes were also noted to be significantly larger ($P < 0.001$) than the fibroblasts, despite having used the same cell count for all experiments (**Figure 29**).

Figure 29: Cell-cell adhesion of fibroblasts is negatively affected by PAO1 BCM compared to keratinocytes in a hanging drop assay. (A) Images of cells taken under 10 X objective. Cell clumps were incubated in the respective conditions (DMEM, 3 % PAO1 BCM and 50 % PAO1 BCM) and suspended in a hanging drop assay. Cells were then resuspended to determine the size of cell clumps. The greater the cell clump after resuspension, the stronger the cell-cell adhesion. Scale bar: 250 µm. Figure is representative of 3 biological and technical replicates (N=3, n=3) for each condition.

(B) Graph of cell clump sizes. Statistical comparisons between the various conditions were conducted using 1-way ANOVA and Tukey post hoc tests. (P-value: NS $P > 0.05$, * $P \leq 0.05$, ** $P < 0.01$, *** $P < 0.001$). Data expressed as mean $\pm$ SD. Figure is representative of 3 biological and technical replicates (N=3, n=3) for each condition.

3.2.7.2 Dysregulated tight junctions and adherens junctions are found in PAO1 BCM-incubated fibroblasts and keratinocytes

Since vinculin (focal adhesion) expression and size were affected by PAO1 BCM, I then wanted to determine if tight junctions (Occludin) and adherens junctions (N-cadherin, E-cadherin, β -catenin) were influenced by BCM. Tight junctions are intercellular barriers between epithelial cells while adherens junctions are important for the maintenance of adhesions between cells (Schneeberger and Lynch., 2004; Yamazaki et al., 2008; Golovkine et al., 2018; Hartsock and Nelson., 2008).

Occludin expression was examined in both fibroblast and keratinocytes. In fibroblasts, occludin expression was significantly decreased ($P < 0.001$) and seems to be lost from the membranes in 50 % BCM-challenged cells compared to controls (**Figure 30**). Low dose BCM (3 %) did not affect ($P > 0.05$) the expression of occludin in fibroblasts (**Figure 30**). In keratinocytes, occludin expression was significantly increased ($P < 0.001$) in low dose BCM-incubated cells, while decreased ($P < 0.001$) in high dose BCM-incubated cells (**Figure 30**).

Figure 30: High dose of PAO1 BCM decreases occludin expression in fibroblasts and keratinocytes. Low dose of PAO1 BCM increases occludin expression in keratinocytes only. (A) Images of cells taken under 40 X oil objective (Blue: DAPI, Red: Occludin). Monolayer of cells were scratched, incubated for 5 h at respective conditions (Control-DMEM, 3 % and 50 % PAO1 BCM) and stained for occludin. Scale bar: 25 μ m. Figure is representative of 3 biological and technical replicates (N=3, n=3) for each condition.

(B) Graph of occludin expression. Statistical comparisons between the various conditions were conducted using 1-way ANOVA and Tukey post hoc tests. (P-value: NS $P > 0.05$, * $P \leq 0.05$, ** $P < 0.01$, *** $P < 0.001$). Data expressed as mean $\pm$ SD. Figure is representative of 3 biological and technical replicates (N=3, n=3) for each condition.

β -catenin is part of the adherens junctions family and it connects cadherins to the actin cytoskeleton (Chattopadhyay et al., 2003). It is involved in the stabilisation of cell-cell contacts and tissue homeostasis and repair (Chattopadhyay et al., 2003; Cheon et al., 2006; Whyte et al., 2012). Immunocytochemistry of β -catenin showed that control keratinocytes had β -catenin localised at the cell membranes, with slightly higher expression at the wound edge (**Figure 31**). In contrast, 50 % PAO1 BCM-challenged keratinocytes had an overall upregulation of β -catenin expression at the membranes (**Figure 31**). Overall, the expression of β -catenin incubated with PAO1 BCM was unchanged compared to controls ($P > 0.05$ for both 3 and 50 % PAO1 BCM) (**Figure 31**).

Conversely, untreated fibroblasts showed that β -catenin expression was uniformly cytoplasmic (**Figure 31**). Upon treatment, there was an overall downregulation of cytoplasmic β -catenin expression, where it was significantly downregulated ($P < 0.01$) in 50 % PAO1 BCM-challenged fibroblasts (**Figure 31**).

Figure 31: PAO1 BCM only decreases β -catenin expression in fibroblasts, while it stabilises membrane localisation of β -catenin in keratinocytes at 50 % dosage. (A) Images of cells taken under 40 X oil objective (Blue: DAPI, Red: β -catenin). Monolayer of cells were scratched, incubated for 5 h at respective conditions (Control-DMEM, 3 % and 50 % PAO1 BCM) and stained for β -catenin. Scale bar: 25 μ m. Figure is representative of 3 biological and technical replicates (N=3, n=3) for each condition. (B) Graph of β -catenin expression. Statistical comparisons between the various conditions were conducted using 1-way ANOVA and Dunnett's post hoc tests. (P-value: NS $P > 0.05$, * $P \leq 0.05$, ** $P < 0.01$, *** $P < 0.001$). Data expressed as mean $\pm$ SD. Figure is representative of 3 biological and technical replicates (N=3, n=3) for each condition.

Other members of the adherens junction family were checked, namely N-cadherin and E-cadherin. Results demonstrated that PAO1 BCM caused a significant decrease in N-cadherin expression in fibroblasts (3 % PAO1 BCM- $P \leq 0.05$, 50 % PAO1 BCM- $P < 0.001$), especially in the membranes, in both low and high doses (**Figure 36**).

Conversely, E-cadherin expression was unaffected by PAO1 BCM incubation ($P > 0.05$) but seemed to be more cytoplasmic than membranous at a dosage of 50 % BCM compared to controls (**Figure 32**).

Figure 32: PAO1 BCM decreases N-cadherin expression in fibroblasts, while it seems to cause E-cadherin to be cytoplasmic at 50 % dosage. (A) Images of cells taken under 40 X oil objective (Blue: DAPI, Green: E-cadherin). Monolayer of cells were scratched, incubated for 5 h at respective conditions (Control-DMEM, 3 % and 50 % PAO1 BCM) and stained for E-cadherin. Scale bar: 25 μ m. Figure is representative of 3 biological and technical replicates (N=3, n=3) for each condition.

(B) Graph of E-cadherin expression. Statistical comparisons between the various conditions were conducted using 1-way ANOVA and Dunnett's post hoc tests. (P-value: NS $P > 0.05$, * $P \leq 0.05$, ** $P < 0.01$, *** $P < 0.001$). Data expressed as mean $\pm$ SD. Figure is representative of 3 biological and technical replicates (N=3, n=3) for each condition.

Collectively, cell-cell adhesion is disrupted in PAO1 BCM-challenged fibroblasts compared to keratinocytes. This is further supported by a reduction in occludin, β -catenin and N-cadherin levels. Whereas keratinocytes saw an increase in occludin levels, coupled with stabilisation of β -catenin at the membranes and cytoplasmic E-cadherin.

3.2.8 Optimisation of wounding model

3.2.8.1 Direct application of BCM on wounds did not cause any significant changes to wound closure at D3 post-wounding

Direct application of BCM to wounds was performed on mice and macroscopic monitoring of the wound size was conducted on D0 (day of wounding) and D3 (day of harvest). This was the most ideal of all BCM application choices as the state of the BCM was unmodified (remained as a liquid form). Macroscopic wound comparisons showed that at D3 post wounding, there was no difference in wound sizes of control wounds and wounds with 100 % PAO1 BCM them (**Figure 33**).

Figure 33: Direct application of BCM on wounds did not see a difference in wound closure macroscopically *in vivo* at day 3 post-wounding. Macroscopic wound images were taken at the day of wounding, day 0 (D0) and at day 3 post-wounding (D3). White dotted lines represent original wound sizes at D0. Scale bar: 0.3 cm.

3.2.8.2 30 % Pluronic in BCM did not cause any significant changes to wound closure at D3 post-wounding

BCM was then applied to wounds in the form of a gel, as a gel provided stability during application and will not flow out of the wound easily. Pluronic F127 was chosen due to its thermoreversible properties which made it easy for application to wounds, and it would stay inside the wound, allowing the volume delivered to the wounds to be consistent (Cunha-Filho et al., 2012). Macroscopically, there was no difference in wound sizes between controls and 100 % PAO1 BCM application (**Figure 34**).

Figure 34: 30 % pluronic in 100 % BCM on wounds showed similar wound closure macroscopically *in vivo* at day 3 post-wounding. Macroscopic wound images were taken at the day of wounding, day 0 (D0) and at day 3 post-wounding (D3). White dotted lines represent original wound sizes at D0. Scale bar: 0.3 cm.

3.2.8.3 Delivery of 100 % PAO1 BCM using plasters preserves the toxicity of BCM on cells

Wound dressings (in the form of a plaster) were used to absorb the BCM and then apply the plasters to the wounds. Plasters that are already in the market were used (Guardian, Watsons and Hansaplast) for the experiment. They were compared for their waterproof ability so that there is no absorption of exogenous liquid and leakage of internal liquid. They were also compared for the absorbance ability of the plaster's absorbent pad so that as much BCM could be applied to the wound.

Results showed that plasters from Guardian showed the best liquid absorbance ability as their absorbent pads were able to absorb the most amount of liquid (300 μ l) compared to the other plasters (**Figure 35**). Moreover, plasters from Guardian were the most waterproof, where liquid absorbed were unable to leak out of the plaster through the adhesive portion of the plaster and liquid is unable to enter the absorbent pad as well (**Figure 35**). Comparatively, plasters from Watsons and Hansaplast had a lot of liquid leakage through the adhesive portion of the plaster (**Figure 35**).

Bioactivity of the BCM that has been absorbed by the plaster was tested through an MTT assay to measure the amount of toxicity present in the BCM. This is to further confirm that the plaster is inert and did not modify any properties of the BCM when absorbed. MTT assay results showed that the BCM absorbed by plasters was still toxic compared to DMEM ($P < 0.001$) and the level of toxicity of the BCM still remained similar ($P > 0.05$) to that of native BCM (**Figure 36**).

Figure 35: Plasters from Guardian absorbs the most liquid and does not allow leakage. Images of plasters from various brands after application of media on the absorbent pads for absorption of the liquid, and of plasters that have been lifted to determine how much liquid leakage there is from the absorbent pads. Graph of amount of liquid absorbed amongst the 3 brands was plotted. 100 µl liquid was applied at a time using a pipette till the liquid starts flowing out of the absorbent pads and the amount being absorbed was recorded.

Figure 36: (A, B) MTT assay revealed that BCM absorbed by plasters still evoke a similar response to cells in terms of cytotoxicity. 100 % DMEM and BCM were added to the absorbent pads of plasters respectively and then collected back and added to cells for testing their bioactivities. For a positive control, the MTT reagent was diluted in DMEM and lysis buffer in a well containing cells, while the negative control was prepared the same way without cells. Treatment results were normalised to the positive control of 1. Statistical analyses using 1-way analysis of variance (ANOVA) and Tukey's post hoc tests were performed against their non-plaster controls (P-value: *** P<0.001, * P<0.05, NS P>0.05). Data has been expressed as mean $\pm$ standard deviation (SD). Figure is representative of 3 biological and technical replicates (N=3, n=3) for each condition.

3.2.9 Wound closure is similar in control and PAO1-BCM challenged wounds

Since the bioactivity of BCM remained the same, *in vivo* experiments on mice were conducted and wound measurements chartered at days 0, 3 and 7 post-wounding. Macroscopic wound comparisons showed that the size of wounds incubated with 1 X 100% PAO1 BCM were similar (P>0.05) at days 3 and 7 post-wounding compared to controls (**Figure 37**). Macroscopic wound comparisons showed that the size of wounds incubated with 10 X 100% PAO1 BCM were similar (P>0.05) at day 3 post-wounding compared to controls (**Figure 37**).

Figure 37: Application of BCM on wounds using plasters showed similar wound closure macroscopically *in vivo* at days 3 (A) and 7 (B) post-wounding. Macroscopic wound images were taken and the graph of percentage wound closure plotted in (C). White dotted lines represent original wound sizes at D0. Scale bar: 0.5 cm. Statistical analyses using 1-way analysis of variance (ANOVA) and Tukey's post hoc tests were performed to compare the differences in decrease or increase in wound closure percentage (P-value: NS $P > 0.05$). Data has been expressed as mean $\pm$ standard deviation (SD). For days 0 and 3, figure is representative of 4 biological replicates (N=3) for control wounds, 6 biological replicates (N=6) for 100 % BCM wounds. For day 7 wounds, figure is representative of 3 biological replicates (N=3) for all conditions.

Although all 3 methods showed that macroscopic wound closure did not differ between control wounds and BCM-challenged wounds, the application of BCM on wounds using plasters was chosen as the way to deliver BCM to wounds in this thesis.

3.2.10 Hampered re-epithelialisation with intact E-cadherin junctions observed in D3 PAO1 BCM-challenged wounds

Re-epithelialisation was used as a measure of wound closure and was determined by the length of epithelial tongue formed. Histological analyses were conducted on control wounds and wounds challenged with BCM. Visual observations show that BCM-challenged wounds had hampered re-epithelialisation ($P < 0.05$ for 10 X 100 % PAO1 BCM wounds) compared to controls (**Figure 38**). Intact E-cadherin junctions were also observed in D3 PAO1 BCM-challenged wounds, similar to controls (**Figure 39**).

Figure 38: Normal re-epithelialisation observed in BCM-challenged wounds compared to controls.
 (A) Images of H&E stained tissues were taken at day 3 post-wounding (D3). Dotted lines represent the epithelial tongue, which is a sign of re-epithelialisation. Scale bar: 200 μ m.
 (B) Graph of epithelial tongue length. Statistical analyses using 1-way analysis of variance (ANOVA) and Tukey's post hoc tests were performed amongst all conditions (P-value: * $P < 0.05$, NS $P > 0.05$). Data has been expressed as mean $\pm$ SD. Figure is representative of 4 biological replicates (N=3) for control wounds and 6 biological replicates (N=6) for 100 % BCM wounds

Figure 39: Intact E-cadherin junctions observed in BCM-challenged wounds compared to controls. (A) Images of tissues stained with E-cadherin and DAPI. Scale bar: 100 μ m. Figure is representative of 4 biological replicates (N=3) for control wounds, 6 biological replicates (N=6) for 100 % BCM wounds.

In summary, PAO1 BCM hampers re-epithelialisation *in vivo*, through a reduced epithelial tongue length compared to controls. This is consistent with 1X PAO1 BCM and 10X PAO1 BCM. However, BCM does not cause disruptions to E-cadherin junctions *in vivo*.

3.3 Discussion

3.3.1 The effects of BCM on cell migration

Migration is hampered at high doses of PAO1 BCM and slightly improved at low doses, albeit not significant. Although Marano et al. (2015) carried out studies on PAO1 BCM as well, they found that PAO1 BCM is toxic to human keratinocytes, so they were unable to study its effects on cell migration. In that study, 5 % BCM over a span of 15 h was sufficient to elicit toxicity so even diluted BCM could not be used for studying cell migration because cell death would complicate the migration assay results. Differences

in BCM toxicity could be caused by Marano et al. (2015) using the MBEC high-throughput assay for BCM cultivation as compared to our technique. Moreover, the IBIDI cell migration assay inserts were used for the migration assay by Marano et al. (2015), which could be slightly different from our setup since an *in vitro* scratch was not induced. The cues for migration (since no scratch was induced) could have then differed and may have caused a deviation in migration results from data from this thesis. Apart from keratinocytes, PAO1 BCM is known to inhibit migration of mesenchymal stromal cells (Ward et al., 2015). These reiterate that in spite of different BCM generation methods and cell types, PAO1 BCM is able to inhibit cell migration. Other than supernatant studies, ExoT of *P. aeruginosa* has been shown to inhibit cell migration via inhibiting the GTPase-activating protein (GAP) domain that affects Rho family GTPases involved in migration (Garrity-Ryan et al., 2000; Kazmierczak and Engel., 2002; Krall et al., 2000). These show that the physical bacterium and its secreted factors have similarities in hampering migration. A more recent study by Karlsson et al. (2012) demonstrated that *P. aeruginosa* 3-oxo-C₁₂-HSL at lower concentrations promote migration, while higher concentrations suppress migration of human epithelial cells in a scratch wound assay. This is reflective of what was observed by PAO1 BCM in this dissertation, where dose-dependent effects could hamper or promote migration. This is further supported by research from Kanno et al. (2011, 2013, 2015) that *P. aeruginosa* itself and its quorum sensing molecules (C₄-HSL and 3-oxo-C₁₂-HSL) are able to promote wound healing. Although studies by Kanno et al. (2011, 2013, 2015) do not deal with varied concentrations, they suggest that both the physical bacterium of *P. aeruginosa* and its secreted factors are able to elicit beneficial effects to wound healing although their presence have always been known to complicate wounds. Collectively, PAO1 BCM is able to achieve both sides of the spectrum in terms of cell migration rates.

Another component of cell migration, focal adhesions, regulate the migratory capacity of cells (DeMali et al., 2002; DeMali and Burridge., 2003; Chorev et al., 2014; Zhang et al., 2004a). Vinculin, a focal adhesion protein, is important for binding to the Arp2/3 complex, and its phosphorylated form at the residue Y1065 (activated state), signals the activation of the adhesion complex (Arp2/3) (DeMali et al., 2002; DeMali and Burridge., 2003; Chorev et al., 2014; Zhang et al., 2004a). Arp2/3 is essential for inducing cell adhesion and migration through actin polymerisation, linkage with integrins and an eventual leading-edge protrusion (DeMali et al., 2002; DeMali and Burridge., 2003; Chorev et al., 2014; Zhang et al., 2004a). Focal adhesion size also biphasically affects cell migration rate, where an increase in cell size increases migration rates until a certain threshold, when the speed declines (Kim et al., 2013). Keratinocytes incubated with BCM had unchanged total and phosphorylated vinculin expression, and vinculin sizes were similar to that of controls. This suggests that cell-surface adhesion of keratinocytes is unchanged compared to controls, and reduced migration rates in keratinocytes were unaffected by vinculin. Results demonstrate that migration capacity in fibroblasts is probably reduced due to reduced phosphorylated vinculin, and the increased vinculin sizes may reduce cell speeds as well. However, as different cells may have different thresholds for focal adhesion sizes, the theory showed by Kim et al. (2013) has to be separately proven for BJ-5ta to fully claim that the increased vinculin sizes decreased migration rates. Low dose BCM-incubated fibroblasts did not really affect vinculin compared to controls, which correlates with the similar migration speeds of low dose BCM and controls. Studies have not shown the relationship between *P. aeruginosa* and vinculin, but have reported that integrins facilitate *P. aeruginosa* entry through facilitating bacterial adherence (Leroy-Dudal et al., 2004; Roger et al., 1999). This

highlights the possibility that BCM may have targeted vinculin expression and size in fibroblasts for facilitating pathogenic adhesion, and also reduced their migrative capability as an added effect.

