Effect of Estrogen on Host-Pathogen Interactions in *ex vivo* and *in vitro* Models of the Inflammatory Phase of Age-Related Impaired Healing

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List of Abbreviations

Aβ	Amyloid β-peptide
Arg-1	Arginase
ATP	Adenosine tri phosphate
BCA	Bicinchoninic acid
BCG	*Mycobacterium bovis bacillus Calmette-Guerin*
BHI	Brain heart infusion
BSA	Bovine serum albumin
CAMs	Cellular adhesion molecules
CF	Cystic fibrosis
CFU	Colony-forming units
CMP	Common myeloid progenitor
CMR	Common myeloid progenitor
CMoP	Committed monocyte progenitor
CLRs	C-type lectin receptors
C5b-9	Membrane attack complex
CR	Complement receptors
CRID	Rac-interacting and binding domain
c-SRC	p60 Src tyrosine kinase
DHT	Dihydrotestosterone
DHEA	Dehydroepiandrosterone
DHEA-S	Dehydroepiandrosterone sulphate
DN	Dominant negative
DPBS	Dulbecco's phosphate-buffered saline
DPN	Diarylpropionitrile
ECM	Extra cellular matrix
E-coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
EGF	Epidermal growth factor
ER	Membrane-bound estrogen receptor
ER-α	Estrogen receptor alpha
ER-β	Estrogen receptor beta
ERE	Estrogen response element
ESC	Endometrial stromal cells
FAK	Focal adhesive kinase
FBS	Foetal bovine serum
FGF-2	Fibroblast growth factor 2
FITC	Fluorescein isothiocyanate
FL1-A	Fluorescence parameter 1
FL2-A	Fluorescence parameter 2
FSC	forward scattered light

FTIR	Fourier Transform Infra-Red
GAP-s	GTPase activating proteins
GCS-F	Granulocyte colony-stimulating factor
GDIs	Guanine nucleotide dissociation inhibitors
GEFs	Guanine nucleotide exchange factors
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GDP	Guanosine diphosphate
HBCCM	Human blood cell culture medium
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HPBM	Human peripheral blood monocytes
HSCs	Hematopoietic stem cells
HRT	Hormone replacement therapy
ICs	Immune complexes
IgG	Immunoglobulin G
IGF-1	Insulin-like growth factor-1
INF-γ	Interferon gamma
IL-1β	Interleukin-1 beta
IL-4	Interleukin-4
IL-13	Interleukin-13
IL-8	Interleukin-8
IL-17	Interleukin-17
IL-6	Interleukin-6
iNOS	Inducible NO synthase
KGF	Keratinocyte growth factor
K. pneumonia	*Klebsiella pneumoniae*
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
LR	Langerhans cells
M	Molar
MBL	Mannose binding lectin
MCS-F	Macrophage colony-stimulating factor
MCP-1	Macrophage chemoattractant protein 1
MDR	Macrophage and dendritic cells precursor
MIF	Migration inhibitory factor
MIP	Macrophage inflammatory protein
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MMPs	Matrix metalloproteinases
mL	Millilitre
MOI	Multiplicity of Infection
MRSA	Methicillin-resistant *Staphylococcus aureus*
MRS11	Methicillin-resistant *Staphylococcus aureus* strain 11
NA	Nutrient agar

NaCl	Sodium chloride
NC	Negative control
NET	Neutrophil extracellular trap
NF-Kβ	Nuclear transcription factor kappa beta
ng	Nanograms
NGF	Nerve-growth factor
NHS	National Health Service
NLRs	Nucleotide-binding oligomerisation domain-like receptors
NO	Nitric oxide
PAK 1	p21-activated kinases
PAMPs	Pathogen-associated molecular patterns
PAO1	*Pseudomonas aeruginosa* strain
PDGF	Platelet-derived growth factor
PKC	Protein kinase-C
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethylsulfonyl fluoride
PPT	Propyl pyrazole triol
PRRs	Pattern recognition receptors
PTK2	Protein tyrosine kinase 2
qPCR	Real-time polymerase chain reaction
RIPA	Radio immunoprecipitation assay
RLRs	RIG-1 like receptors
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
rpm	Revolutions per minute
RNAseq	RNA sequencing
RT	Room temperature
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
SERDs	Selective estrogen receptor degraders
SERMs	Selective estrogen receptor modulators
SSC	Side scattered light
TBS-tween	Tris-buffered saline tween
TGF β	Transforming growth factors β
THAM	Tris (hydroxymethyl) aminomethane hydrochloride
THC	T helper cells
Th1	Type 1 helper T-cell
Th2	Type 2 helper T-cell
Tris-HCl	Tris-hydrochloride
TLR4	Toll-like receptors 4
TLRs	Toll-like receptors
TNF-α	Tumour necrosis factor alpha
TPBM	Theophylline, 8-[(benzylthio)methyl]
VEGF	Vascular endothelial growth factor

WASP	Wiskott Aldrish Syndrome Protein
µg	Microgram
µm	Micrometre
µL	Microliters
γδ	Gamma delta
°C	Degree Celsius

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Abstract

Chronic wounds in the elderly often become infected, leading to substantial morbidity and mortality. Impaired healing in the elderly is mediated by age-related changes in steroid hormones, particularly declining levels of estrogen with increasing age. Although the anti-inflammatory activity of estrogen has been defined, very little is known about the effects of estrogen deprivation (ageing processes) on bacterial clearance. The aim of this study was to determine the effect of ageing (estrogen deprivation) on the ability of *in vitro* human U937-derived macrophages and *ex vivo* human peripheral blood monocyte (HPBM)-derived macrophages to eliminate bacteria via phagocytosis.

Host-pathogen assays were used to measure macrophage-mediated phagocytosis of two major wound pathogens, methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa*, under *in vitro* and *ex vivo* conditions that model estrogen levels in the elderly, young adults and following exogenous estrogen supplementation. Epifluorescence, confocal and scanning electron microscopy were used to visualise host-pathogen interactions and protein mediators of phagocytosis were measured by immunoblotting. Estrogen at concentrations typical of youth or supraphysiological levels significantly (P<0.05) increased the phagocytosis and effective killing of MRSA and *P. aeruginosa* in a dose-dependent manner compared to estrogen deprivation with significantly enhanced clearance of bacteria by M1 macrophages compared to M2 or M0 macrophages. Epifluorescence, confocal and scanning electron microscopy confirmed estrogen increases co-localisation of fluorescent GFP-*S. aureus* or mCherry-*P. aeruginosa* within macrophages and promotes bacterial internalisation. Activation of estrogen receptor (ER)-alpha (ER-α) mirrored the stimulatory effect of estrogen on phagocytosis whilst ER-α antagonism significantly (n=6; P<0.05) blocked the phagocytic effect of estrogen. In contrast, activation of ER-beta (ER-β) had no significant (n=6; P>0.05) effect on phagocytosis, confirming estrogen mediates bacterial clearance via specifically through ER-α. Immunoblotting analysis demonstrated that enhanced phagocytosis by estrogen is associated with altered levels of mediators involved in the actin

cytoskeleton of phagocytes including increased levels of FAK, Rac1, Cdc42 and RhoG, but reduced levels of RhoA.

Collectively the findings suggest estrogen may promote the resolution of wound bacterial infections during youth but this protection is lost as estrogen levels decline with increasing age, resulting in increased propensity and progression of wound infections in the elderly. Thus, novel wound dressings that provide local estrogen supplementation or selective activation of ER-α and/or specific targeting of downstream mediators of the actin cytoskeleton may provide effective treatment options for infected wounds in the elderly.

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Declaration

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The author has successfully published the following manuscripts during the PhD research course at MMU:

Nicola R. Sproston, **Mohamed El Mohtadi**, Mark Slevin, William Gilmore and Jason J. Ashworth; The Effect of C-Reactive Protein Isoforms on Nitric Oxide Production by U937 Monocytes/Macrophages. *Frontiers in Immunology.* (2018) 9:1500.