Golgi repositioning towards the leading edge was used as a measure of migration capability, as it causes cells to be polarised for directional migration and secretion (Kupfer et al., 1983; Yadav et al., 2009). Despite this, studies showed that Golgi positioning depends on geometrical constraints as freely-migrating fibroblasts do not display Golgi polarity that correlates with the migration direction, unlike cells at the wound edge (Pouthas et al., 2008; Uetrecht and Bear., 2009). Cells can re-orientate their Golgi from as early as 3 h to 5 h post-wounding (Kupfer et al., 1983). A study by Natividad et al. (2018) highlighted that the stabilisation of the Golgi position corresponds to enhanced migration speed and persistence. A high dose of PAO1 BCM inhibited Golgi repositioning in both keratinocytes and fibroblasts at 3 and 5 h post-wounding. This suggests a persistence in the inhibition as the reorientation was not recovered at 5 h post-wounding. For cells incubated with low dose BCM, keratinocytes and fibroblasts displayed Golgi reorientations similar to controls. Based on the current literature, this is the first time *P. aeruginosa* has been shown to inhibit reorientation of the Golgi apparatus. Other pathogens such as *Escherichia coli*, *Shigella flexneri* and *Citrobacter* negatively affect Golgi function by fragmenting the Golgi apparatus through binding GTPases and kinases (Olsen et al., 2013; Selyunin and Alto., 2011; Dong et al., 2012). This inhibits secretion of cytokines and cell surface receptors, culminating in dampened inflammation (Olsen et al., 2013; Selyunin and Alto., 2011; Dong et al., 2012; Burnaevskiy et al., 2013). Although fragmentation was not observed in PAO1 BCM-incubated cells, these studies show that Golgi apparatus may be a

bacterial target in the pathogenesis of wound infections. Collectively, these studies highlight the possibility that PAO1 BCM targets Golgi repositioning as a mechanism to impede migration in keratinocytes and fibroblasts, perturbing the wound healing process.

Lamellipodia area was measured as a determinant of migration rates as the bigger the lamellipodia area, the faster the migration (DeMali et al., 2002). High dose BCM negatively regulated lamellipodia protrusion in keratinocytes and fibroblasts after 5 h of incubation, correlating to the reduced migration rates compared to controls. Comparatively, low dose BCM result in lamellipodia sizes similar to controls, which concurs with the similar migratory rates in the scratch wound assay. The quorum sensing molecule of *P. aeruginosa*, 3-oxo-C₁₂-HSL, was shown to interact with a possible integrator of Rac1 to inhibit Rac1 (Karlsson et al., 2012). Rac1 regulates lamellipodia protrusions and membrane ruffles, so an inhibition leads to impaired cell migration (Evers et al., 2000). *P. aeruginosa* ExoS has also been characterised to inhibit lamellipodia protrusions in macrophages (Rocha et al., 2003; Holm et al., 2015; Holm et al., 2016). These studies show that various secreted factors of *P. aeruginosa* are able to inhibit lamellipodia protrusions in a variety of cells, and this agrees with the results shown in this dissertation.

In summary, high doses of BCM cause hampered migration through delayed Golgi re-orientation and reduced lamellipodia protrusion. For fibroblasts, downregulated phosphorylated vinculin (Y1065) was another additional factor.

3.3.2 The effects of BCM on cell viability

Cell viability was compromised in a dose- and time-dependent manner in both keratinocytes and fibroblasts. Keratinocytes became apoptotic, whereas fibroblasts were resistant to apoptosis and necrosis upon incubation with 50 % BCM for 5 h. Similarly, Marano et al. (2015) reported keratinocyte cytotoxicity caused by PAO1 BCM, through cell shrinkage and clumping. However, the cell death pathway was not examined. This study shows that the apoptosis by PAO1 BCM was not just confined to immortalised cells, but also in primary keratinocytes. In contrast, Bowler showed that PAO1 BCM was not cytotoxic to human lung epithelial cells (unpublished thesis, 2015). Non-apoptotic toxicity of PAO1 supernatant to murine RAW 264.7 macrophages was also observed (Roy et al., 2013). In other studies, *P. aeruginosa* has been shown to cause apoptosis through virulence factors such as ExoT and ExoS and necrotic responses through ExoU (Kaminski et al., 2018; Wood et al., 2015a,b; Sato et al., 2015). Collectively, these studies were either infection studies or conducted using planktonic supernatant in different cell types, which could explain for the differences in cell death responses. Chronic wounds have also been correlated with high levels of apoptosis due to the production of high levels of reactive oxygen species (ROS) (Arya et al., 2011; Bhan et al., 2013; Brubaker et al., 2011). Jiang et al. (2014) demonstrated that elevated caspase 3 levels were present in pressure ulcers, especially in the ulcer centers compared to ulcer margins, causing delayed wound healing. These studies show that apoptotic keratinocytes caused by a high dose of PAO1 BCM is similar to what is observed in patients.

Since PAO1 BCM compromises cell viability, the possibility of a rescue of insulted cells after a short exposure of BCM was questioned. Keratinocytes displayed

compromised cell viability in the first 24 h with an eventual wound closure after 2 days. In contrast, fibroblasts displayed severe, reversible cell shrinkage and closed the wound within a day. The 5 h induction was chosen as it was the maximum time allowed for cell viability of the cells to be compromised without lifting off from the wells during media change at 50 % dosage (data not shown). It is noted that the experiments were qualitative because PI signals fade upon cell lysis, leading to inaccurate quantification. Also, the experiment was stopped after 2 days due to confluency which may complicate results observed. Since cell shrinkage was reversed, fibroblasts experienced a complete rescue after a transient insult compared to keratinocytes. Moreover, both cell types had an eventual wound closure. This also hints that only upon clearance of secreted factors of biofilms can the wound heal properly. Keratinocytes were shown to have compromised cell membranes (via PI signal) at 12 h post media replenishment, which is a feature of reduced cell viability. This concurs with a characterisation conducted by Rehm et al. (2003) showing that mitochondria depolarisation and caspase activation take place as early as 30 mins, while nuclear condensation and formation of apoptotic bodies range from 3- 48 h.

Keratinocytes were more susceptible to PAO1 BCM at high doses compared to fibroblasts. One of the reasons include the choice of cell type. HaCaTs have been found to be more susceptible to apoptosis, perhaps due to aberrant signalling, having harboured two mutant p53 alleles compared to normal keratinocytes (Bowen et al., 2003; Chaturvedi et al., 2001; Petit-Frère et al., 2000). Reversible cell damage occurred in fibroblasts, causing them to be morphologically compromised. Fibroblasts have also been shown to have lower levels of enzyme systems for drug metabolism, so it may have taken a longer time to metabolise PAO1 BCM, resulting in differences compared to

keratinocytes in their cell viability responses (Chiang et al., 2012; Wang et al., 2002). Moreover, neither apoptosis nor necrosis occurred in fibroblasts incubated with 50 % PAO1 BCM for 5 and 7 h respectively, unlike keratinocytes which apoptosised. Perhaps other cell death mechanisms took place in fibroblasts, which could be confirmed in future studies.

As cell death signals can be transmitted through gap junctions, Cx43 expression was checked in both cell types (Udawatte et al., 2005; Cusato et al., 2006; Krysko et al., 2005). As Cx43 expression is dynamic and highly time-dependent, 3 and 5 h post-incubation timings were assessed. It was hypothesised that Cx43 would be upregulated in both cell types as it is representative of a non-healing wound (Sutcliffe et al., 2015). However, in keratinocytes, Cx43 levels were unchanged upon incubation with 50 % PAO1 BCM and upregulated with low dose of 3 % BCM. In contrast, Cx43 levels in fibroblasts were only upregulated at 5 h post-incubation with 3 % BCM and downregulated at 5 h post-incubation with 50 % BCM. These observations are different from improved wound closure caused by Cx43 downregulation in wound edge keratinocytes and fibroblasts (Qiu et al., 2003; Mori et al., 2006; Cogliati et al., 2015). The differences between studies could be attributed to pathogenic modulation of the gap junctions, causing Cx43 levels to not be correlated with wound healing statues. Current literature has not shown relationships between connexins and infectious wound healing caused by *P. aeruginosa*. Based on reports where *P. aeruginosa* is capable of both decreasing and increasing Cx43 expression in airway cells, it suggests that gap junctions may play multiple roles in the pathogenesis of infections (Yeh et al., 2005; Martin and Prince., 2008; Sargedine et al., 2009; Losa et al., 2014; Cannon et al., 2003). Cx43 is also known for mediating apoptosis in *P. aeruginosa*-infected cells through upregulated

Cx43 expression (Losa et al., 2014; Sarieddine et al., 2009). This seems to be a host response towards the infection as Cx43 upregulation was also linked to neutrophil recruitment (Losa et al., 2014; Sarieddine et al., 2009). However, apoptosis of 50 % PAO1 BCM-challenged keratinocytes had no change in Cx43 levels. It is proposed that the upregulation of Cx43 in 3 % BCM-challenged cells and downregulation in 50 % BCM-challenged fibroblasts act as a form of sensing conducted by the host to detect the presence of pathogenic substances, thereby causing increased communication channels between cells to notify downstream host mechanisms to prepare for a pathogenic attack, similar to a study by Martin and Prince (2008). This possibility is relevant since *P. aeruginosa* is known to colonise deeper tissues near blood vessels (Høiby et al., 2011; Schaber et al., 2007; Soave et al., 1978; Plotkowski et al., 1994). It is then suggested that the unchanged expression of Cx43 protein in 50 % BCM-challenged keratinocytes is due to either the pathogen's attempt to invade the host through gap junctions, or a host strategy to prevent the pathogen from developing mechanisms to hijack the communication channels by maintaining Cx43 expression levels (Eugenin., 2014).

In conclusion, secreted factors of *P. aeruginosa* biofilms reduce cell viability in the host through apoptosis in keratinocytes and a non-apoptotic and non-necrotic pathway in fibroblasts. This may be a strategy which the pathogen undertakes to compromise the host and induce the conditions (non-viable nature of the wound bed) for re-colonisation and infection of *P. aeruginosa* after debridement. It could also be a host response to get rid of cells that have been infected by the pathogen and to trigger protective mechanisms as a host survival response (Ashida et al., 2011).

3.3.3 The effects of BCM on cell adhesion

In single cell migration, upregulation of cell adhesion inhibits migration as cells are bound and unable to move (Mayor and Etienne-Manneville., 2016). However, in collective migration (such as in wound healing), cell adhesion is important as these forces provide traction for the cells to move together as a sheet (Mayor and Etienne-Manneville., 2016). In the hanging drop assay, the bigger the cell clump, the harder it is for external forces to disrupt the cell-cell adhesion, signifying that these adhesions are stronger. 50 % PAO1 BCM causes cell-cell adhesion to be maintained in keratinocytes, shown by unchanged levels of β -catenin and E-cadherin. An exception was that occludin levels in keratinocytes decreased after incubation with a high dose of BCM. Incubation with 3 % BCM caused the cell-cell adhesion of keratinocytes to be stronger (albeit insignificant) and is supported by an increase in occludin levels. Whereas in fibroblasts, cell-cell adhesion was disrupted when challenged with 50 % PAO1 BCM, supported by decreases in occludin, β -catenin and N-cadherin levels. The incubation with 3 % BCM in fibroblasts caused no changes in cell clump sizes compared to controls, reflective of unchanged occludin and β -catenin levels. However, the gaps inside the cell clumps reveal that cell junctions may be compromised, which is demonstrated by the loss of N-cadherin. Similarly, Bower (unpublished thesis, 2015) showed a loss of occludin and unchanged E-cadherin levels caused by PAO1 BCM in human lung epithelial cells. This shows that the modulation of cell junctions by PAO1 BCM is robust enough to be reproduced although the BCM generation method and cell types used are different. This also agrees with findings in this dissertation and other studies that the effects of PAO1 BCM and *P. aeruginosa* may be different on various cell junctions. Tight junctions have been shown to decrease in response to virulence factors of *P. aeruginosa* such as LasB, 3-oxo-C₁₂ -HSL and ExoS (Beaufort et al., 2013; Vikström et al., 2009; Vikström et al.,

2010; Soong et al., 2008). β -catenin, N-cadherin and E-cadherin have been shown to be degraded by virulence factors of *P. aeruginosa* such as lectin (LecB), ExlA and 3-oxo-C₁₂-HSL (Vikström et al., 2009; Reboud et al., 2017; Cott et al., 2016). E-cadherin gaps found in newly formed junctions were also exploited by *P. aeruginosa* to gain access to the basal domain (Golovkine et al., 2016). These studies are similar to result of fibroblasts incubated with 50 % PAO1 BCM and show that various secreted factors of *P. aeruginosa* are able to elicit cleavage of adherens junctions, probably to gain access to deeper tissues, as what Golovkine et al. (2016) have suggested. In contrast, studies also show that E-cadherin was unaffected by *P. aeruginosa* T2SS effectors and its elastase did not alter β -catenin and E-cadherin expression (Golovkine et al., 2014; Nomura et al., 2014). Moreover, β -catenin was stabilised in membranes of keratinocytes, further supporting that migration was hampered as stabilised β -catenin was shown to inhibit migration (Stojadinovic et al., 2005). In contrast, stabilised β -catenin promotes migration in fibroblasts, reiterating that migration was suppressed by PAO1 BCM (Cheon et al., 2002). The modulation of junctional proteins may be for transmigration of the pathogen and shows the multifaceted functions of cell junctions in pathogenesis (Golovkine et al., 2018). In summary, biofilm exudates of *P. aeruginosa* cause dysregulation of tight junctions and adherens junctions.

3.3.5 Optimisation and results of *in vivo* wounding model

In this thesis, a full thickness excisional wound model in mice was chosen. PAO1 BCM was applied onto wounds by allowing the absorbent pad in the plasters to absorb the media and applying the plasters to the wounds. This is the first time that biofilm secreted factors in the form of BCM has been applied onto mice wounds as biofilms are usually used instead. Direct application of BCM was not favoured as the liquid would flow out

of the wounds when the animal is slightly tilted, causing the volume that was applied to the wounds to be inconsistent. BCM dissolved in pluronic was not favoured as well due to the difficulty in dissolving pluronic into a 10 X concentrated BCM. When dissolved, the BCM becomes a paste-like structure and the amount to be applied on wounds is unable to be determined. As *P. aeruginosa* is known to secrete surfactants such as rhamnolipids, surfactant-surfactant interactions between pluronic and BCM are unknown and may alter BCM properties (Davey et al., 2003). All the methods of BCM application on wounds did not show significant differences in macroscopic wound closure, but the delivery of the BCM through the plaster is advantageous due to reproducibility and the preservation of BCM toxicity. Additionally, it mimics the delivery of biofilm exudates on wounds and allows a larger volume of media to be easily handled and localised at the wounds.

Herein, application of 100 % PAO1 BCM (both 1 and 10 X) to wounds on mice had similar wound closure and intact E-cadherin junctions. Only re-epithelialisation was hampered compared to controls. BCM may have hampered re-epithelialisation but not wound contraction, causing any difference in wound closure to be minimal. This could be due to rodents healing largely through wound contraction instead of re-epithelialisation and granulation tissue formation (Seaton et al., 2015; Schierle et al., 2009). Wound contraction and re-epithelialisation each accounted for around 40-60 % of the initial wound closure in rodents, so both processes have to be hampered for differences to be observed macroscopically (Chen et al., 2015). PAO1 BCM could have promoted wound contraction more than controls, overpowering the hampered migration phenotype. This can only be confirmed through future experiments through initial markings on the surrounding skin of the mice to deduce if PAO1 BCM had a more

significant effect on wound contraction than re-epithelialisation. Another possibility is that a dilution of BCM effects could have occurred since there is no constant delivery of BCM apart from the first application at the day of wounding. However, hampered re-epithelialisation and intact E-cadherin junctions when challenged with PAO1 BCM concur with the *in vitro* results, so diluted effects may not have occurred. Furthermore, variances in the amount of media applied on the wounds did not result in macroscopic differences in wound closure, so a dilution of BCM effects cannot be concluded. This can only be confirmed through future experiments using a dosage even higher than 10 X concentrated BCM to determine if wound closure is still similar to controls.

As this is the first study that investigated the effects of BCM on wound healing *in vivo*, there are no prior studies for comparison. In contrast, infection studies of *P. aeruginosa* showed hampered wound healing *in vivo* in diabetic mice (Zhao et al. 2010; Zhao et al., 2012; Goldufsky et al., 2015). The results obtained in these studies could have been complicated by the presence of diabetes in the animals, causing wound healing to be hampered as none of the infection studies included control, non-diabetic mice as part of their study except for Goldufsky et al. (2015). However, Goldufsky et al. (2015) did not compare between the wound closure of control and diabetic wounds without *P. aeruginosa* infection. Based on graphs of wound size measurements, a reduction in 80 % of original wound size in controls compared to a 50 % in diabetic wounds may be significant, so the enlarged wounds observed could have been complicated by diabetes (Goldufsky et al., 2015). Moreover, normal wounds were unaffected by *P. aeruginosa* PA103 infection (Goldufsky et al., 2015). These studies agree with results observed in this dissertation and collectively show that *P. aeruginosa* and its biofilm exudates can heal similarly to controls in the absence of diabetes. Roy et al. (2014) also showed that

percentage wound closure in biofilm-infected wounds was similar to controls in a chronic porcine burn wound model. This further reiterates that a chronic wound model with *P. aeruginosa* or its biofilm exudates in the absence of diabetes is able to achieve similar healing rates in both biofilm-infected and non-infected wounds. Interestingly, Kanno et al. (2011, 2013, 2015) showed that *Pseudomonas aeruginosa* itself and its quorum sensing molecules (C₄-HSL and 3-oxo-C₁₂-HSL) are able to promote acute wound healing in Sprague-Dawley rats. Studies that involved the quorum sensing molecules showed that neutrophil infiltration (but not TNF- α synthesis) was coupled to better healing (Kanno et al., 2013; 2015). Collectively, both chronic and acute wound models are capable of having similar wound closure rates when challenged with *P. aeruginosa* or its quorum sensing virulence factors. However, due to the varied *P. aeruginosa* strains used in these studies and the size discrepancies between mice, pigs and rats relative to the CFU/ml bacterial load, studies cannot be compared directly to one another. Although these studies do not deal with biofilm exudates specifically, they suggest that both the physical bacterium of *P. aeruginosa* and its virulence factors are able to elicit similar or beneficial effects to wound healing although their presence have always been known to complicate wounds. These also suggest that due to the varied responses that biofilms can have on wound healing, there is more to be understood from their interactions with the wound microenvironment.

3.4 Conclusion

In this chapter, PAO1 BCM was used to determine the effects of secreted factors of *P. aeruginosa* biofilms on wound healing responses. PAO1 BCM in high doses inhibit migration through suppressed Golgi repolarisation, hampered lamellipodia protrusion and reduced p-vin (in fibroblasts). It also reduces cell viability by inducing apoptosis in

keratinocytes. PAO1 BCM was unable to elicit apoptosis nor necrosis in fibroblasts although cell viability was compromised. Finally, it reduces cell-cell adhesion in fibroblasts through decreased β -catenin and N-cadherin levels. Occludin levels were reduced in both cell types. Despite this, keratinocytes had unchanged cell-cell adhesion through maintaining β -catenin and E-cadherin levels. Low doses of BCM may have the ability to improve migration, albeit insignificant. Additionally, this dissertation reveals a novel way of delivering secreted factors of biofilms in an *in vivo* setting. This was achieved using the conventional full-thickness excisional wound model in mice and then applying a BCM-filled bandage to mimic the presence of biofilm exudates. Wounds with PAO1 BCM-challenge were noted to be similar in sizes at days 3 and 7 post-wounding with intact E-cadherin junctions. Only re-epithelialisation was hampered compared to controls. Taken together, these results show that *P. aeruginosa* biofilm exudates are capable of perturbing wound healing responses

4. IMPLICATIONS OF PAO1 BIOFILM SECRETOME ON INFLAMMATION AND KERATINOCYTE DIFFERENTIATION

4.1 Introduction

Infectious pathogens are known to elicit inflammatory host responses (Lemaitre et al., 1996; Medzhitov and Janeway., 2002; Medzhitov et al., 1997; Ruco et al., 1978). They have also evolved to be able to immunomodulate the host system to evade immune surveillance, as a strategy for survival in the host (Reddick and Alto., 2014). Based on current literature, *Pseudomonas aeruginosa* biofilms can immunomodulate the host system through strategies such as the production of rhamnolipids and toxins that inhibit host translational machinery (Jensen et al., 2007; Bjarnsholt et al., 2005; Dunbar et al. 2012, McEwan et al., 2012). However, the mechanisms are not as well understood compared to other pathogens like *S. flexneri* or *Yersinia pestis* (Matsuura., 2013; Rebeil et al., 2004; Montminy et al., 2006; Paciello et al., 2013; Burnaevskiy et al., 2013; Dong et al., 2012). Further, the immunomodulation capacity of bacterial exudates in general have not yet been elucidated. This information is crucial given that the exudates are able to propagate their effects in the host even though debridement has occurred, as shown in results in the previous chapter. Moreover, due to the importance that inflammation has on wound healing, investigating the effects of *P. aeruginosa* biofilm exudates on host inflammatory responses would give us greater insights on the prognosis of wound healing.

Multiple studies have indicated that the NF- κ B signalling pathway is important for proper skin development and function, but the different experimental models used have caused much confusion about its significance in the skin (Kaufman and Fuchs., 2000). The literature suggests that *P. aeruginosa* biofilms impede wound healing and re-

epithelialisation (Zhao et al., 2010; Watters et al., 2013; Goldufsky et al., 2015). However, there has been minimal focus on the effects of pathogens (including *P. aeruginosa*) on keratinocyte differentiation although bacteria were reported to indirectly enhance keratinocyte differentiation through β -defensins (Lai et al., 2010). Keratinocyte differentiation is required for proper re-epithelialisation and wound closure (Pastar et al. 2014). There is a close affiliation between the inflammation (NF- κ B) and keratinocyte differentiation pathways. Uncontrolled NF- κ B activation or inactivation causes disorders such as inflammation and other autoimmune diseases, making the pathway an attractive target for therapeutic interventions (Rahman and McFadden., 2011). Disruption of the NF- κ B signalling pathway may prevent the establishment of bacterial immunity against the host. This study investigates the possible bacterial modulation of the NF- κ B pathway and the effect(s) on keratinocyte differentiation. The focus is on *P. aeruginosa* biofilm exudates and how it influences the epidermal barrier and re-epithelialisation.

This chapter aims to characterise the *in vitro* and *in vivo* effects of PAO1 BCM on wound healing responses, namely inflammation and keratinocyte differentiation, and the pathways that are involved. In light of previous studies, I hypothesise that PAO1 BCM will increase inflammation through the NF- κ B signalling pathway and elevate expression of keratinocyte differentiation markers.

Analysis of *in vivo* effects were conducted using a murine full-thickness excisional wound model. The effects of PAO1 BCM were observed over a span of 1 and 7 days post-wounding. Immunohistochemical staining of the tissue samples were carried out to observe morphological differences and quantify potential changes in protein levels.

ELISA was then conducted on wound fluid and homogenised tissue samples to determine effects of PAO1 BCM on cytokine secretion. Analysis of *in vitro* effects were conducted with immortalised human fibroblasts (BJ-5ta) and keratinocytes (HaCaT), the 2 main cell types in the skin. Immunocytochemistry, real time-quantitative polymerase chain reaction (RT-qPCR) and Western blot analyses were conducted to determine the possible effects that PAO1-BCM could exert.

4.2 Results

4.2.1 PAO1 BCM reduced dermal immune cell infiltration at D1-post wounding *in vivo*

Contrary to the hypothesis of this chapter, haematoxylin and eosin (H&E) staining showed a significant reduction ($P<0.05$) in immune infiltrate in the dermal area of wounds with 100 % PAO1 BCM compared to that of control wounds (100 % DMEM) (**Figures 40, 41, 42, 45**). This reduction was also significant ($P<0.05$) in wounds that had 10 X-concentrated 100 % PAO1 BCM compared to that of control wounds (100% DMEM) (**Figures 40, 41, 43, 45**). Using **Figure 44** as a guide for macroscopic identifications, control wounds were noted to have the most leukocytes, whilst 100 % PAO1 BCM wounds had more fibroblasts compared to controls, with occasional leukocytes (**Figures 41 and 42**). 10 X- concentrated 100 % PAO1 BCM had some leukocytes (**Figure 43**). Overall, there were fewer leukocytes based on macroscopic observations in wounds with PAO1 BCM application compared to controls (**Figures 41-43**).

Figure 40: Representative images chosen in figures 41-43. 1 X 100 % PAO1 BCM represents 100 % dosage of BCM, while 10 X 100% PAO1 BCM represents 10 X-concentrated 100 % BCM. Representative image of the wound edge (indicated by black box) in the context of the entire wound H&E image. Scale bar: 2000 μ m.

Figure 41: Control H&E stained wound edges have more dermal immune infiltrate (purple dots) compared to PAO1 BCM-challenged wounds. Representative images of the dermal areas are indicated by black boxes. Arrows depict the possible infiltrating immune cells. Scale bar: 100 μ m (L), 20 μ m (M), 20 μ m (R).

Figure 42: 1 X 100 % PAO1 BCM H&E stained wound edges have fewer dermal immune infiltrate (purple dots) compared to PAO1 BCM-challenged wounds. Representative images of the dermal areas are indicated by black boxes. Arrows depict the possible infiltrating immune cells. Scale bar: 100 μ m (L), 20 μ m (M), 20 μ m (R).

Figure 43: 10 X 100 % PAO1 BCM H&E stained wound edges have fewer dermal immune infiltrate (purple dots) compared to PAO1 BCM-challenged wounds. Representative images of the dermal areas are indicated by black boxes. Arrows depict the possible infiltrating immune cells. Scale bar: 100 μ m (L), 20 μ m (M), 20 μ m (R).

Figure 44: Representative images of leukocytes (lymphocyte, basophil, neutrophil) and fibroblast. Cell types based on guide from Ross et al., (2015).

Figure 45: PAO1 BCM reduces immune infiltrates *in vivo*. Graph of immune filtrate count per field of view per mouse. Statistical analyses using 1-way analysis of variance (ANOVA) and Tukey's post hoc tests were performed amongst all conditions (P-value: * $P < 0.05$, NS $P > 0.05$). Data has been expressed as mean $\pm$ SD. Figure is representative of 4 biological replicates (N=4) for each condition.

As **figure 44** was used as a guide to identify the immune infiltrate, further analysis was conducted through myeloperoxidase (MPO) immunostaining of the tissues. Myeloperoxidase is a heme-containing enzyme that is abundantly expressed in neutrophils, macrophages and monocytes (Aratani., 2018). It was confirmed that control wounds had more leukocytes ($P < 0.01$) than 1 X PAO1 BCM wounds and 10 X PAO1 BCM wounds (**Figure 46**). There was no significant difference ($P > 0.05$) between 1 and 10 X PAO1 BCM on leukocyte count (**Figure 46**). We can also appreciate that not all the inflammatory infiltrates highlighted by the H&E staining were positive for myeloperoxidase (**Figure 46A**).

Figure 46: 1 X 100 % PAO1 BCM reduces MPO staining *in vivo*. (A) Images of H&E stained wound edges were taken at day 1 post-wounding and tissues stained with **MPO** and **DAPI**. 1 X 100 % PAO1 BCM represents 100 % dosage of BCM, while 10 X 100% PAO1 BCM represents 10 X-concentrated 100 % BCM. Representative images of the dermal areas stained with **MPO** and **DAPI** are indicated by the black boxes. White arrows indicated areas of **MPO** stain. Negative controls of the MPO-stained tissues are at the right. Scale bar: 100 μ m.

(B) Graph of MPO-stained immune filtrate count per field of view per mouse. Statistical analyses using 1-way analysis of variance (ANOVA) and Tukey's post hoc tests were performed amongst all conditions (P-value: * P<0.05, NS P>0.05). Data has been expressed as mean $\pm$ SD. Figure is representative of 4 biological replicates (N=4) for each control and 1 X 100% PAO1 BCM, and 2 biological replicates (N=2) for 10 X 100 % PAO1 BCM.

4.2.2 Downregulation of pro-inflammatory cytokine secretion by PAO1 BCM *in vitro*

To determine if there was a reduced immune cell infiltration, ELISA was conducted to check for pro-inflammatory and anti-inflammatory cytokine secretion. Tumour necrosis factor-alpha (TNF- α), interleukin-6 (IL-6) and interleukin-10 (IL-10) were chosen as they are target genes regulated by NF- κ B, of which the latter cytokine is anti-inflammatory, while the rest are pro-inflammatory (Dinarello., 2000; Opal and DePalo., 2000). *In vitro* analyses of keratinocyte supernatants revealed that the pro-inflammatory cytokines TNF- α ($P \leq 0.05$) and IL-6 ($P \leq 0.01$) were downregulated around 3 and 1.5 times respectively upon incubation with 50 % PAO1 BCM compared to controls (**Figure 47A**). Incubation with 50 % PAO1 BCM also upregulated the secretion of anti-inflammatory cytokine IL-10 around two-fold, albeit insignificant ($P > 0.05$) compared to controls (**Figure 47A**). *In vivo* analyses of wound fluid revealed that the pro-inflammatory cytokines TNF- α and IL-6 were relatively unchanged ($P > 0.05$) in PAO1 BCM-challenged wounds compared to controls (**Figure 47B**).

Figure 47: Downregulation of pro-inflammatory cytokines upon incubation of keratinocytes with PAO1 BCM *in vitro*. (A) Downregulation of pro-inflammatory cytokine levels by PAO1 BCM *in vitro*. Cytokine levels of TNF α , IL-6 and IL-10 were measured via ELISA. Complete DMEM and 50 % PAO1 BCM were incubated respectively on keratinocytes for 5 h, and supernatants collected for ELISA. Mann-Whitney test were performed to compare between each condition (P-value: ** P<0.01, * P<0.05, NS P>0.05). Data has been expressed as mean $\pm$ SD. Figure is representative of 3 biological and technical replicates (N=3, n=3) for each condition. (B) Unchanged pro-inflammatory cytokine levels by PAO1 BCM application. Cytokine levels of TNF α and IL-6 were measured via ELISA. Complete DMEM and 100 % PAO1 BCM were applied on mice wounds, and wound fluid collected for ELISA at day 1 post-wounding. Mann-Whitney test were performed to compare between each condition (P-value: NS P>0.05). Data has been expressed as mean $\pm$ SD. Figure is representative of 6 biological (for TNF- α) and 3 biological (for IL-6) replicates.

4.2.3 PAO1 BCM decreases total p50 and p50 *in vitro* and *in vivo*, with no change in I κ B- α levels

Since PAO1 BCM reduced dermal immune cell infiltration at day 1 post-wounding *in vivo* and reduced pro-inflammatory cytokine secretion *in vitro*, the inflammatory NF- κ B pathway was checked for negative effects as well. Results of the immunostaining of the PAO1 BCM-incubated NIH-3T3 mouse fibroblasts stained with p50 displayed significant (P<0.01) *in vitro* downregulation in total p50 (**Figure 48**).

Figure 48: Total p65 is downregulated in PAO1 BCM-challenged mouse fibroblasts (NIH-3T3). Images of cells taken under 40 X oil objective (Blue: DAPI, Green: Total p65, Red: Actin). Monolayer of cells were scratched, incubated for 5 h at respective conditions (Control-DMEM and 50 % PAO1 BCM) and stained for p65. Scale bar: 25 μ m. Statistical comparisons between the various conditions were conducted using Mann-Whitney test. (P-value: ** P<0.01). Data expressed as mean $\pm$ SD. Figure is representative of biological and technical replicates (N=4, n=2) for controls and (N=3, n=1) for 50 % PAO1 BCM.

To determine if this downregulation was clinically relevant, human fibroblasts and keratinocytes incubated with PAO1 BCM were also probed for total p65. Results confirmed the downregulation observed in the mouse fibroblasts, where both types of PAO1 BCM-incubated human cells experienced a marked decrease ($P \leq 0.001$) in p65 levels compared to control (**Figure 49**).

Figure 49: Total p65 is downregulated in PAO1 BCM-challenged human fibroblasts and keratinocytes. Images of cells taken under 40 X oil objective (Blue: DAPI, Red: Total p65). Monolayer of cells were scratched, incubated for 5 h at respective conditions (Control-DMEM and 50 % PAO1 BCM) and stained for p65. Scale bar: 25 μ m. Statistical comparisons between the various conditions were conducted using Mann-Whitney test. (P-value: *** $P \leq 0.001$). Data expressed as mean $\pm$ SD. Figure is representative of 3 biological and technical replicates (N=3, n=3) for each condition.

To ascertain if this downregulation of p65 in both human fibroblasts and keratinocytes was related to our *in vivo* results (see in **4.2.1 PAO1 BCM reduced dermal immune cell infiltration at D1-post wounding in vivo**), p65 was stained in the mouse wound tissues. The results demonstrated that p65 levels were globally downregulated in both keratinocytes and fibroblasts *in vivo* in both 1 X ($P < 0.01$) and 10 X-concentrated ($P < 0.01$) 100% PAO1 BCM-incubated wounds compared to control wounds (**Figures 50 and 51**). To determine if this effect was only observed at the wound edge, the

epidermis behind the wound edge was also investigated for p65 levels. The levels of p65 protein in PAO1 BCM-challenged wounds were comparable ($P>0.05$) to that of controls (Figures 50 and 52).

Figure 50: Images of H&E stained wound edges taken at day 1 post-wounding. Complete DMEM and PAO1 BCM were absorbed by plasters and applied on the backs of mice. 1 X 100 % PAO1 BCM represents 100 % dosage of BCM, while 10 X 100% PAO1 BCM represents 10 X-concentrated 100 % BCM. Representative images of the wound edge areas stained with p65 and DAPI are indicated by the black boxes. Scale bar: 200 μ m. Figure is representative of 4 biological replicates (N=4) for each condition.

Figure 51: Total p65 is downregulated in PAO1 BCM-challenged wound edges at day 1 post-wounding. Images of the respective areas demarcated in (A) stained with p65 and DAPI. White dotted lines indicated wound edges and WB represents wound bed. Negative controls of the p65-stained tissues are at the last column. Scale bar: 100 μ m. Figure is representative of 4 biological replicates (N=4) for each condition.

Figure 52: Total p65 is comparable to controls in PAO1 BCM-challenged epidermis at day 1 post-wounding. (A) Images of the respective areas demarcated in (A) stained with **p65** and **DAPI**. White dotted lines indicated epidermis behind the wound edge and WB represents wound bed. Negative controls of the p65-stained tissues are at the last column. Scale bar: 100 μ m. Figure is representative of 4 biological replicates (N=4) for each condition.

(B) Graph of p65 pixels per field of view. Statistical analyses using 1-way analysis of variance (ANOVA) and Tukey's post hoc tests were performed amongst all conditions (P-value: ** P<0.01, * P<0.05, NS P>0.05). Data has been expressed as mean $\pm$ SD. Figure is representative of 4 biological replicates (N=4) for each condition.

To ascertain if this downregulation was limited to p65 only, or if it was a global downregulation of the components of the NF- κ B pathway, cells were stained for p50 and I κ B- α . Results revealed that p50 levels of PAO1 BCM-incubated cells were also downregulated compared to that of control ($P < 0.01$), while I κ B- α levels remained unchanged ($P > 0.05$) (**Figure 53**). These observations were similar in both human fibroblasts and keratinocytes (**Figure 53**).

Figure 53: Total p50 is downregulated in PAO1 BCM-challenged human fibroblasts (BJ-5ta) and keratinocytes (HaCaT), while total I κ B- α is relatively unchanged. Images of cells taken under 40 X oil objective (Blue: DAPI, Red: Total p50, Green: Total I κ B- α). Monolayer of cells were scratched, incubated for 5 h at respective conditions (Control-DMEM and 50 % PAO1 BCM) and stained for p65. Scale bar: 25 μ m. Statistical comparisons between the various conditions were conducted using Mann-Whitney tests. (P-value: ** $P < 0.01$, NS $P > 0.05$). Data expressed as mean $\pm$ SD. Figure is representative of biological and technical replicates (N=5, n=1) for each condition.

4.2.4 Decreased p65 and p50 levels were not attributed to decreased mRNA expression

To determine if the downregulation of total p65 and p50 protein levels was brought about by a downregulation of mRNA expression, RT-qPCR was conducted on keratinocytes. Normalised mRNA levels showed that any differences between cells incubated with 50 % PAO1 BCM for 5 h and the controls were statistically insignificant ($P > 0.05$) (**Figure 54**). An exception was observed where p65 expression of 50 % PAO1 BCM incubated cells were significantly higher ($P \leq 0.05$) than that of the control (**Figure 54**).

Figure 54: mRNA expression of total p65, total p50 and total IκB-α are relatively unchanged. Complete DMEM and 50 % PAO1 BCM were incubated respectively on cells for 5 h, and RNA extracted for RT-qPCR. Mann-Whitney test were performed to compare between the different conditions and their respective controls (P-value: *: $P \leq 0.05$, NS: $P > 0.05$).

4.2.5 p65 is possibly degraded via proteasomal degradation

The next possibility was to determine if protein degradation of p65 had occurred. Various pre-treatment timings and drug concentrations were tested, and pre-treatment with 10 μ M Carbobenzoxy-Leu-Leu-leucinal (MG132) gave the highest increase ($P \leq 0.001$) in p65 signal compared to the rest of the conditions tested (**Figure 55**). The amount of p65 signal after rescue was not as significant ($P \leq 0.001$) as that of the control (**Figure 55**).

Figure 55: Highest signal intensity rescue is observed when keratinocytes were pre-incubated with 10 μ M MG132 for 1 h. Images of cells taken under 40 X oil objective (Blue: DAPI, Red: Total p65). Monolayer of cells were scratched, incubated for 5 h with either MG132 or Bafilomycin A1 (BafA1) and stained for p65. Scale bar: 25 μ m. Graph of normalised p65 pixels:nuclei ratio was plotted. Statistical comparisons between the various conditions were conducted using 1-way ANOVA with Dunnett's post-hoc test (P-value: *** $P \leq 0.001$, ** $P < 0.01$, NS $P > 0.05$). Data expressed as mean $\pm$ SD. Figure is representative of biological and technical replicates (N=5, n=1) for each condition.

4.2.6 PAO1 BCM impairs keratinocyte differentiation *in vitro*

To determine if PAO1 BCM has an impact on keratinocyte differentiation, early and late differentiation markers were tested. Keratin 10 (K10) was chosen as an early differentiation marker, while involucrin was chosen as a late differentiation marker (Ekanayake-Mudiyanselage et al., 1998; Watt., 1983). Results showed that the amount of K10 and involucrin in keratinocytes were significantly reduced ($P \leq 0.001$) respectively following incubation with 50 % PAO1 BCM (**Figures 56A, B**). Moreover, the number of cells expressing K10 and involucrin were significantly lower in 50 % PAO1 BCM incubated cells than controls ($P \leq 0.001$) (**Figures 56A, C**).

Figure 56: K10 and involucrin are downregulated in PAO1 BCM-challenged human keratinocytes (HaCaT). (A) Images of cells taken under 40 X oil objective (Blue: DAPI, Green: K10/Involucrin). Monolayer of cells were scratched, incubated for 5 h at respective conditions (Control-DMEM and 50 % PAO1 BCM) and stained for K10/Involucrin. Scale bar: 25 μ m.

(B) Graphs of K10-expressing cell areas and number of K10-expressing cells. Statistical comparisons between the control and 50 % PAO1 BCM were conducted using Mann-Whitney tests. (P-value: *** $P \leq 0.001$). Data expressed as mean $\pm$ SD. Figure is representative of biological and technical replicates (N=3, n=3) for each condition.

(C) Graphs of involucrin-expressing cell areas and number of involucrin-expressing cells. Statistical comparisons between the control and 50 % PAO1 BCM were conducted using Mann-Whitney tests. (P-value: *** $P \leq 0.001$). Data expressed as mean $\pm$ SD. Figure is representative of biological and technical replicates (N=3, n=3) for each condition.