Anthony J. Slate, David J Wickens, **Mohamed El Mohtadi**, Nina Dempsey-Hibbert, Glen West, Craig E. Banks and Kathryn Whitehead (2018); Antimicrobial Activity of Ti-ZrN/Ag coatings for use in biomaterial applications. *Scientific Reports*. (2018) 8:1497.

Samuel J. Davidson, Lisa I. Pilkington, Nina C. Dempsey-Hibbert, **Mohamed El Mohtadi**, Shiying Tang, Thomas Wainwright, Kathryn A. Whitehead and David Barker. Molecular Synthesis and Biological Investigation of 5-Hysroxymethyl Dibenzyl Butyrolactones and Related Lignans. *Molecules* 2018, 23, 3057.

Abdulmannan Fadel, Andrew Plunkett, Jason Ashworth, Ayman M. Mahmoud, Yazan Ranneh, **Mohamed El Mohtadi** and Weili Li; The effect of extrusion screw-speed on the water extractability and molecular weight distribution of arabinoxylans from defatted rice bran. *Journal of Food Science and Technology*. (2018) 55(3): 1201-1206.

The author attended the 2017 eleventh Cold Spring Harbor Laboratory meeting on Microbial Pathogenesis and Host Response in New York, USA. In addition, the author has attended several national, international conferences and scientific meetings including the 2018 fifth European Congress of Immunology in Amsterdam, Netherlands, and the 2017 International Biodeterioration and Biodegradation Society's Symposium 'IBBS 17' in Manchester, UK.

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Dedication

I dedicate chapter 6 in my thesis to Professor Kathryn Whitehead and Dr Nina Dempsey.

To my Almighty God, to all my family and close friends, to my lovely mum and dad, to Mina and Thabet, to everyone who believed in me, especially my sister Farah, this thesis is for you….

Chapter 1 : Introduction

1.1 Acute Wound Healing

Acute wound healing is a complex and dynamic biological process divided into four sequential, overlapping phases; haemostasis, inflammation, tissue proliferation and remodelling of the tissue scar (Figure 1.1). Immediately after trauma, healing initiates with haemostasis (coagulation). This phase is characterised by the formation of a fibrin clot within minutes following injury to prevent further blood loss and provide a temporary, protective barrier over the wound. The inflammatory phase occurs within minutes after injury, with neutrophils being the first inflammatory cells recruited from circulation, followed by the recruitment of monocytes. The primary objective of inflammation is to localise and eradicate the causative irritant or stimuli (e.g. bacteria). Neutrophils peak in numbers at 24 to 36 hours post wounding whereas monocytes peak at around 5 to 7 days after injury. Monocytes undergo a series of changes to differentiate into tissue macrophages. Macrophages carry out phagocytosis and release cytokines that encourage the recruitment and activation of leukocytes at the injury site. Upon the clearance of apoptotic cells, macrophages undergo a phenotypic change from a pro-inflammatory to a reparative state that encourages the initiation of the proliferation phase (Mosser and Edwards, 2010).

Three to ten days after injury, the proliferation phase starts enabling granulation tissue formation, re-epithelialisation and angiogenesis. Proliferation is characterised by the creation of a new extracellular matrix (ECM) by fibroblasts, angiogenesis by endothelial cells and re-epithelialisation by keratinocytes. The final phase is remodelling of a mature tissue scar, which can take several months or, in some cases, up to a year post-injury. It is characterised by the remodelling of collagen and the vascular maturation of newly formed capillaries, allowing vascular density to return to normal within the wound (Guo and DiPietro, 2010).

For successful healing, wound repair requires progression through all four phases in the correct order and timeframe (Singer and Clark, 1999; Guo and DiPietro, 2010).