4.2.7 Impaired keratinocyte differentiation seems to be attributed to downregulation of the JNK pathway

To determine which pathways were involved in the regulation of keratinocyte differentiation caused by p65 downregulation, Western Blot analyses were conducted on the markers of various pathways. Results revealed that the JNK pathway was involved as JNK1 and transcription factor AP-1 (c-Jun) were both downregulated or undetectable compared to that of their controls (**Figure 57**).

Figure 57: JNK pathway is negatively affected by PAO1 BCM. Western Blot analyses were conducted on keratinocytes. Complete DMEM and 50% PAO1 BCM were incubated respectively on cells for 5 h. GAPDH levels are unchanged.

4.2.8 PAO1 BCM-challenged wounds suffer from dysregulated keratinocyte differentiation at D7-post wounding *in vivo*

To determine if the impaired differentiation was observed *in vivo* as well, mouse wounds were challenged with PAO1 BCM and harvested at day 7-post wounding. The PAO1 BCM treatment downregulated ($P \leq 0.001$) K10 levels in non-nascent epidermis around the wound edge (**Figures 58-60**). The K10 levels were comparable to controls in non-nascent epidermis behind the wound edge (**Figures 58-60**). Wound-edge keratinocytes also displayed a loss of K10 ($P \leq 0.001$) in PAO1 BCM-challenged wounds compared to controls (**Figures 58-60**). Nascent epidermis had a slight increase ($P > 0.05$) in K10 levels following PAO1 BCM application compared to controls (**Figures 58-60**). A

gradient in K10 levels was observed, with the epidermal tongue having the least amount of K10, followed by the wound edge area and then the nascent epidermis behind the wound edge (**Figures 58-60**). Generally, there seems to be an increase in epidermal thickness in PAO1 BCM-challenged wounds compared to controls, especially in the spinous layer (**Figures 58-60**). Apart from K10, the PAO1 BCM downregulated ($P \leq 0.001$) involucrin levels in non-nascent epidermis (**Figure 61**).

Figure 58: Complete DMEM and 100 % PAO1 BCM were absorbed by plasters and applied on the backs of mice. (A) Images of H&E stained tissues were taken at day 7 post-wounding. Representative image of the wound edge (indicated by black box) in the context of the entire wound H&E image. Scale bar: 2000 μ m.

(B) Representative image of selected K10-stained areas shown in (C) and (D) indicated by the black boxes. Scale bar: 200 μ m and 50 μ m. Figure is representative of 3 biological replicates (N=3) for each condition.

Figure 59: K10 levels in control wounds. (A) Images of the respective areas demarcated in Figure 62 for controls stained with **K10** and **DAPI**. White dotted lines indicated epidermal tongue/wound edges/epidermis behind wound edge and WB represents wound bed. Negative controls of the **K10**-stained tissues are at the bottom row. Scale bar: 100 μ m. Figure is representative of 3 biological replicates (N=3) for each condition.

Figure 60: K10 is downregulated in PAO1 BCM-challenged epidermis and upregulated in epidermal tongue at day 7 post-wounding. (A) Images of the respective areas demarcated in Figure 62 for 100% PAO1 BCM stained with K10 and DAPI. White dotted lines indicated epidermal tongue/wound edges/epidermis behind wound edge and WB represents wound bed. White arrow represents the point where K10 levels return back to normal compared to controls. Negative controls of the K10-stained tissues are at the bottom row. Scale bar: 100 μ m. Figure is representative of 3 biological replicates (N=3) for each condition.

(B and C) Graph of K10 levels per field of view. Statistical analyses using 1-way analysis of variance (ANOVA) and Tukey's post hoc tests were performed amongst all conditions (P-value: *** $P \leq 0.001$, NS $P > 0.05$). Data has been expressed as mean $\pm$ SD. Figure is representative of 3 biological replicates (N=3) for each condition.

Figure 61: Invulucrin is downregulated in PAO1 BCM-challenged epidermis at day 7 post-wounding. (A) Murine wound tissues stained with **Invulucrin** and **DAPI**. White dotted lines indicate epidermal tongue/epidermis behind wound edge, orange dotted lines indicate wound edges and WB represents wound bed. Negative controls are same as the K10-stained tissues. Scale bar: 100 μ m. Figure is representative of 3 biological replicates (N=3) for each condition.

(B) Graph of **Invulucrin** levels per field of view. Statistical analyses using 1-way analysis of variance (ANOVA) and Tukey's post hoc tests were performed amongst all conditions (P-value: * $P < 0.05$, NS $P > 0.05$). Data has been expressed as mean $\pm$ SD. Figure is representative of 3 biological replicates (N=3) for each condition.

4.3 Discussion

This chapter arose from an observation made in the *in vivo* work conducted in **Chapter 3**, where the effects of 100 % PAO1 BCM on wound healing in a murine full thickness-excisional wound model were determined. The BCM was noted to reduce dermal

leukocyte infiltration at D1 post-wounding *in vivo* through macroscopic H&E observations and MPO staining. Myeloperoxidase is a heme-containing enzyme that is abundantly expressed in neutrophils, macrophages and monocytes (Aratani., 2018). This was opposite of the proposed hypothesis that PAO1 BCM in high doses should promote inflammation and suggests that BCM is able to reduce inflammation. We can also appreciate that not all the inflammatory infiltrates shown by the H&E staining were positive for MPO, suggesting other cell types (e.g. fibroblasts, basophils). However, since the wounds were harvested at day 1 post-wounding, their identities were highly likely to be neutrophils, as they are recruited first to the wound site (Shi and Parmer., 2011; Koh and Di Pietro., 2011; Schauer et al., 2014). Taken together, PAO1 BCM possibly reduces leukocyte infiltration at day 1 post-wounding *in vivo*.

In vitro and *in vivo* pro-inflammatory and anti-inflammatory cytokine levels were then checked to determine if the decreased immune cell infiltration affected cytokine secretion. Day 1 wound fluid was only collected on day 1 as the amount of wound fluid collected is highest on day 1 (data not shown). and the inflammatory infiltration in the dermal area was observed at day 1 post-wounding. Tumour necrosis factor-alpha (TNF- α), interleukin-6 (IL-6) and interleukin-10 (IL-10) were chosen as they are target genes regulated by NF- κ B, of which the latter cytokine is anti-inflammatory, while the rest are pro-inflammatory (Dinarello., 2000; Opal and DePalo., 2000). ELISA analyses showed that there was dampened pro-inflammatory cytokine secretion *in vitro*. Although insignificant, there was increased IL-10 secretion *in vitro* as well. Perhaps increased study (n) numbers would help to balance out variances between samples. There was unchanged cytokine secretion *in vivo*, probably due to the filter paper in the wound bed causing a foreign body reaction. As wound fluid collected reflected the

immune response from the entire wound, while the reduced leukocyte infiltration was only localised to the wound edge area, this could explain for the discrepancies in results as well.

There have been no published studies to date on the inflammatory responses of PAO1 BCM, but an unpublished thesis by Bowler (2015) showed that PAO1 BCM immune responses were elevated compared to controls. This could be due to differences in BCM generation methods since the biofilms generated by Bowler (unpublished thesis, 2015) were only 4 h old, which may not be as mature, causing virulence factors secreted to vary. This is in comparison to the biofilms generated after 2 days in this dissertation. Additionally, human lung epithelial cells may have different cytokine profiles compared to HaCaT keratinocytes. Moreover, the cells were incubated with BCM for 1 day by Bowler (unpublished thesis, 2015), which differs from a 5-h setup from this thesis. This could result in different responses compared to Bowler's results (unpublished thesis, 2015). There was no indication of BCM dosage in Bowler's results, so differences due to dosages could not be correlated. However, dosage studies were conducted and a lower dose of PAO1 BCM at 3 % could lead to reduced p65 levels in keratinocytes, albeit not as significant as 50 % (data not shown). Fibroblasts, however, had a complete absence of p65 levels when incubated with 3 % PAO1 BCM, which was similar to that of the 50 % dose. Since the *in vivo* observations were based on the high dose of 100 % BCM, dose-dependent effects were not highlighted in this thesis. In other studies using BCM, *P. aeruginosa* SAMMC-418 BCM induced inflammation in human mesenchymal stromal cells (MSCs) as shown by increased cytokine secretion such as TNF- α and IL-6 (Ward et al., 2015). A difference in the bacterial strain could be the reason for different inflammatory responses between the results of Ward et al. (2015) and this dissertation.

BCM of *S. aureus* ATCC 6538 elicited inflammation, further supporting that variations in bacterial strains can also contribute to differences in inflammation phenotype, (TNF- α , chemokine (C-X-C motif) ligand 2 (CXCL-2) and IL-6) in primary keratinocytes (Tankersley et al., 2014). Oral bacteria (*S. oralis* and *A. naeslundii*) BCM also elicited inflammation through TLR2 expression (Petridis et al., 2018). Despite many pathogenic bacteria showing that inflammation is induced upon BCM incubation, perhaps the low levels of cytokine production in PAO1 BCM is similar to *S. aureus* 10943 BCM as it elicits sustained low levels of cytokine production in human foreskin keratinocytes (Secor et al., 2011). As persistent inflammation is a hallmark of chronic wounds, PAO1 BCM could be responsible for this effect if future studies prove that the low cytokine secretion is sustained (Loots et al., 1998; Diegelmann., 2003; Nunan et al., 2014). As *P. aeruginosa* is known to be immunomodulatory, these novel findings of downregulating inflammation could be effective for host infection. To further characterise the effects of PAO1 BCM on the activation of different types of immune molecules, other cytokines and chemokines could be examined for using bio- or multi-plexed assays. This will give us a more complete picture of the types of immune molecules being up- or down-regulated.

A downregulation of *in vitro* total p65 and p50 protein levels in keratinocytes and fibroblasts was observed, with unchanged I κ B- α levels compared to controls when incubated with 50 % PAO1 BCM. Similarly, a downregulation of p65 protein levels *in vivo* was observed in keratinocytes and fibroblasts when 100 % PAO1 BCM and 10 X concentrated 100 % PAO1 BCM were used. The NF- κ B family transcription factors p65 and p50 bind to each other and get translocated into the nucleus from the cytoplasm when the inflammatory pathway is activated (Hayden and Ghosh., 2012; Ben-Neriah.,

2002). On the other hand, I κ B- α is an inhibitor of the NF- κ B pathway (Sun, 2011). Degradation of I κ B- α by the proteasome is essential for the p65/p50 complex to translocate into the nucleus and bind DNA for downstream transcriptional machineries to be activated (Ben-Neriah., 2002; Hayden and Ghosh, 2008). Taken together, these results suggest that the lack of I κ B- α degradation is sequestering the p65/p50 complex in the cytoplasm and somehow causes the downregulation of p65/p50. This suggests three reasons for the downregulated p65 and p50 levels: first, a downregulation of mRNA; second, a defective translational machinery; third, protein degradation.

mRNA levels of p65, p50 and I κ B- α were unchanged compared to controls, showing that the decreased total p65 and p50 protein levels was not caused by a decrease in mRNA expression. An exception was observed, where p65 expression in 50 % PAO1 BCM-incubated cells was significantly higher than that of the control. However, the increase in mRNA expression was not logically accountable for the significant downregulation in p65 protein levels.

Since the decreased p65 protein levels is not attributed to the decrease in mRNA expression, protein degradation was checked. The 2 main protein degradation pathways in eukaryotic cells are the ubiquitin-proteasome pathway and the autophagy-lysosome pathway (Cooper, G., 2000). The drug MG132 was used to inhibit the ubiquitin-proteasome pathway (Goldberg., 2012). MG132 reversibly acts on the chymotrypsin-like and caspase-like sites in the β subunit of the 20S proteasome that is a component of the 26S proteasome (Goldberg., 2012; Vivier et al., 2008). Blocking these sites prevent the proteolytic cleavage of proteins to small peptides by the 26S proteasome (Goldberg., 2012).

The drug bafilomycin A1 (BafA1) was used as a reversible inhibitor of the autophagy-lysosome pathway (Bowman et al., 1988; Yoshimori et al., 1991; Yamamoto et al., 1998). BafA1 is a vacuolar-type (H⁺)-ATPase (V-ATPase) inhibitor that reduces vesicle or vacuolar acidification, by blocking the passing of protons into the lysosomal lumen. This process is necessary for autophagosome maturation and fusion with lysosomes (Zhang et al., 1994; Yoshimori et al., 1991; Yamamoto et al., 1998; Shacka et al., 2006). Initial incubation of cells with BafA1 inhibits the autophagy pathway but longer incubation periods result in apoptotic cells (González-Polo et al., 2005). Due to the toxicities of both drugs and the uncertain effects that the drugs have on BCM properties, they were incubated with cells prior to the incubation of BCM and removed when BCM was added (Yuan et al., 2008; Han and Park., 2010; Möller Westerberg et al., 2012). Moreover, other studies have shown that drugs can be used as a pre-treatment before the intended product application/treatment in various studies (Yan et al., 2014; Feoktistiova et al., 2011).

Incubation of keratinocytes with 10 μ M MG132 for 1 h prior to 50 % BCM incubation resulted in the highest rescue in p65 signal intensity compared to BafA1 and other concentrations and timings of MG132 incubation. In light of MG132's function, this result suggests that p65 is degraded mainly via the proteasome pathway and could explain for the decreased p65 levels. Although there was some rescue, the amount of increase in p65 was not complete. This could be because MG132 is a reversible inhibitor and the amount of p65 signal rescued could have been suppressed by the effects of PAO1 BCM after inhibitor removal. As the binding partner of p65, p50, is also downregulated, there is a possibility that the entire heterodimer (with the exception of I κ B α) is being

degraded by the proteasome. Further experiments would need to be conducted to confirm this. Fibroblasts could also be tested for p65 signal as the mechanisms of protein downregulation could differ between cell types.

Apart from mRNA downregulation and protein degradation, the function of p65 could have been perturbed. There are several ways that this could occur, such as inhibiting nuclear import of p65 through nuclear accumulation of importin- α (adaptor required for nuclear import) as observed in *Salmonella* T3SS effector SpvD; alternatively through toxin or caspase-mediated cleavage of p65 (Rolhion et al., 2016; Coiras et al., 2008; Silva et al., 2013). Dimer exchange could have also occurred where NF- κ B heterodimers change on the same promoter due to the different binding affinities and transcriptional activities (Huxford et al., 1999; Lin et al., 1995; Chen et al., 1998). Defective post-translational modifications could have also occurred as the phosphothreonine lyase (OspF) effector causes the irreversible removal of phosphate groups on ERK due to its phosphothreonine lyase activity (Li et al., 2007a). Moreover, modifications such as acetylation and phosphorylation of p65 are common and modulate transcriptional activity, subcellular localisation, I κ B interaction and its DNA-binding capacity (Perkins., 2006; Chen et al., 2001; Kiernan et al., 2003; Zhong et al., 2002). These may have caused p65 levels to be low. However, in these cases, p65 (or its fragments, or modified p65) would have been detectable in the cytoplasm or nucleus, instead of not being detected at all. Should dimer exchange be occurring, the expression of p65/p50 heterodimers should still be maintained instead of being downregulated. Dimers that end up binding to the promoters of the gene targets would not be p65/p50. For further confirmation, immunoblotting with truncated fragments of various sizes like p40 and

p55 could be performed. In summary, the downregulation of p65 protein could not be attributed to p65 functional loss, post-translational modifications or dimer exchange.

Pseudomonas aeruginosa has been reported to target elongation factor EF2 for endocytosis and subsequent ribosylation through the pathogen's exotoxin A (ExoA) in *Caenorhabditis elegans* intestinal epithelial cells (McEwan et al., 2012; Dunbar et al., 2012). This results in the upregulation of a significant group of genes that are *P. aeruginosa* infection-related, resulting in heightened inflammation as a host stress response (McEwan et al., 2012). It was hypothesised that *C. elegans* may be using translational modification as a form of surveillance strategy towards pathogens (McEwan et al., 2012). Although this was a strategy to upregulate inflammation, the same could happen to downregulate inflammation. Apart from the fact that the pathogen can modulate host translational machinery, p65 itself is capable of regulating host translational proteins. The p65 positively regulates the transcription of a regulatory subunit of the RNA helicase eukaryotic initiation factor 4H (eIF4H), that is involved in translation initiation (Fiume et al., 2013). This positive regulation results in increased translation of eIF4A and increased global protein synthesis (Fiume et al., 2013). A downregulation of p65 due to proteasomal degradation could initiate a negative feedback loop where eIF4H is not as highly transcribed to initiate protein translation. A combination of defective protein translation and proteasomal degradation may have culminated in reduced p65 protein, which could explain the incomplete p65 signal intensity rescue observed in MG132 incubated cells compared to controls.

PAO1 BCM was shown to downregulate p65. Similarly, in a study by Kim et al.(2010), 4-hydroxy-2-alkylquinolines secreted by *P. aeruginosa* decreased TNF- α and IL-6

secretion and downregulates the NF- κ B pathway through delayed I κ B degradation in primary bronchoalveolar lavage cells and mouse monocyte/macrophage cells. The T3SS effector of *S. flexneri* probable E3 ubiquitin-protein ligase (IpaH4.5) is able to modulate the immune response by interacting with p65 as an E3 ubiquitin ligase, causing the proteasomal degradation of the p65 subunit (Wang et al., 2013a). Four independent studies have shown that the metalloprotease, NleC of *E. coli*, degrades both free cytoplasmic p65 and p65 that is complexed with I κ B α (Muhlen et al., 2011; Pearson et al., 2011; Yen et al., 2010; Baruch et al., 2011). It was also reported that during *Edwardsiella piscicida* infection, phosphothreonine lyase-dependent p65 proteasomal degradation occurs through de-phosphorylation of p65 (Hou et al., 2018). Generally, downregulating the NF- κ B pathway through p65 degradation seems to be common among pathogens for hijacking the pathway. This could be a way that *P. aeruginosa* uses to increase host survival and pathogen persistence simultaneously while switching to a chronic infection mode (Monack et al., 2004; Diacovich and Gorvel., 2010; Hajishengallis and Lambris., 2011).

Since I κ K- α is closely affiliated to both the NF- κ B and the keratinocyte differentiation pathways, the effects on differentiation were checked (Liu et al., 2012). In PAO1 BCM-challenged wounds, both early and late keratinocyte differentiation were impaired *in vitro*, while early keratinocyte differentiation was hampered in intact epidermis behind the wound edge *in vivo*. These suggest that PAO1 BCM may have dedifferentiated keratinocytes, causing an absence of K10 and involucrin protein *in vitro* and a slight downregulation of K10 in the intact epidermis *in vivo*. Calcium ions are major regulators of keratinocyte differentiation and proliferation (Yuspa et al., 1989; Hennings et al., 1980; Pillai et al., 1988). In low calcium concentrations (0.03 mM), keratinocytes

proliferate but are unable to differentiate (Wilson., 2013). However, past the threshold of 0.1 mM, differentiation is initiated (Hennings et al., 1980). As a reference, high calcium growth media contain around 2.8 mM calcium (Wilson., 2013). In DMEM, the calcium concentration (from calcium chloride) is 1.8 mM and the presence of FBS contains high levels of calcium, so keratinocytes would have been induced to differentiate. However, this was not observed in PAO1 BCM-incubated keratinocytes. Despite being terminal, keratinocytes are able to dedifferentiate into their precursor cells through the help of basic fibroblast growth factor (Wikaramanayake et al., 2014; Sun et al., 2011; Mannik et al., 2010). Fibroblasts are also able to reversibly differentiate from one state to another via myoblast determination protein 1 (MyoD), so there may be a possibility that keratinocytes use this mechanism for switching between differentiated and de-differentiated states (Driesen et al., 2014; Satoh et al., 2008; Hecker et al., 2011).