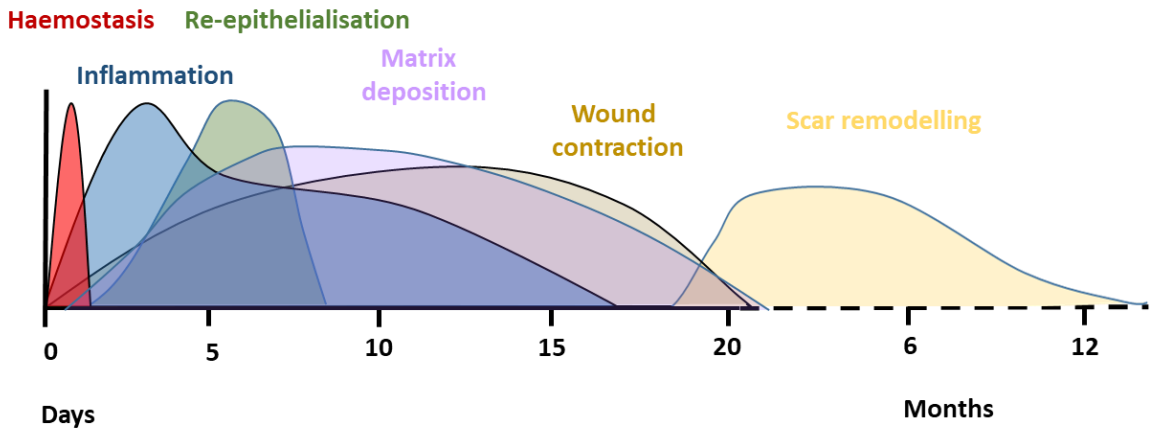

Figure 1.1 Diagrammatic illustration of typical timescale and phases of acute wound healing stages. 0 = day of wounding/injury. Figure drawn based on information in Gosain and DiPietro (2004) and Olczyk *et al*. (2014).

1.1.1 Haemostasis

Immediately after injury, degranulating platelets adhere to damaged blood vessels and start a haemostatic reaction, increasing the coagulation cascade to prevent extreme blood loss and to provide temporary protection for the wound against foreign bodies (Figure 1.2). This is accomplished by vasoconstriction and the creation of a platelet plug (Vaughan *et al*., 2000; Weyrich and Zimmerman, 2004; Gilliver *et al*., 2007). When the platelets come into contact with exposed collagen, they become adherent and activated, producing a platelet plug at the wound site. This involves a coagulation cascade in which fibrinogen is transformed to fibrin by thrombin, creating a clot of insoluble fibres bound to platelets (Fang *et al*., 2005). The clot forms a provisional matrix rich in fibronectin that acts as a scaffold over which cells can migrate during the wound healing process. Platelets in the clot release a variety of pro-inflammatory cytokines and growth factors including platelet-derived growth factor (PDGF), transforming growth factors β (TGF-β), fibroblast growth factor 2 (FGF-2), vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) (Bauer *et al*., 1985; Guo and DiPietro, 2010). These factors induce the recruitment of inflammatory cells from circulation and cells involved in the proliferative phase of wound repair including keratinocytes, endothelial cells and fibroblasts (Singer and Clark, 1999; Guo and DiPietro, 2010).