Wound-edge keratinocytes displayed a loss of K10 in PAO1 BCM-challenged wounds compared to controls. Stojadinovic et al. (2008) have reported that early differentiation markers such as keratins 1, 10 and small proline-rich proteins were downregulated along with filaggrin (late differentiation marker) in non-healing edge venous ulcers compared to healthy, unwounded skin. However, late differentiation markers such as involucrin, loricrin and transglutaminase 1 and other small proline-rich proteins were still expressed (Stojadinovic et al., 2008). Keratinocytes in diabetic foot ulcer wound edges were also shown to have reduced expression of K10, indicative of an impaired differentiation state (Usui et al., 2008). These reiterate that PAO1-BCM-challenged wounds have some features in common with chronic wounds.

PAO1 BCM was found to affect K10 levels in different parts of the wound differently. Nascent epidermis found in the newly formed epidermal tongue had slight increase in K10 levels compared to controls, suggesting that it has started to differentiate. It is proposed that the BCM reduces proliferation in the keratinocytes epidermal tongue to keep infected wounds open, resulting in delayed healing. This is because differentiation and proliferation have inverse relationships (Ruijtenberg and van den Heuvel., 2016). Based on current literature, this is the first report on the relationship between *P. aeruginosa* and keratinocyte differentiation in epithelial tongues. An increase in epidermal thickness in PAO1 BCM-challenged wounds compared to controls was noted, especially in the spinous/basal layer. Although there are no prior studies on the effects of *P. aeruginosa* secreted factors on pathogen invasion, *P. aeruginosa* is known to attach to the stratum corneum for biofilm formation (Garcia et al., 2018; Mihai et al., 2014; Roy et al., 2014; Brandenburg et al., 2015); Jensen et al., 2017). Since the pathogen uses virulence factors to alter cell junctions such as occludin, e-cadherin and β -catenin (shown in Chapter 3), it is suspected that these junctional breaches may have altered the thickness of the epidermal layers for facilitating pathogen entry (Golovkine et al., 2018). Roy et al. (2014) have also shown that biofilm-infected wounds can be closed but TEWL was increased, signifying that there was impaired skin barrier function restoration. This was found to be due to a loss of tight junction proteins (ZO-1 and 2). Similarly, wounds challenged with PAO1 BCM saw a decrease in K10 and involucrin expression in the epidermis, suggesting that skin barrier function is also impaired. Together with results from Chapter 3 where a loss of occludin was observed in cells incubated with 50 % PAO1 BCM, this reiterates the possibility that impaired keratinocyte differentiation and tight junction protein loss lead to improper skin barrier repair. Another explanation for this is the elevation of IL-31 expression that is observed in allergic and inflammatory

diseases (Cornelissen et al., 2012). IL-31 has been shown to cause epidermal thickening through basal cell proliferation, which is similar to observations in this dissertation (Singh et al., 2016). However, this is unlikely since inflammation was suppressed, so IL-31 would have been reduced.

Figure 62: Expression of various keratinocyte differentiation markers in the different skin layers.
Image by Erica Teo Mei Ling. Information adapted from Sandilands et al. (2009).

Although there are no studies on the relationship between *P. aeruginosa* and keratinocyte differentiation, *S. aureus*, which is prevalent in chronic wounds (often co-existing with *P. aeruginosa*) is well known to affect keratinocyte differentiation (Bur et al., 2013; Son et al., 2014). The extracellular adherence protein of *S. aureus* (Eap) heightened expression of keratinocyte differentiation markers K10 and involucrin, as a possible mechanism to hide from the host during skin infections (Bur et al., 2013). Another study using neonatal human keratinocytes and reconstructed human epidermis reported that *S. aureus* reduces markers such as K10 and filaggrin by increasing IL-6 secretion and STAT3 activation (Son et al., 2014). In contrast, decreased K10 and IL-6 levels were observed with PAO1 BCM. Perhaps the impaired keratinocyte differentiation observed is also caused by IL-6 and STAT3. The effects of *P. aeruginosa*

on keratinocyte differentiation is not well researched. Studies should be conducted as the differentiation process is crucial for maintenance of skin integrity. Other studies have shown the impairment of mesenchymal stromal cell differentiation by *P. aeruginosa* BCM and osteogenic differentiation by *S. aureus* BCM (Ward et al., 2015; Sanchez et al., 2013a). Although they are differentiation processes in different cell types, it concurs with the results in this dissertation that the changing of cell states can be hampered by PAO1 BCM.

Herein, JNK pathway was downregulated by PAO1 BCM, with a concomitant decrease in c-Jun levels. Similar to this dissertation, *P. aeruginosa* has been reported to exploit kinase pathways as well by inducing apoptosis in human conjunctiva epithelial cells and human megakaryocytes through JNK pathway activation (Gopala Krishnan et al., 2019; Jendrossek et al., 2001). Additionally, Losa et al. (2014) have reported that apoptosis induced in airway epithelial cells by *P. aeruginosa* is caused by increased gap junction expression and this increase in gap junction is regulated by JNK signalling inhibition. The T3SS in *P. aeruginosa* is also involved in activation of the JNK pathway to increase apoptosis and IL-8 production (Cuzik et al., 2006; Jendrossek et al., 2001; Jia et al., 2003; Wood et al., 2015a). Taken together, the induction of apoptosis in PAO1 BCM-challenged keratinocytes (seen in Chapter 3) suggested that the JNK pathway should have been upregulated.

In contrast, other studies show that epidermal c-Jun activation promotes keratinocyte differentiation and inflammation through epidermal growth factor (EGF) and increased pro-inflammatory cytokine expression respectively in various *in vitro* and *in vivo* models (Schonthaler et al., 2011; Park et al., 2008; Zhao et al., 2016a; Ip and Davis.,

1998; Ying et al., 2015). Activation of c-Jun also leads to increased keratinocyte differentiation in human neonatal foreskin keratinocytes and c-Jun itself is present in all stages of differentiation (Gazel et al., 2006; Jang and Steinert., 2002; Mehic et al., 2005). Moreover, JNK1, JNK2 and JNK-dependent soluble factors are required for keratinocyte differentiation in primary human keratinocytes (Schumacher et al., 2014). In agreement to these studies, we observed a decrease in c-Jun protein, with a decrease in keratinocyte differentiation and inflammation. It has also been shown that p65 inhibits JNK signalling and Jun kinase activity in the epidermis and various cell lines, which is what we have observed in this dissertation (Zhang et al., 2004b; Reuther-Madrid et al., 2002). This study also suggests that the downregulated p65 observed could be linked to the decreased JNK and c-Jun levels. Reasons for the discrepancy between JNK1 and c-Jun levels could be that JNK participates in crosstalk with other pathways since it is one of the major signalling kinases. Moreover, c-Jun is downstream of JNK1 and is more targeted in inactivating involucrin, which directly controls late keratinocyte differentiation, so it is less likely to be influenced by possible activation of multiple pathways simultaneously. Differences in results could also be attributed to different cell types being used and the use of biofilm conditioned media instead of inoculation with live bacteria. Moreover, as confluency contributes to keratinocyte differentiation *in vitro*, the difference in cell monolayer confluency between studies causes the induction of differentiation to be varied as well.

It is proposed that the global downregulation of genes involved in the NF- κ B pathway may be attributed to changes in kinases even more upstream of JNK such as MAPK, TAK and PI3K. As the link between inflammation, keratinocyte differentiation, JNK and *P. aeruginosa* biofilm secreted factors has not yet been clearly established, an array

of kinase inhibitors can be used to determine the regulators of the downregulated NF- κ B signalling and impaired keratinocyte differentiation.

Figure 63: Keratinocyte differentiation pathway involving K10 and involucrin.

Image by Erica Teo Mei Ling.

4.4 Conclusion

In this chapter, the biofilm exudate of *P. aeruginosa* was demonstrated for the first time to be capable of inducing a downregulation of the immune response through the NF- κ B pathway. This is achieved through possible proteasomal degradation of the p65 subunit. This chapter has also illustrated the implications of decreased p65 on wound healing responses such as inflammation and keratinocyte differentiation. Fewer leukocytes were seen to infiltrate into wounds with PAO1 BCM application compared to controls at day 1 -post wounding. This was coupled together with an anti-inflammatory cytokine profile *in vitro* and *in vivo*. Both early and late keratinocyte differentiation were also impaired *in vitro* and *in vivo* at day 7 post-wounding. Based on current results, this dampening of the NF- κ B pathway seems to be regulated by the JNK pathway. Seen in this light, *P. aeruginosa* may have hijacked the NF- κ B pathway to potentiate its colonisation and subsequent infection of wounds. Finally, this chapter presents the potential of rescuing

the decreased expression of p65 subunit of the NF- κ B pathway as a therapeutic strategy to alleviate infections caused by *P. aeruginosa*.

5. DETERMINATION OF PATHOGENIC PAO1 BCM COMPONENTS AND THE IMPLICATIONS ON PATHOGENESIS

5.1 Introduction

Previous experimental chapters have shown the range of wound healing features which the secreted factors of *Pseudomonas aeruginosa* biofilms affect, namely, cell migration, cell viability, cell adhesion, inflammation and keratinocyte differentiation. In this chapter, I now explore the various known or novel pathogenic factors present in PAO1 BCM that are involved in eliciting effects on the host cells. I propose that knowledge of these factors would facilitate development of effective treatments for infected wounds.

Debridement is one of the gold standards of wound therapy for removal of necrotic and infected tissue (Kirshen et al., 2006). However, debridement has its limitations. *P. aeruginosa* biofilms were regenerated and returned to pre-debridement levels by some bacterial remnants in a porcine burn model within 48 h (Roy et al., 2014). Only a temporary decline in bacterial bioburden was observed (Roy et al., 2014). Additionally, new microcolonies were found in deeper tissue layers post-debridement, raising concerns that sharp debridement may lead to inoculation into deeper tissues and culminate in persistent infections (Roy et al., 2014). Apart from bacterial colonies that can remain in the wound, secreted factors could also aggravate wound healing. *Pseudomonas aeruginosa* biofilms produce secretory factors that hamper wound healing, however the exact molecules in biofilm conditioned media that are causing this effect are unknown (Marano et al., 2015). *Pseudomonas aeruginosa* is well-known for its secretory systems and some of the known factors include proteins such as alkaline protease, elastase and exotoxins (Bleves et al., 2010). With the presence of LPS and eDNA, *P. aeruginosa* biofilms are capable of converting infiltrating phagocytes into

cells that destroy host tissue even without direct contact with the bacteria (Ciszek-Lenda et al., 2019). Biofilm matrix components of *P. aeruginosa* such as EPS and eDNA also elicit low-grade chronic inflammation (Ramirez et al., 2019). The negative impact of PAO1 BCM (e.g. compromised cell viability) on wound healing *in vitro* and *in vivo* was already shown in previous chapters (Chapters 3 and 4). However, the identities of the components in this BCM that cause these effects are currently unknown. Moreover, there are currently no known *P. aeruginosa* virulence factors that are reported to have these negative effects on keratinocyte differentiation, focal adhesions and inflammation. Identifying these secreted components are important as they have the capability to cause host destruction and so become future drug targets to improve wound healing.

Contrary to what is known about pathogenic bacteria, low concentrations of PAO1 BCM may exert positive effects *in vitro* on human keratinocytes and fibroblasts, where a slight increase in cell migration rate was observed in a scratch wound assay (Chapter 3). The identities of what causes this phenomenon are also currently unknown. Identifying these components would create potential therapeutic options for promoting faster wound closure.

I hypothesise that PAO1 BCM contains secretory factors that both assist and interfere with the healing process, in a concentration dependent fashion. These factors are probably proteins or metabolites given the wide spectrum of virulence factors that *Pseudomonas aeruginosa* is reported to produce (Gellatly and Hancock., 2013; Bleves et al., 2010). Given these research gaps, this chapter aims to identify the factors involved.

Currently, we do not know if it is one or a multitude of factors that are involved in affecting the different wound healing responses. Since high concentrations (50-100 %) of PAO1 BCM reduced viability in keratinocytes and fibroblasts (see Chapter 3), I decided to determine the possible factors that could cause cytotoxicity before moving on to the other wound healing responses. The MTT assay was used widely in this chapter to determine if cell viability was improved or worsened after certain properties of PAO1 BCM have been altered.

Some common mutants of PAO1 (The *lasR* mutant is defective in AHL-based quorum sensing, the *lasB* mutant is defective in the elastase gene and the *rhIA* mutant is unable to produce heat-stable hemolysins, which are important for rhamnolipid synthesis (Mun et al., 2009; Kuang et al., 2011; Alcorn et al., 2004; Held et al., 2012; Whiteley and Greenberg., 2001; Johnson and Boese-Marrazzo., 1980; Deziel et al., 2003)) were grown to generate BCM, which was tested at high concentrations of 50 % and 75 % to determine any activity on cell viability. Next, an array of biochemical tests was used to decipher if the secretory factor involved was protein. Finally, endotoxin testing was used to determine if the common virulence factor of *P. aeruginosa*, LPS, was the main player in exerting toxicity *in vitro* and *in vivo*. A non-confluent monolayer cell cultures for timelapse videos was used as a qualitative measure of cytotoxicity to prevent over-confluency from influencing cytotoxicity. The temperatures chosen for heat-inactivated media represented low, moderate and high heat. Currently, there is no standardisation in heating temperatures nor duration provided in the literature. For example, Marano et al.(2015) used 75 °C for 20 mins to denature proteins, while Bowler (unpublished thesis-2015) used 100 °C for 5 mins. Lastly, 50 µg/ml proteinase K was used for digestion at 50 °C for 1 h as it is the optimal temperature for PK activity (Farrell., 2010; Ebeling et

al., 1974). However, there is also little standardisation in the proteinase K concentration and digestion timings provided in the literature. For example, the concentrations of proteinase K that have been used range from 50-200 µg/ml, the timings range from 30 mins- 2 h, and the temperature used is usually 37 °C (Jandu et al., 2006; Shime et al., 2008; Hauck et al., 2008). Due to the variability in proteinase K digestion temperatures, an intermediate combination of 37 °C for 1 h was used in our study. Trypsin was used at 37 °C as that is its optimal digestion temperature (Babu., 2013). Apart from PMSF, literature has provided other options of inhibition of proteinase K and trypsin activity, which include use of the serine protease inhibitor Pefabloc, centrifugation and α_2 -macroglobulin (Jandu et al., 2006; Rossi et al., 2012; Hauck et al., 2008). *Escherichia coli* LPS was used instead of *P. aeruginosa* LPS as the latter activates cells slower and is less purified, causing increased chances of false positive responses compared to *E. coli* LPS (Raoust et al., 2009). The scope of the work was limited to biochemical testing to decipher the identity of the factors involved, but identification via advanced biochemical (e.g. mass spectrometry) or genetic (e.g. mutant library screen) methods is recommended in **7. FUTURE PERSPECTIVES** of this dissertation.

5.2 Results

5.2.1 The toxic effect observed in PAO1 BCM is not strain specific

First, I investigated if the cytotoxicity, morphological changes and PI uptake observed from PAO1 were applicable in another *P. aeruginosa* strain. In this case, a clinical strain PA 15159 of diabetic foot ulcer origin was chosen, its BCM collected and effects tested on HaCaT cells. I then checked if the biofilm-forming capacity of both strains differed, as biofilm-formation could have implications on the toxicity level of the BCM. I observed similar results ($P > 0.05$) between the wild-type PAO1 BCM and PA 15159

BCM (**Figure 64**). Time-lapse widefield microscopy showed that the morphological changes (rounded cells with PI uptake indicating possible cell death and shrinkage of cytoplasm and formation of large holes between the cells indicating loss of cell-surface adhesion) were similar to that of the wild-type PAO1 BCM (**Figure 65**).

Figure 64: Crystal violet assay on filter wools of PAO1 and PA15159 at day 5 BCM harvest. Crystal violet assay measured biofilm forming capacity. No significant differences were found between the amount of biofilms formed on the filter wool (quantified via $A_{580\text{ nm}}$) of PAO1 and PA15159. Statistical comparison between groups were performed using 1-way ANOVA and Tukey post hoc analysis. (P-value: NS $P>0.05$). Data has been expressed as mean $\pm$ SD. Figure is representative of 3 biological and technical replicates ($N=3$, $n=3$) for each condition.

Figure 65: Toxic effects of PAO1 BCM are not strain specific. 30 % PAO1 BCM and PA15159 BCM were incubated with HaCaTs for 24 h in a time-lapse experiment. Scale bar: 75 μm . Figure is representative of 3 biological and technical replicates ($N=3$, $n=3$) for each condition.

5.2.2 Toxicity not caused by some common virulence genes

I investigated if the observed toxicity of PAO1 BCM was caused by some known common virulence genes. In this case, *lasR*, *lasB* and *rhlA* genes were checked for contributions to the toxicity of PAO1 BCM. The *lasR* mutant is defective in AHL-based quorum sensing, the *lasB* mutant is defective in the elastase gene and the *rhlA* mutant is unable to produce heat-stable hemolysins, which are important for rhamnolipid synthesis (Mun et al., 2009; Kuang et al., 2011; Alcorn et al., 2004; Held et al., 2012; Whiteley and Greenberg., 2001; Johnson and Boese-Marrazzo., 1980; Deziel et al., 2003). Using an MTT assay to test for cell viability of the HaCaTs, none of the mutant BCMs rescued the reduced cell viability ($P > 0.05$) of the wild type PAO1 BCM (**Figure 66**). The *rhlA* mutant was actually more toxic than control and demonstrated decreased cell viability ($P < 0.001$) compared to the wild type PAO1 BCM (**Figure 66**).

Figure 66: Mutant PAO1 BCM do not rescue cell viability loss induced by wild-type BCM in an MTT assay. For a positive control, the MTT reagent was diluted in DMEM and lysis buffer in a well containing cells, while the negative control was prepared the same way without cells. Treatment results were normalised to the positive control of 1. Statistical analyses using 2-way analysis of variance (ANOVA) and Dunnett's post hoc tests were performed between all the conditions and the control (DMEM+10% FBS) (P-value: *** $P < 0.001$, NS $P > 0.05$). Data has been expressed as mean $\pm$ SD. Figure is representative of 3 biological and technical replicates (N=3, n=3) for each condition.

5.2.3 Toxic components may be heat-sensitive

As it was hypothesised in this thesis that PAO1 BCM toxicity could be attributed to secreted protein factors, the PAO1 BCM was heated to a range of temperatures and tested for its subsequent biological effect on HaCaT cell viability by an MTT assay. Heating for 1 h at 37 °C and 50 °C did not result in a rescue from the toxicity ($P < 0.001$), however 100 °C was effective ($P < 0.01$) in rescuing from the toxic effects (**Figure 67**).

Figure 67: Heating at high temperature of 100 °C rescues cell viability loss seen in non-heated (non-HI) BCM in an MTT assay. Conditions that are 75 % of heat-incubated (HI) DMEM/BCM are topped up with normal, complete DMEM. For a positive control, lysis buffer with cells was used, while the negative control was without cells. Treatment results were normalised to the positive control of 1. Statistical analyses using 1-way analysis of variance (ANOVA) and Tukey's post hoc tests were performed between all the conditions (P -value: *** $P < 0.001$, ** $P < 0.01$, NS $P > 0.05$). Data has been expressed as mean $\pm$ SD. Figure comprises 3 each of biological and technical replicates ($N=3$, $n=3$) for each condition.

5.2.4 Toxic components are not proteinaceous

To further ascertain if the toxic components were proteinaceous, proteinase K (PK) and trypsin were incubated with BCM to enzymatically digest any proteins present. Proteinase K and trypsin are broad-spectrum serine proteases that cleaves peptide bonds in proteins (Ebeling et al., 1974; Morihara and Tsuzuki., 1975). Phenylmethylsulfonyl fluoride (PMSF) was used as an inhibitor of proteinase K (Koffman et al., 1991; Babu et al., 2013). Proteinase K digestion at 37 °C ($P < 0.001$) and 50 °C for 1 h, and trypsin

digestion ($P < 0.05$) did not rescue the cytotoxic effect of PAO1 BCM on HaCaTs when compared to their respective controls (**Figure 68**).

Figure 68: Protease digestion does not rescue cell viability from BCM in an MTT assay using (A) proteinase K and (B) trypsin. All digestions took place for 1 h at 37 and 50 °C for (A) and at 37 °C for (B). For a positive control, the MTT reagent was diluted in DMEM and lysis buffer in a well containing cells, while the negative control was prepared the same way without cells. Treatment results were normalised to the positive control of 1. Statistical analyses using 1-way analysis of variance (ANOVA) and Tukey's post hoc tests were performed between all the conditions (P-value: *** $P < 0.001$, * $P < 0.05$, NS $P > 0.05$). Data has been expressed as mean $\pm$ SD. Figure is representative of 3 biological and technical replicates ($N=3$, $n=3$) for each condition.

5.2.5 Toxicity is not LPS-related

Since the toxic factor causing a loss of cell viability seemed to be non-proteinaceous, I decided to check if LPS is the main cause for toxicity in PAO1 BCM. I first quantified the amount of LPS in the BCM to see if there was any present in the media and then determined if the observed LPS amount caused toxicity in human keratinocytes (HaCaTs). Results showed that there was only 0.0135 ng/ml of LPS present in the BCM. Through the scratch wound assay, I showed that this amount did not cause toxicity to the human keratinocytes compared to PAO1 BCM at 100 % concentration after 5 h incubation with the cells (**Figure 69**).

Figure 69: Lipopolysaccharide is not responsible for the toxic effects in PAO1 BCM on cells. Time-lapse images of scratch wound assay after 5 h of incubation with 100 % PAO1 BCM and 0.0135 ng/ml LPS on HaCaTs. Scale bar= 75 μ m. Figure is representative of 3 biological and technical replicates (N=3, n=3) for each condition.

5.2.6 FBS is needed during BCM generation for cytotoxin production

To determine if FBS was needed by the pathogen to produce the cytotoxic factors, I grew up another batch of PAO1 BCM without supplementing with FBS in the growth media. Results showed that the cell viability differences at 1 and 4 h post-incubation ($P > 0.05$) were insignificant (**Figure 70**). However, PAO1 BCM at 50 % dose created without FBS showed a rescue in cell viability ($P < 0.01$) at 24 h post-incubation compared to that of normal BCM (**Figure 70**). It is noted that the difference in cell viability of PAO1 BCM created without FBS still remains significantly ($P > 0.05$) lower than control at 24 h (**Figure 70**).

Figure 70: PAO1 BCM grown without 10 % FBS supplementation attenuated the reduced cell viability compared to wild-type BCM in an MTT assay. For a positive control, the MTT reagent was diluted in DMEM and lysis buffer in a well containing cells, while the negative control was prepared the same way without cells. Treatment results were normalised to the positive control of 1. Statistical analyses using 2-way analysis of variance (ANOVA) and Tukey's post hoc tests were performed between all the conditions (P-value: *** $P < 0.001$, ** $P < 0.01$, NS $P > 0.05$). Data has been expressed as mean $\pm$ SD. Figure is representative of 3 biological and technical replicates ($N=3$, $n=3$) for each condition.

5.3 Discussion

In this chapter, I have conducted preliminary tests to determine the nature of the cytotoxic factors present in PAO1 BCM. *lasR*, *lasB* and *rhlA* genes were not the cause of toxicity of PAO1 BCM. All the BCMs derived from bacteria with these mutant genes do not ameliorate cell death. From this I was able to conclude that factors related to AHL-based quorum sensing elastase B and rhamnolipid are not causing cytotoxicity respectively (Moradali et al., 2017; Mun et al., 2009; Kuang et al., 2011; Alcorn et al., 2004; Held et al., 2012; Whiteley and Greenberg., 2001; Johnson and Boese-Marrazzo., 1980; Deziel et al., 2003). Known effectors of apoptosis in *P. aeruginosa* are the T3SS cytotoxins such as ExoT and ExoS, and the quorum sensing autoinducer 3-oxo-C12-HSL (Kaufman et al., 2000; Shafikhani et al., 2008; Wood et al., 2015a,b; Li et al., 2004; Kaminski et al., 2018). Host-cell contact allows T3SS to be activated, which is absent from my system since biofilms were not cultured together with host cells, so *lasB* should not be one of the genes causing cytotoxicity in my model. Albumin could have inhibited quorum sensing in *P. aeruginosa* through sequestering homoserine lactones,

resulting in the similar cell viability of wild-type and mutant *lasR* BCM (Smith et al., 2017). The *rhlA* mutant showed a decreased cell viability compared to the wild type PAO1 BCM, suggesting that the loss of rhamnolipid production may have affected other genes that drive an increase in toxicity towards keratinocytes. Since rhamnolipids are able to induce necrosis in polymorphonuclear leukocytes, they may be one of the indirect players involved in contributing to PAO1 BCM cytotoxicity in keratinocytes (van Gennip et al., 2009; Jensen et al., 2007). This is supported by a study showing that human neonatal fibroblasts and keratinocytes undergo cell death (apoptosis and necrosis) when incubated with BAC3, a *Pseudomonas* rhamnolipid (Stipcevic et al., 2005).

However, the absence of virulence factors may not equate to reduced pathogenic virulence. Virulence is important for establishing infections and acute virulence may be lost or reduced to induce chronicity (Granato et al., 2016; Smith et al., 2006; Bragonzi et al., 2009; Winstanley et al., 2016; Turner et al., 2014; Sousa and Pereira., 2014; Nguyen and Singh., 2006). Studies demonstrated that *P. aeruginosa* bacterial fitness (the ability of bacterial replication in a given environment) in chronic wounds is regulated by the capacity for high-density bacterial growth *in vitro* (in the absence of host) and metabolic factors such as long-chain fatty acids, but not virulence factors or biofilm functions (Morgan et al., 2019; Turner et al., 2014). Some virulence factors could be dispensable as well, as the accumulation of mutations in genes related to quorum sensing, drug efflux and Gac signalling system in *P. aeruginosa* are prevalent in cystic fibrosis patients (Smith et al., 2006; Turner et al., 2014; Winstanley et al., 2016). Clinical isolates from chronic human wounds were often defective in twitching motility, rhamnolipids and proteases, and could even lack both quorum sensing systems (Morgan et al., 2019). *Pseudomonas aeruginosa* 2-aminoacetophenone induces apoptosis and

oxidative stress in murine skeletal muscle through downregulating mitochondrial membrane potential *in vivo* (Bandyopadhyaya et al., 2016; Tzika et al., 2013). This quorum-sensing excreted small volatile molecule is usually found in chronic infections as it promotes adaptation to hypoxic conditions in the *Drosophila* infection model through inducing *lasR* mutations (Bandyopadhyaya et al., 2012; Kesarwani et al., 2011; Hoffman et al., 2010). Taken together, the similar cell viability of mutant *lasB* and *lasR* compared to wild-type could be due to them being dispensable and mutations in these genes may create a chronic infection phenotype. Improvements to the study include testing out BCM of other metabolic- or EPS-related mutant genes to see if cell death is elicited. This may further narrow down possible genes involved in host cytotoxicity.

Cytotoxicity was prevented after heating the BCM at 100 °C but not 37 °C and 50 °C, suggesting that the factors are heat sensitive only at higher temperatures of 100 °C. Proteins are not considered as they are slowly denatured as temperature rises (as low as 25 °C), and becomes irreversible beyond 80 °C (Schön et al., 2017; Cavagnero et al., 1998; Potheikin et al., 2000; Wrba et al., 1990; Klump et al., 1992; McAfee et al., 1996; Pfeil et al., 1997; Pappenberger et al., 1997; Ogasahara et al., 1998). They can be denatured at 95 °C within 10 mins (Qian et al., 2017). A rescue in cell viability should have been observed when BCM was heated if the factors involved were proteinaceous. Lipids have not been ruled out despite their thermal degradation taking place above 100 °C as some lipids can remain stable at temperatures around 200 °C depending on fatty acid composition (Charuwat et al., 2018; Chen et al., 2018; Nawar., 1969). The duration of heating in my study was 1 h, which may not be sufficient for complete degradation of lipids present in BCM. For example, Nawar (1969) has shown that glycerides heated at 200 °C for 3 h led to the release of short and long chain fatty acids, which raises the

possibility that lipids may have contributed to cytotoxicity. As for carbohydrates, they degrade over a range of temperatures depending on the type of sugar (e.g. glucose, fructose), from as low as 100 °C in the presence of liquid, to as high as 160 °C, where caramelisation occurs and volatile diacetyls are released (Luning et al., 1994; Golon and Kuhnert., 2012). Due to the thermal stability of carbohydrates, there is a possibility that the cytotoxic factors involved are carbohydrate-related. In terms of nucleic acids, DNA in aqueous solutions degrades between 100 °C and 110 °C under pressure around 5 mins and above 90 °C under *in vitro* conditions, so there is a possibility that eDNA is one of the cytotoxins (Karni et al., 2013; Nguyen-Hieu et al., 2012; Kozyavkin et al., 1995). Metabolites are also one of the possible toxins because they demonstrated very altered properties at high temperatures of at least 100 °C for as little as 5 min in a study by Fang et al. (2015). A recent study by Song et al. (2019) showed that the quorum sensing autoinducer (a metabolite), *N*-(3-oxo-dodecanoyl) homoserine lactone, triggers apoptosis in lymphocytes. This suggests a possibility that metabolites may be one of the possibilities in inducing apoptosis in keratinocytes. Currently, there is a lack of evidence correlating eDNA and *P. aeruginosa* secreted carbohydrates to host cell death, so existing studies were unable to be compared. In summary, the thermal stabilities of lipids, carbohydrates, nucleic acids and metabolites are consistent with the results from my study where these cytotoxic factors are only sensitive to high temperatures around 100 °C.

The cleavage of peptide bonds by proteinase K and trypsin did not prevent cytotoxicity. This confirms that the cytotoxic factors are non-proteinaceous. Digestion by proteinase K at 50 °C led to an increased cell death compared to 37 °C, which could be due to the increased activity of proteinase K at 50 °C (Farrell., 2010; Ebeling et al., 1974).

Arguably, the presence of a biofilm matrix-associated serine protease inhibitor (a protein), ecotin, may cause the inhibitor to act on proteinase K and trypsin instead of BCM (Tseng et al., 2018). However, the amount of ecotin available may not be in high amounts, as BCM generation did not involve lysis of the biofilm matrix. Tseng et al. (2018) also showed that ecotin was only present within bacterial biofilm cells and not in the extracellular matrix at 48 and 96 h post-biofilm growth, and is only present in the extracellular matrix in older biofilms formed after 144 h. Moreover, if this inhibitor was present, it would cause results of both proteinase K and trypsin to be the same, which was not observed. Additionally, since ecotin is a protein, it would have been denatured as digestion studies were conducted at 37 and 50 °C. Lastly, a major difference is in the way biofilms were grown. Tseng et al. (2018) used tube biofilms grown under continuous flow. Experiments could be performed to confirm if ecotin is present in BCM.

LPS was present in BCM but the amount did not elicit any morphological changes in cells. Being a major virulence factor of gram-negative bacteria, LPS is encased and secreted through membrane vesicle trafficking in outer membrane vesicles (OMV) that may contain other virulence factors (Kulp and Kuehn., 2010). For years, biofilms have been studied using *in vitro* and *in vivo* models, but it remains debatable whether *in vitro* models resemble *in vivo* biofilms in chronic infections (Lebeaux et al., 2013; McBain., 2009; Joo and Otto., 2012; Coenye and Nelis., 2010; Bjarnsholt et al., 2013). As LPS is used as a form of immune stimulation and is usually exogenously introduced to the host, it is difficult to find references for comparing the relevance and amount of LPS detected in BCM (Raoust et al., 2009; Brunetti et al., 2016). Future improvements could include measuring LPS amounts *in vivo* and use of LPS from *P. aeruginosa* to further confirm results obtained in the current study. Studies have shown that LPS of these 2 species are

comparable in terms of macrophage stimulation despite structural differences, with 80 % of *P. aeruginosa* LPS cores are uncapped compared to an estimated 10 % found in *E. coli* (Goldman and Leive., 1980; Raoust et al., 2009; Wilkinson and Galbrath., 1975).

Lastly, FBS components are involved in contributing to PAO1 BCM cytotoxicity in keratinocytes. FBS contains bovine serum albumin as a major component, with other proteins such as glycoproteins and complements (Issaq et al., 2007; Treadwell and Ross., 1963; Anderson and Anderson., 2002; Anderson et al., 2004). FBS also contains amino acids, lipids, carbohydrates, nucleic acids and hormones (Treadwell and Ross., 1963; Wei et al., 2016; Anderson and Anderson., 2002; Psychogios et al., 2011; Anderson et al., 2004). Since cell viability was slightly increased when FBS supplement to the growth media during BCM generation was removed, its presence contributed to increased virulence. The pathogen could have used FBS components to synthesise the secreted factors in PAO1 BCM, thereby contributing to increased cytotoxicity. Insignificant differences in cell viability at 4 and 8 h were noted between normal PAO1 BCM and PAO1 BCM created without FBS. This may be due to PAO1 BCM created without FBS containing cell-survival factors that are of larger molecular weight, causing cell uptake to be slower. Although cell uptake timings differ based on cell type and particle size, Behzadi et al. (2017) claim that cell uptake via phagocytosis range from 30 mins to several h. A study by Wei et al. (2016) demonstrated that RNA in FBS can be taken up by glioma cells, so there may be a chance that *P. aeruginosa* uses the RNA present in FBS for synthesising secreted factors. Serum also promotes virulence factor production in *P. aeruginosa* by increasing production of quorum sensing genes in late phase growth and upregulating iron-related genes such as sigma factor *pvdS* and exotoxin A regulatory gene *regA* (Kruczek et al., 2014; Kruczek et al., 2012). Other

pathogens have also been known to upregulate virulence in serum conditions. For example, the presence of calf serum significantly increased expression of enterotoxins, proteases, haemolysins and iron acquisition factors in *S. aureus* (Oogai et al., 2011). Additionally, *A. baumannii* cultivated in human serum had an upregulation of virulence-related genes involved in bacterial attachment, twitching motility, DNA uptake, drug efflux transporters and host invasion (Jacobs et al., 2010; Jacobs et al., 2012; Quinn et al., 2018). This concurs with results from my study where serum components contribute to PAO1 BCM cytotoxicity.

Generally, my results concur with the study of Marano et al. (2015) where heat treatment (at 75 °C for 20 mins) could not abrogate the cytotoxic effect of *P. aeruginosa*. *Pseudomonas aeruginosa* activity was attributed to a small molecule (unidentified) that was < 3 kDa (Marano et al., 2015). Molecular weight cut-off filters were also used in my study, but a clean cut-off was not achieved despite showing cytotoxic effects at the flow through of the < 3 kDa cut-off (data not shown). Should the cytotoxicity of the pathogen be attributed to the flow through of the < 3 kDa cut-off, metabolites and perhaps lipids are most likely. This is because the size of *P. aeruginosa* eDNA is around 30 kbp (1110 kDa) (Tetz et al., 2009; Tetz and Tetz., 2010). This does not match with the < 3 kDa profile. On the other hand, some secreted lipids such as BAC3 (heat-stable hemolysin (glycolipid in nature)) have a molecular weight of 650 Da (Johnson and Boese-Marrazzo., 1980). Other studies have also tried to identify factors found in *P. aeruginosa* supernatant. Phenazine-1-carboxylic acid, a secondary metabolite found in *P. aeruginosa* supernatant, induces IL-8 secretion and decreases regulated on activation, normal T Cell expressed and secreted (RANTES) expression in human airway epithelial cells (Denning et al., 2003; Leidal et al., 2001). However, the group

did not characterise if the supernatant was planktonic or biofilm in nature, but since it was grown for 72 h under shaking conditions, microaggregates could have been formed, depending on the shaking speed used. In a recent study, soluble, small metabolites from both planktonic and biofilm CM elicited uncoupling of the citric acid (TCA) cycle, enhanced glycolysis, and selective amino acid uptake and metabolism in macrophages (Ammons et al., 2017). The results in these studies may be related to- or the identities of our unknown cytotoxic factors.

In contrast, heat sensitive secreted proteases (LasB and LasA) in *P. aeruginosa* conditioned media were found to inhibit non cystic fibrosis airway epithelial repair by impairing directed cell migration (Ruffin et al., 2016). Moreover, unpublished data from the thesis of Bowler (2015) proposed that the active factor present in PAO1 BCM causing human lung epithelial cell morphological changes is possibly elastase (*lasB*). She found that the heat labile factor is controlled by quorum sensing and the LasI operon (unpublished data- Bowler., 2015). Of the secreted proteins identified in her study that were unique to BCM, the majority were related to metabolism (unpublished data- Bowler., 2015). This concurs with studies by Morgan et al. (2019), Turner et al. (2014), James et al. (2016) and my revisited hypothesis that metabolites may be involved (**Table 19**). Despite this, the thesis did not show a direct link between the *lasB* gene with cell death or apoptosis (unpublished data- Bowler., 2015). Moreover, heat inactivation was conducted at boiling for only 5 mins and effectiveness may be variable depending on the type of proteins present. The BCM was cultivated using the Calgary biofilm device shaking at 150 rpm in serum-free DMEM, which does not recapitulate *in vivo* settings as much as our model due to the lack of serum. This also suggests that the absence of serum may have altered the biofilm landscape during BCM generation, causing Bowler

(unpublished thesis- 2015) to identify a protein instead of a non-protein responsible for cell morphological changes. They grew biofilms for 4 h, which may not be sufficient for biofilm development as *P. aeruginosa* has been shown to form microcolonies around 8 h and microcolonies resembling biofilms develop around 1-day post-infection in mice and other models (Schaber et al., 2007; Maurice et al., 2018; Chen et al., 2018). Notably, only proteins were identified in her study, making the identification of secreted metabolites, carbohydrates and lipids of paramount importance in future studies.

Table 19: Secreted proteins identified by LC/MS/MS that are unique to PAO1 BCM (Obtained from unpublished thesis- Bowler., 2015)

Functional Class	Accession	Description
Virulence and Protection	gi 15599566	Insulin-cleaving metalloproteinase outer membrane protein [Pseudomonas aeruginosa PAO1].
Virulence and Protection	gi 15597895	hypothetical observed protein PA2699 [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi 15597648	hypothetical observed protein PA2452, partial [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi 15595992	methylecitrate synthase [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi 15597221	glutathione reductase [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi 15598377	keto-hydroxyglutarate-aldolase/keto-deoxy-phosphogluconate aldolase [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi 15597498	protein AmbE [Pseudomonas aeruginosa PAO1].
Flagella and Pili	gi 15596277	flagellar hook protein FlgE [Pseudomonas aeruginosa PAO1].
Flagella and Pili	gi 15596279	flagellar basal body rod protein FlgG [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi 15599104	extracellular DNA degradation protein EddB [Pseudomonas aeruginosa PAO1].
Metabolism and Growth	gi 15595599	aspartate carbamoyltransferase [Pseudomonas aeruginosa PAO1].
Virulence and Protection	gi 15595320	hypothetical observed protein PA0122 [Pseudomonas aeruginosa PAO1].
Virulence and Protection	gi 15595460	secreted protein Hep [Pseudomonas aeruginosa PAO1].
Other	gi 15597647	hypothetical observed protein PA2451 [Pseudomonas aeruginosa PAO1].
Other	gi 15599821	hypothetical observed protein PA4625 [Pseudomonas aeruginosa PAO1].

Host cell death signals may also contribute to cytotoxicity apart from BCM factors. Some well-known signals which are non-proteinaceous are acetyl-coenzyme A (CoA), ATP and non-essential amino acids. Coenzyme A activates Ca^{2+} /calmodulin-dependent protein kinase II (CamKII), which phosphorylates caspase 2, causing cell sensitisation to various stress signals, resulting in cell death (Nutt et al., 2009). Several non-essential amino acids also promote cell death/survival, such as asparagine, serine, glycine, arginine and glutamine (Galluzzi et al., 2013).

In summary, the revisited hypothesis is that the cytotoxic factors may be known or unknown lipids (but not LPS or rhamnolipid), carbohydrates (such as Pel and Psl from the biofilm matrix), eDNA or metabolites (e.g. autoinducers, pseudoverdine, paerucumarin). Apart from known factors, unknown or uncharacterised factors may be identified and characterised for better insights on how secreted factors in PAO1 BCM may negatively affect wound healing responses. Future work could involve better fractionation of the BCM to decipher the molecular weight range of the factors that induced cytotoxicity in keratinocytes. Apart from these biochemical tests, other virulence identification tests could be considered as listed in **table 20**. Collectively, the discovery of novel virulence factors of *P. aeruginosa* is essential for the deeper understanding of its pathogenesis and the progression of treatments.

Table 20: List of virulence tests for secreted, non-proteinaceous virulence factors

Virulence test	Virulence factor identified	Reference
Ethyl acetate extraction	Low molecular weight metabolites	Dossa et al. (2014)
DNase digestion with Neomycin I as DNase inhibitor	DNA	Woegerbauer et al. (2000)
Sheep blood agar	Heat-stable hemolysin (glycolipid): di-rhamnolipid, α -L-rhamnopyranosyl-(1-2) α -L-rhamnopyranosyl-3-hydroxydecanoyl-3-hydroxydecanoic acid, also known as BAC3	Edberg et al. (1996); Ejiofor et al. (2018)
Pyoverdine quantification	Pyoverdine	Das et al. (2016); Pericolini et al. (2018); Adonizio et al. (2008)
Pyocyanin extraction and quantification using chloroform	Pyocyanin	Essar et al. (1990)
Incubation with propidium iodide	eDNA quantification	Pericolini et al. (2018)
EPS extraction and quantification	EPS	Tribedi and Sil (2014); Das et al. (2016)

5.4 Conclusion

Being an opportunistic pathogen, *P. aeruginosa* produces a wide arsenal of virulence factors for its pathogenesis in a variety of clinical settings. In this chapter, factor(s) in

PAO1 BCM capable of inducing cytotoxicity were shown to be non-proteinaceous and sensitive to heat. Based on my investigations to date of various mutant bacterial strains, there was no supportive evidence for them being one of the more common virulence factors (i.e. LasR, LasB and RhlA) nor was it due to LPS. I propose that the cytotoxic factors are likely to be a combination of lipids, carbohydrates, metabolites or nucleic acid products (such as eDNA). Notably, they may be synthesised by the pathogen in the presence of FBS components. This is the first time that other biochemical tests have been used to tease out the nature of the cytotoxic factors found in PAO1 BCM as Bowler (2015) and Marano et al. (2015) have only conducted heat inactivation treatments of the BCM. Further studies are needed to elucidate the identities of these factors and other factors that are responsible for inducing the negative phenotypes and increased migration illustrated in previous chapters. Apart from their identification, understanding how they work together to potentiate pathogenesis in chronic wounds will be important. *Pseudomonas aeruginosa* may use biofilm secreted factors to increase persistence in chronic wounds. These may serve as biomarkers or therapeutic targets for more effective treatments.

6. CONCLUDING DISCUSSION

6.1 General experimental model and selection justifications

In this thesis, the method of growing surface-attached biofilms in a shaking culture by Schleheck et al. (2009) was modified by a media switch to DMEM. DMEM supplemented with 10 % FBS contains components present in wound exudates such as serum and growth factors, giving it more relevance compared to general bacterial media such as TSB or lysogeny broth (LB). Moreover, the presence of serum is relevant for chronic *Pseudomonas aeruginosa* conditions such as cystic fibrosis (with hemoptysis) and chronic wounds due to the presence of blood (Flume et al., 2005). To date, there is no standardised medium that is of ‘wound-exudate’ grade and it is difficult to define which media is most reflective of chronic wound conditions (Cutting et al., 2003). BCM was generated with biofilms that were evenly grown across the entire surface as the distribution of bacteria is non-random in chronic wounds and even growth would make the BCM generation more reproducible (Bjarnsholt., 2013; Fazli et al., 2009).

The BCM that was generated in this thesis comprised of a high-density biofilm ($\approx 10^{10}$ CFU per filter), it had microaggregates present in the culture media and had pathogenic functions on the host (Schleheck et al., 2009). A notable study by Morgan et al. (2019) stated that bacterial fitness in chronic wounds is attributed to the capacity for high-density growth (in the absence of a host) and not biofilm functions or virulence. They have also acknowledged that the study of biofilm growth and chronic infection pathogenesis is difficult and pointed out that stationary phase cultures and colonies represented the general selective environment of wounds. Other studies have also highlighted the importance of bacterial aggregates in biofilm formation and virulence (Kragh et al., 2016; Visaggio et al., 2015; Niederdorfer et al., 2016). It has also been

stated by Robson (1997) and Bowler (2003) that infected chronic wounds contain more than 10^5 CFU bacteria per gram of tissue. In my case, a high inoculum helped to restrict my studies to effects of high bacterial bioburden, and this reduced complications from both low (start of infection) and high inocula effects. With this evidence, it was justified that the BCM used in this thesis was relevant for experimental studies and appropriate as a preliminary screen for therapeutic targets.

Moreover, BCM was not grown in the presence of any host components, which could change the properties of BCM and make it difficult to elucidate effects. Additionally, studying biofilms in the presence of host cells leads to massive cell death and detachment, which impedes longer term biofilm studies (Anderson et al., 2008; Moreau-Marquis et al., 2008a; Moreau-Marquis et al., 2008b). My BCM was generated in a closed system (no fluid flow, not much air-liquid interface nor shear stress) at 37 °C, with shaking conditions. Nutrient depletion was minimised through media changes and 37 °C resembles the temperature of chronic leg ulcers range between 25-37 °C (Fierheller and Sibbald., 2010; Romanelli et al., 2002). The justification for using a shaking to a static culture is because the shaking culture represents the dynamism of the fluid flow in the host. Albeit without constant media replenishment, this mimics the reduced blood flow and nutrient exchange to the wound bed. The presence of a filter wool provides mechanical support for biofilm attachment, together with its fibrous and porous nature that provides complexity, similar to the ECM matrices and other cellular components found in wound beds for biofilms to attach. Having more layers of PE fibres probably led to a lack of nutrient availability, so biofilm growth was not optimal. It remains questionable whether the filter wool would evoke similar responses in gene expression and resistance compared to biofilms on biotic surfaces. With all these

justifications, our model is as shown in the earlier segment **2.1.2 Generation of PAO1 Biofilm Conditioned Media (BCM).**

PAO1 is a wound isolate from a patient in Australia and is currently a well-defined laboratory reference strain (Holloway., 1955; Stover et al., 2000). PAO1 was chosen as the strain for this study as there are mutants widely available for identifying the virulence factors of interest through genomics (Held et al., 2012; Jacobs et al., 2003; Lewenza et al., 2005). PAO1 has been reported to have a similar genome to other *Pseudomonas* strains such as PAK and CF181 (Ernst et al., 2003; Wolfgang et al., 2003). It was also suggested that its virulence is not attenuated through extensive passaging, but rather it is originally of moderate virulence (Lee et al., 2006). Despite this, studies have shown that it has undergone evolution and differs in genomic content and gene expression when compared to other strains (Morgan et al., 2019; Bragonzi et al., 2009; Klockgether et al., 2011; Frydenlund et al., 2015). Nevertheless, PAO1 is known to be less virulent than other clinical strains such as PA14 in a variety of hosts including *C. elegans* and mice (Wiehlmann et al., 2007; Choi et al., 2002; Rahme et al., 1995; Tan et al., 1999; Mahajan-Miklos., 2000). PA14 consists of genes residing on large pathogenicity islands such as PAPI-1 and 2 that are not present in PAO1 (He et al., 2004; Harrison et al., 2010). The *pilA* (type 4 pilus protein) and *pilC* (type 4 fimbrial assembly protein) genes also differ in sequences from those found in PAO1 (Choi et al., 2002). Moreover, PA14 contains a mutation in the *ladS* gene, resulting in increased cytotoxicity and type 3 secretion system (T3SS) activity compared to PAO1 (Mikkelsen et al., 2011). A recent study by Crespo et al. (2017) has shown that PAO1 contains mutated class III ribonucleotide reductase (RNR) promoter region that prevents it from undergoing anaerobic growth, thereby reducing virulence. Despite these differences, this

dissertation showed that the effects of PAO1 BCM are not strain specific as the clinical strain PA 15159 (isolated from a diabetic foot ulcer) had similar toxic effects on human keratinocytes. This is further supported by Lee et al. (2006), who have found that virulence-related genes in one strain is not required or is predictive of virulence in other strains. Using microarrays, the group identified genes in PA14 that are absent in PAO1 that did not contribute to PA14's virulence in *C. elegans* (Lee et al., 2006). In the future, other clinical isolates could be tested to compare with PAO1 to determine similarities between the BCMs generated.

Given the polymicrobial settings of chronic infections, the presence of different bacteria in the same microenvironment could cause the types of virulence factors produced to be dynamic (Fugère et al., 2014). In this thesis, a monoculture of *P. aeruginosa* was used as monomicrobial wounds are also present (Basu et al., 2009; Shettigar et al., 2016). A study also showed that 57 % of chronic wound samples received were monomicrobial, reiterating the clinical relevance (Rhoads et al., 2012). This also helps to study the monoculture effects before moving on to polymicrobial ones for comparison. However, does the dynamism and interaction between pathogens also contribute to virulence? In the case of chronic wound pathogens *S. aureus* and *P. aeruginosa*, Phalak et al. (2016) demonstrated that spatial partitioning of the 2 bacteria occurred in the biofilm due to the differences in their needs for oxygen and this also resulted in antagonistic metabolic effects of *P. aeruginosa* on *S. aureus*. *Pseudomonas aeruginosa* outcompetes *S. aureus* in a polymicrobial culture (Mashburn et al., 2005; Filkins et al., 2015; Nguyen and Oglesby-Sherrouse., 2016). Despite this, a recent study by Woods et al. (2019) managed to maintain *S. aureus* biofilms in co-culture with *P. aeruginosa*, so future work in polymicrobial BCM may be worth considering. Cross-feeding may occur where

different bacteria can provide nutrients to one another for enhanced virulence (Ramsey et al., 2011). These show that polymicrobial growth of pathogens in the same environment could cause competition for nutrient resources or symbiotic relationships and hence may alter the virulence profile from monomicrobial growth. In summary, the monospecies culture method was a compromise from several published methods. To date, no studies have generated polymicrobial biofilm conditioned media and this could be considered as future work for comparisons with monomicrobial cultures.

The *in vitro* monolayer cell culture model was chosen to determine the effects of PAO1 BCM on a small scale on keratinocytes and fibroblasts. Arguably, primary cells would be a better choice as immortalised cells have modifications in their genome for continual passaging. However, primary cells are costly and will not be able to be sustained in culture as long as that of immortalised cells, so the effects elicited may not be as reliably reproduced. Moreover, *in vivo* work would encompass the other factors that the *in vitro* setup is lacking due to the presence of the immune system, other cell types in the skin allowing for cell-cell signalling, and longer experimental timings. Nevertheless, *in vitro* monolayer cultures are used as they are cheap, easy, fast in obtaining results and gives a good starting platform for observing effects before proceeding to *in vivo* experiments (van den Broek et al., 2014).

6.2 Aiding wound healing by *Pseudomonas aeruginosa*

In this thesis, the potential of secreted factors of *P. aeruginosa* biofilms in improving wound healing was displayed through slightly increased migration rates *in vitro* (with the low doses of PAO1 BCM) and similar wound closure rates *in vivo*. This is opposite of the proposed, initial hypothesis that PAO1 BCM hampers migration and wound

closure. Despite most studies on microbiome improving wound healing focusing on skin microflora and probiotics, pathogenic bacteria in low levels have been demonstrated to promote wound healing (Johnson et al., 2018; Linehan et al., 2018). As early as 1983, Levenson et al. reported an acceleration of wound healing when *S. aureus* was inoculated in rats. Laato et al.(1998) then proved that non-infective levels of *S.aureus* improve wound healing by increasing rate of re-epithelialisation, granulation tissue formation, enhancing blood flow and collagen deposition. These reiterate that even though these pathogenic bacteria are prevalent in chronic wounds, at controlled amounts, they are capable of helping wounds to heal. Apart from the physical bacteria, secreted factors have shown potential in improving cell migration such as *Streptococcus salivarius* that promotes gingival re-epithelialisation through a secreted serine protease (Fernandez-Gutierrez et al., 2017). More recently, a series of studies by Kanno et al. (2011, 2013, 2015) analysed the effects of PAO1 in promoting wound healing. The authors first reported that biofilm formation on full-thickness wounds of Sprague Dawley rats did not delay epithelialisation in acute wound healing (Kanno et al., 2011). The group then showed that 3-oxo-C₁₂-HSL, a quorum sensing autoinducer, accelerated wound healing at day 3 post-wounding through inflammation and TNF- α production (Kanno et al., 2013). Epidermal cell proliferation and neo-vascularisation were promoted (Kanno et al., 2013). Kanno et al. (2015) reported that *P. aeruginosa* C₄-HSL, another quorum sensing autoinducer, promotes wound healing in rats as early as day 3 post-wounding through neutrophil infiltration. This concurs with reports showing that elevated local inflammation promotes wound healing (Niebuhr et al., 2008). Karlsson et al. (2012) also demonstrated that *P. aeruginosa* 3-oxo-C₁₂ -HSL at lower concentrations promote migration, while higher concentrations suppress migration of human epithelial cells. This is reflective of this dissertation and reiterates that *P.*

aeruginosa is capable of promoting wound healing in appropriate amounts. Collectively, the results by Kanno et al. (2011, 2013, 2015) show that although virulence mechanisms such as quorum sensing are capable of inducing host destruction, they are also capable of promoting wound closure. As *P. aeruginosa* is also a skin commensal, perhaps it does have mechanisms that aid in host healing, but research has only focused on the negativities. Unknowingly, the microenvironment of chronic wounds could have caused *P. aeruginosa* to switch serotypes and become virulent and activate its host-destruction mechanisms.

6.3 Parallels with *Staphylococcus aureus* biofilm exudates

Similar to *P. aeruginosa*, *S. aureus*, the other pathogenic biofilm-forming bacteria prevalent in chronic wounds, is also negatively implicated in various cellular processes (Gjødtsbol et al., 2006). Both bacteria seem to be negatively affecting the host wound healing responses in similar ways. For example, *S. aureus* BCM was shown to inhibit proliferation and migration in human tympanic membrane derived keratinocytes and human epidermal keratinocytes (Marano et al., 2015). *S. aureus* BCM was also found to induce apoptosis and reduce wound closure by inhibiting fibroblast and keratinocyte migration (Secor et al., 2011; Kirker et al., 2012; Tankersley et al., 2014). This is similar to this dissertation where reduced migration in fibroblasts and keratinocytes and induction of apoptosis in keratinocytes were observed. An exception is in terms of the inflammatory responses, in which *S. aureus* was shown to elicit inflammation (Ward et al., 2015; Tankersley et al., 2014; Kirker et al., 2012; Secor et al., 2011). Kirker et al.(2012) also reported high levels of TNF- α expression in *S. aureus* BCM. Comparatively, our results showed that *P. aeruginosa* BCM downregulates inflammation, which could be a different way that *P. aeruginosa* immunomodulates

compared to *S. aureus* (Binsker et al., 2018). Apart from fibroblasts and keratinocytes, *S. aureus* BCM was shown to promote apoptosis and inhibit differentiation in osteoblasts and MSCs (Sanchez et al., 2013a; Ward et al., 2015). Although in a different cell type, this is similar to this dissertation where PAO1 BCM inhibits keratinocyte differentiation, showing the robustness of the effects of PAO1 BCM on various cellular responses. Due to the similar nature of their pathogenic responses on the host, this may be the reason for them to be highly prevalent in chronic wounds (Gjødtsbol et al., 2006).

6.4 p65 degradation as a host response

In this thesis, the downregulation of p65 was viewed as a negative pathogenic response. However, it can also be a host-induced response as proteasomal degradation of p65 by the host has been shown to be necessary for termination of inflammatory responses when needed, if not the NF- κ B pathway continues to be activated (Saccani et al., 2004). This is to prevent excessive inflammation leading to chronic inflammatory diseases through homeostatic regulation of the NF- κ B pathway. Apart from proteasomal degradation, p65 autophagic degradation is also useful for controlling the innate immunity. Leucine rich repeat containing 25 (LRRC25), an important innate immune system protein that is essential for mediating protein-protein interactions (e.g. detection of PAMPs via pattern recognition receptor (PRRs)), functions as an inhibitor of the NF- κ B signalling pathway through the promotion of autophagic degradation of the p65/p50 heterodimer complex (Ng et al., 2011; Feng et al., 2017). As it was also hypothesised from this dissertation that the p65/p50 heterodimer complex could be degraded due to their simultaneous downregulation in protein levels, these studies suggest the possibility that the downregulation of p65 could be a host response as well. Chang et al. (2013) has also shown that TLR-2 signal can induce sustained phosphorylation of ERK1/2, thus

culminating in autophagy-dependent degradation of p65. These studies reiterate that p65 degradation could be an automated regulation by the host to prevent further propagation of infections. It was also hypothesised from this dissertation that protein translation of p65 may have been inhibited. Should the translation inhibition be proven to be true, this may be due to a selective synthesis of key proteins, or to promote stress resistance and avoidance of pathogens, as a possible surveillance mechanism that the host has for pathogen recognition (Li et al., 2011; Melo and Ruvkun., 2012).

Since *P. aeruginosa* is capable of both up- and down-regulating inflammation, the question of whether inflammation is advantageous or disadvantageous to the host wound healing responses arises. Although chronic inflammation dysregulates the wound healing response, inflammation is important and necessary for proper wound resolution (Cowin et al., 1998; Martin and Leibovich., 2005). As inflammation is the first stage of the wound healing cycle, the process is crucial for clearing cellular debris and infectious pathogens (Tian et al., 2016b). Reports have also shown that basal levels of inflammation are needed for proper wound closure (Rieder et al., 2007). However, if inflammation is downregulated at the start of the wound healing process, the first stage of the wound healing cycle is not activated, which may then hamper other processes such as re-epithelialisation and tissue remodelling as necrotic tissue in the wound bed and pathogenic bacteria are not cleared away by the inflammatory cells (Swift et al., 2001; Tian et al., 2016b). This will result in hampered wound closure, which is characteristic of non-healing wounds (Tomic-Canic et al., 2004). However, there are studies showing the other side of the coin, where inflammation promotes faster healing (Qiu et al., 2003; Mori et al., 2006; Kanno et al., 2011). Collectively, these studies reiterate that inflammation is a double-edge sword in the wound healing response.

6.5 Crosstalk between the various wound healing responses

Studies have shown that NF- κ B is involved in migration, inflammation, cell survival, keratinocyte differentiation and proliferation (Hayden and Ghosh., 2004; Ghosh and Karin., 2002; Karin and Lin., 2002). The association between NF- κ B and migration is usually related to cancer metastasis, where NF- κ B promotes tumour cells to undergo epithelial-mesenchymal transition (EMT) and metastasis (Jana et al., 2017; Pires et al., 2017; Helbig et al., 2003). A more direct relation to non-tumorigenic cell migration would be the association of NF- κ B with Rac1 (highly involved in cell migration), as inhibition of Rac1 dampens NF- κ B activity (Gastonguay et al., 2012). Although the study was conducted in lung carcinoma, Rac1 activation by RhoG is crucial for regulating cell migration, as Rac-deficient cells had a strong reduction in wound closure and cell migration (Steffen et al., 2013; Katoh et al., 2006). This makes Rac1 and its association to NF- κ B relevant to a non-tumorigenic setting. Although the link between NF- κ B and migration is not as established as the other wound healing responses, these studies suggest that there are interactions and NF- κ B is able to affect cell migration.

Cell viability is also linked to NF- κ B as promotion of NF- κ B signalling through I κ K- β leads to increased cell survival (Luo et al., 2005). NF- κ B signalling inhibits cell death (both apoptosis and necrosis) by induction of pro-survival proteins such as FLICE (FADD-like IL-1 β -converting enzyme)-inhibitory protein (FLIP) and cellular inhibitor of apoptosis (c-IAP) (Kucharczak et al., 2003; Karin and Lin., 2002). However, under circumstances, NF- κ B activation may promote cell death, through activating some proapoptotic genes such as Fas ligand (FasL) and Fas/CD95 (Kimura et al., 2003; Wiener et al., 2004). Collectively, these studies demonstrate that cell viability is also linked to NF- κ B signalling.

Studies have shown conflicting results in the correlation between the NF- κ B and keratinocyte differentiation pathway. Mice lacking p65 and c-Rel have been shown to have reduced differentiation rate and expression of K10 and loricrin (early and late differentiation markers), while other studies have shown that the absence of p50 and p65, and I κ K- β and γ deficient embryos had no significant effects on epidermal development and differentiation (Gugasyan et al., 2004; Zhang et al., 2004b; Grinberg-Bleyer et al., 2015; Seitz et al., 1998; Makris et al., 2000; Schmidt-Supprian et al., 2000). Despite the conflicting studies on NF- κ B's effects on keratinocyte differentiation, we can appreciate that there are interactions between these 2 pathways. Additionally, the common molecule that is present in both the NF- κ B and keratinocyte differentiation pathway is I κ K- α . Nuclear localisation of I κ K- α induces the exit of the cell-cycle into terminal differentiation by regulating Smad2/3 (Liu et al., 2012). A lack of I κ K- α causes the lack of the stratum granulosa and corneum, and the formation of a thickened stratum spinosum, which impairs keratinocyte proliferation and differentiation, but studies have also indicated that this phenomenon is NF- κ B-independent (Hu et al., 1999; Li et al., 1999; Takeda et al., 1999; Hu et al., 2001). It has been proven that I κ K- α controls epidermal differentiation through the production of keratinocyte differentiation-inducing factor (kDIF) (Tomic-Canic et al., 1998). These studies also inform us about the connections that the common molecule, I κ K- α , has on both NF- κ B and keratinocyte differentiation.

In terms of cell adhesion, the NF- κ B pathway has been shown to induce endothelial adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (Collins et al., 1995; Perfetto et al., 2003).

However, it has not been shown if the NF- κ B pathway has any effects on non-endothelial cell adhesion, and this could be a subject for future investigations. Additionally, the most prominent link of *P. aeruginosa* with the NF- κ B pathway in wound healing is inflammation. Taken together, this highlights the possibility that NF- κ B pathway may be the main pathway involved in affecting these main cellular processes. This also suggest possibilities of crosstalk between these wound healing responses and the NF- κ B pathway.

6.6 Possible identities of the other factors that have not undergone biochemical testings

Although the results of this dissertation did not cover biochemical tests of PAO1 BCM to decipher the identities of the factors affecting the other wound healing responses apart from cell viability, there are some possible identities that could be investigated on. For example, the autoinducer 3-Oxo-C₁₂-HSL is one of the major possibilities because it has been shown to accelerate wound closure and promote fibroblast differentiation (Nakagami et al., 2011; Karlsson et al., 2012; Kanno et al., 2013). Karlsson et al. (2012) demonstrated that *P. aeruginosa* 3-oxo-C₁₂-HS at lower concentrations promote migration, while higher concentrations suppress migration of human epithelial cells in a scratch wound assay. This is reflective of what was observed by PAO1 BCM in this dissertation, where dose-dependent effects could hamper or promote migration. Kanno et al. (2013) also showed that 3-Oxo-C₁₂-HSL promotes acute wound healing through increased neutrophil infiltration and TNF- α secretion in rats. Although increased neutrophil infiltration was not observed *in vivo*, it was observed that PAO1 BCM slightly increased TNF- α and wound closure was promoted. This reiterates the similarities between 3-Oxo-C₁₂-HSL and PAO1 BCM in their effects on wound closure. Another study showed that the autoinducer 3-Oxo-C₁₂-HSL accelerates wound closure

by promoting fibroblast differentiation to myofibroblasts and inducing COX2 (Nakagami et al., 2011). Although fibroblast differentiation cannot be directly compared to keratinocyte differentiation, the autoinducer seems to be capable of changing the cell from one state to another. This suggests that there may be a chance that it has an effect on keratinocyte differentiation, but is currently unknown.

A possible factor that attributed to NF- κ B downregulation would be 2-aminoacetophenone (2-AA), a quorum sensing regulated volatile molecule (Bandyopadhyaya et al., 2012). However, the resemblance of this factor to the effects of PAO1 BCM was not to the extent that was shown in this dissertation, as 2-AA stimulation activates inflammation by activating key immune pathways such as NF- κ B and MAPK (Kesarwani et al., 2011). Pro-inflammatory cytokines are also secreted (Kesarwani et al., 2011). It is only pre-treatment with 2-AA that NF- κ B and JNK activation were downregulated, together with ERK activation (Bandyopadhyaya et al., 2012). This resembles the results of this dissertation, where JNK1, p65 and p50 were noted to be downregulated. The downregulation of the various kinases led to the formation of the c/EBP β -p65 complex and activation of CCAAT/enhancer-binding protein- β (c/EBP β) that prevents activation of the NF- κ B pathway (Bandyopadhyaya et al., 2012). 2-AA was also reported to induce oxidative stress and apoptosis in murine skeletal muscle, which may favour chronic infections (Bandyopadhyaya et al., 2016). Although not in keratinocytes, apoptosis is a common feature between the study by Bandyopadhyaya et al. (2016) and this dissertation, which suggests the possibility of the robustness of 2-AA should it be the one eliciting apoptosis in PAO1 BCM.

7. FUTURE PERSPECTIVES

7.1 Creation of improved *in vitro* and *in vivo* models

Despite the importance of translating results obtained in the laboratory to the bedside, future perspectives should include advancements in experimental methods. In this thesis, an *in vitro* model was improved for producing biofilm secreted factors and an *in vivo* model for checking the effects of these factors in a simple and reproducible way. These pave the way for improved versions of the models to better mimic the complexity found in *in vivo* biofilms and secretome. Although technically challenging, future models of the *in vitro* system could look towards incorporating host elements and commensals into the system. For example, Sun et al. (2008) developed the *in vitro* multi-species LCWB model to recapitulate conditions in chronic wounds using Bolton broth and other host components. Methods to collect the secretome of biofilms established in the LCWB model could then be established in future models. Polymicrobial cultures of other pathogens and even commensals could also be added to the model for greater complexity and relevance to chronic wounds. Methods to investigate the host-pathogen secretome *in vivo* could also be developed so that studies can reflect clinical manifestations more accurately. Clinical samples such as wound fluid, in the case of chronic wounds, could be used as benchmarks for profiling and comparing it with *in vitro* and *in vivo* secretomes. Correlations between commensals, pathogenic bacteria, host and clinical prognosis could also be drawn from having such models.

In this thesis, the effects of PAO1 BCM on a local wound microenvironment was investigated. Future studies could also look towards how this local wound microenvironment differs from other local or global environments in the host since chronic infections are systemic. It is hypothesised that local and systemic conditions are

probably similar given that chronic conditions such as diabetic foot ulcers are caused by underlying problems such as neuropathy, poor vascular flow and hyperglycaemia, which are systemic issues (Kranke et al., 2012; Tomic-Canic et al., 2004). This would reveal potential similarities or differences that are important in controlling the chronic infection, which may pave way for more effective therapies.

Other chronic wound models such as diabetic model, pressure ulcer model and venous ulcer model can be considered as future work. Research could be focused on other pathogenic complications of *Pseudomonas aeruginosa* such as sepsis and cystic fibrosis as well. This will allow us to compare the similarities and differences of the effects of PAO1 BCM on different wound models. This information would highlight clinicians to make more informed decisions during medicine prescription for treating pathogenic conditions.

7.2 A newfound role of bacteria in wound healing?

Literature has shown that biofilms in chronic wounds are eradicated through debridement, antibiotics and negative pressure therapy (Han and Ceilley., 2017; Jones et al., 2018). However, *P. aeruginosa* biofilms were regenerated and returned to pre-debridement levels by some bacterial remnants in a porcine burn model within 48 h (Roy et al., 2014). Only a temporary decline in bacterial bioburden was observed (Roy et al., 2014). Additionally, new microcolonies were found in deeper tissue layers post-debridement, raising concerns that sharp debridement may lead to inoculation into deeper tissues and culminate in persistent infections (Roy et al., 2014). Kalan et al. (2019) have also found that debridement only reduces the anaerobes (*P. aeruginosa* is an aerobe) in actively healing wounds but not for non-healing wounds such as diabetic foot ulcers,

suggesting the persistence of aerobic bacterial growth in chronic wounds. Antibiotic treatment helps to suppress infections, but due to evolution and a multitude of multi-drug efflux pumps in *P. aeruginosa*, antibiotic resistance is on the rise (Aeschlimann., 2003; Li et al., 2015). On the other hand, negative pressure therapy helps by reducing biofilm thickness and suppressing *P. aeruginosa* motility, but does not affect bacterial cell viability and does not really contribute in biofilm removal (Wang et al., 2018; Guoqi et al., 2018; Tahir et al., 2018). Despite these various treatments, biofilms still complicate the chronic wound (Schaber et al., 2007; James et al., 2008).

Collectively, results from this dissertation reveal the possibility that bacteria may be able to aid wound healing since low doses of PAO1 BCM slightly increases migration rates *in vitro*, while wound closure is similar to controls *in vivo* (Chapter 3). Results from this dissertation suggests that the current understanding of the involvement of these pathogens in complicating chronic wounds is still limited and we may be looking at these pathogens in the wrong way. Are they really foes or our friends? Perhaps it is time to look at these pathogens in a different light. Since *P. aeruginosa* is a commensal under normal circumstances, there may be a chance that the microbiome is here to help us survive and we have evolved and co-existed with them for a reason as seen in studies by Linehan et al. (2018), Chen et al. (2018) and Johnson et al. (2018). Other wound healing responses (e.g. fibroblast activation, keratinocyte activation and ECM deposition) could be checked both *in vitro* and *in vivo* via transcriptomics to determine effects of PAO1 BCM on these wound healing responses. Future directions could consider embracing the presence of *P. aeruginosa* biofilms instead of thinking of ways to eradicate it. Hampering signalling pathways such as the inflammatory pathway to dampen immune responses could be the alternative to treating chronic wounds.

Additionally, since PAO1 BCM is able to dampen immune responses, it could be considered for use as a therapeutic intervention in which its controlled application on chronically infected wounds could suppress inflammation to basal levels so as to reduce collateral damages on the host and promote improved wound closure. Lastly, apart from thinking of the dampened immune system as a pathogenic response, it could also be a host response elicited to reduce collateral damage. Knowledge of the pathways being affected by PAO1 BCM will help in the development of more effective and targeted treatments for *P. aeruginosa*-related wound infections.

7.3 Translating PAO1 BCM components as possible chronic wound biomarkers

The presence of *P. aeruginosa* is widely known to be highly associated with chronic wounds (Gjødtsbol et al., 2006). Studies have shown that this opportunistic pathogen can delay wound healing in both acute and diabetic wounds (Trøstrup et al., 2018; Zhao et al., 2010). Its wealth of virulence-inducing secretion systems and LPS contribute to its pathogenicity. However, can *P. aeruginosa* be indicative of whether the wound heals? In this thesis, the secreted factors of *P. aeruginosa* were demonstrated to have a role in complicating the chronic wound by having a multitude of negative effects on wound healing. Other groups have also reported the negative effects of *P. aeruginosa* secreted factors on migration and cell morphology in various cell types (Marano et al., 2015). Despite compelling evidence that would make this pathogen a wise choice for an indicator of wound healing outcomes, a study by Kalan et al.(2019) has chosen *S. aureus* as an indicator instead due to its association with poor healing outcomes as debridement of diabetic foot ulcers led to the depletion of anaerobic bacteria which correlated to improved wound healing (Cardinal et al., 2009). Other studies have highlighted that anaerobes are prevalent in non-healing wounds (Sloan et al., 2019; Citron et al., 2007).

However, this does not explain the association of aerobes such as *P. aeruginosa* and other facultative anaerobes with chronic wounds (Gjødtsbol et al., 2006; Davies et al., 2004; Kirketerp-Møller et al., 2008). This suggests that the prevalence of *P. aeruginosa* in chronic wounds could be correlated with chronic wound etiologies, and components present in PAO1 BCM could be potential biomarkers for *P. aeruginosa* infections.

Biomarkers can be classified into various types, namely, diagnostic, predictive, prognostic and therapeutic (Durães et al., 2014; Lin et al., 2012). Even though a plethora of chronic wound biomarkers are present to date, not all of them may be suitable biomarkers in all chronic wound types as polymicrobial infections could cause biomarker profiles to differ (Lindley et al., 2016). This highlights the need for more biomarkers for various diseased states. Biomarkers can be used as array-based methods for diagnosis of various clinical conditions that occur due to pathogen infection and for monitoring of diseases, which are more specific and efficient compared to conventional diagnostic tools (Aytur et al., 2006; Kastenbauer et al., 2005; Chaerkady and Pandey., 2008). A volatile organic compound associated with the “grape-like” smell of *P. aeruginosa*, 2-AA, has been hypothesised as a potential breath biomarker for pathogenic growth in cystic fibrosis patients (Scott-Thomas et al., 2010). Pyocyanin is also another biomarker of *P. aeruginosa* infections and has been used for detection in various studies (Elkhawaga et al., 2019; Žukovskaja et al., 2017). These potential biomarkers could also be indicators of wound healing outcomes in *P. aeruginosa* wound infections in the future as well.

In comparison with the work from an unpublished thesis of Bowler (2015), the absence of FBS in her study made her factor of interest a protein, which is different from the

factor of interest in this dissertation, a non-protein. This is despite both studies finding factors that negatively affected host cell viability. This raises the question that perhaps focusing on proteins as target factors was not in the right direction to curing *P. aeruginosa* related infections although secretory systems are well-established and aplenty. Together with evidence provided by other studies, perhaps metabolites or lipids or factors that are non-protein in general were more important for targeting *P. aeruginosa* than secreted proteins (Turner et al., 2014; Morgan et al., 2019). To determine the exact identities of the factors present in PAO1 BCM, proteomics such as mass spectrometry and high-performance liquid chromatography (HPLC) could be performed concurrently with genetic identification techniques such as transposon mutant library screening and microarray. NMR-based metabolite profiling could be used to check the identities of metabolites and HPLC/MS analyses of chloroform extracted BCM to determine identities of hydrophobic small molecules. Additionally, based on results of this thesis, eDNA (isolated from *P. aeruginosa* biofilms) or any of the known quorum sensing autoinducers or metabolites reduced cell viability in keratinocytes and fibroblasts. Identification of these factors could potentially serve as biomarkers of *P. aeruginosa* chronic infections. This could translate into biomarkers or targets for more effective therapies.

8. CONCLUSIONS

This dissertation illustrated the various complications that biofilm secreted factors of *P. aeruginosa* elicit on wound healing through the use of PAO1 BCM. Based on the results of this thesis, the proposed pathogenesis of *P. aeruginosa* biofilm exudates in complicating wound healing is as shown in **figure 71**.

The overarching aim of this thesis was to understand how biofilm secreted factors of *P. aeruginosa* impact wound healing through the use of BCM. This was achieved in Chapter 3, where an improved method of BCM generation was created, together with an *in vivo* model of BCM application on full-thickness excisional wounds. It was then hypothesised that PAO1 BCM would negatively affect cell migration, adhesion and viability. The hypothesis was proven right, with the exception that low doses of PAO1 BCM had the potential to slightly increase migratory rates *in vitro*. Additionally, similar wound closure rates were observed in PAO1 BCM-challenged wounds compared to controls *in vivo*. A revisited hypothesis in the aspect of migration would be that high doses of PAO1 BCM potentially delays re-epithelialisation but increases wound contraction.

In the aspect of cell viability, the revisited hypothesis would be that the apoptotic keratinocytes observed in this study pave way for further infections by *P. aeruginosa* and provides attachment of the bacteria to dead cells, as Capasso et al.(2016) showed that the pathogen tends to bind to apoptotic and necrotic cells. This could aid in transmigration through the epidermis since *P. aeruginosa* has been shown to bind to epithelial surfaces to form bacterial aggregates that can locally alter the apical surface

of cells to recruit adherens junction proteins instead of disrupting junctions (Lepanto et al., 2011; Kierbel et al., 2007; Tran et al., 2014).

Another exception was that cell adhesion in keratinocytes was maintained *in vitro* and *in vivo* compared to fibroblasts after 50 % PAO1 BCM challenge. Only occludin levels were decreased in keratinocytes after 50 % PAO1 BCM challenge. Most of the cell communication and adhesion junctions Cx43, occludin, β -catenin and N-cadherin levels were reduced in fibroblasts after 50 % PAO1 BCM challenge. Collectively, these results suggest that open wounds may be sealed through proper cell adhesion and junctions of keratinocytes, but complications arise from allowing pathogenic infection to ensue beneath the skin since the wounds are visually observed to be “healed”. This is supported by a study from Roy et al. (2014), where the authors reported that *P. aeruginosa* infected wounds were healed macroscopically, but biofilms regenerated to pre-debridement levels within 48 h (Roy et al., 2014). Taken together, the propagation of infections beneath keratinocytes with intact cell junctions could explain for recurrence in chronic wounds in about 60 to 70 % of the patients, reiterating the hypothesis that complications beneath the wound could still occur despite macroscopic observations indicative of wound closure (Richmond et al., 2013). This could also be the reason for debridement not being able to be a standalone method for chronic wound treatment as the wounds are healed macroscopically but not internally (Gordon et al., 2012).

In Chapter 4, it was hypothesised that high doses of BCM will promote inflammation and keratinocyte differentiation. However, the reverse was observed, suggesting possible immunomodulatory effects on the host. 50 % PAO1 BCM downregulated upstream kinases of the NF- κ B pathway (JNK1 and c-Jun) and dampened inflammation

by downregulating p65 and p50 protein levels. The revisited hypothesis is that the entire heterodimer (without I κ B- α) could have been degraded and this possibly helps protect *P. aeruginosa* by immune evasion. This may also be one of the reasons why wounds after debridement are still re-colonised by pathogenic bacteria within h and maintain high numbers of CFU counts (Kim et al., 2018; Hiebert and Robson., 2016). Impaired early and late keratinocyte differentiation were also observed, probably resulting in TEWL and potential regeneration of biofilm infections as shown in Roy et al. (2014), where leaky skin resulted in TEWL (Roy et al., 2014). This shows that a lack of proper epithelial barrier may impede proper healing when incubated with the secreted biofilm exudates.

Finally in Chapter 5, the secreted factors involved were hypothesised to be proteinaceous, or metabolites given the wide spectrum of virulence factors that *P. aeruginosa* is reported to produce (Gellatly and Hancock., 2013; Bleves et al., 2010). Attempts at identification of the secreted factors were conducted but future work is required to do that. PAO1 BCM components could be identified as biomarkers and indicators of wound healing outcomes. In conclusion, this study further enlightens us on the limitations that debridement has in *P. aeruginosa*-infected wounds and filling these research gaps will allow for a more effective treatment.

Figure 71: Summary of PAO1 BCM pathogenesis

9. REFERENCES

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