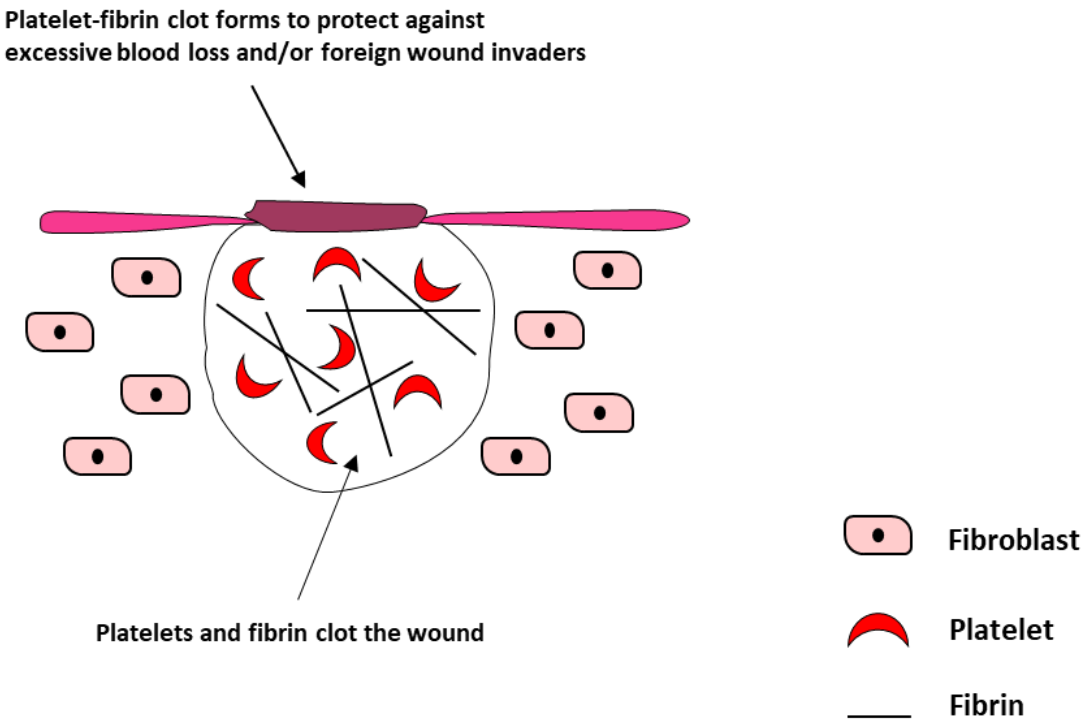

Figure 1.2. Schematic illustration of haemostasis in acute wound healing. Following an injury, platelets adhere to damaged blood vessels and start a haemostatic reaction, increasing the coagulation cascade to prevent extreme blood loss. A platelet plug forms that provides temporary protection against colonisation of the wound by microorganisms. The provisional fibrin matrix also provides a scaffold over which cells can migrate during the wound healing process. Figure drawn based on information in Singer and Clark (1999) and Guo and DiPietro (2010).

1.1.2 Inflammatory Phase

Following injury, the release of cytokines and chemokines by platelets and resident immune cells attracts inflammatory cells from circulation to the wound site (Figure 1.3). Large numbers of circulating neutrophils are recruited to the wound site by chemokine and cytokine expression of endothelial surface cellular adhesion molecules (CAMs), such as CD11 and CD18 (Ley *et al*., 2007). Neutrophils are the first immune cells to arrive at the wound site within minutes of injury. They peak in numbers at 24 to 36 hours post-injury (Mosser and Edwards, 2010). Neutrophils start to remove foreign substances and invading microorganisms, such as bacteria, via the release of reactive oxygen species (ROS) and lysosomal enzymes, and degrade damaged matrix tissues by collagenases and proteinases (Singer and Clark, 1999). The majority of neutrophils are enclosed in the wound clot and are either eliminated with the eschar or by macrophages via phagocytosis (Newman *et al*.,

1982). In response to chemoattractants such as TGF-β, macrophage chemoattractant protein 1 (MCP-1), and macrophage inflammatory protein (MIP), monocytes from the bloodstream subsequently arrive at the wound area and differentiate into tissue macrophages, peaking in number around days 5 to day 7 post-injury (Lorenz and Longaker, 2003; Sen and Roy, 2008). Growth factors mainly TGF-β and residues of the remaining extra cellular matrix (ECM) including collagen, elastin, fibronectin, and thrombin induce circulating monocytes to adhere to the endothelium of blood vessels and migrate into the tissue. Macrophages replace neutrophils as the predominant inflammatory cells at the wound site and carry out the process of phagocytosis of invading microorganisms, removal of damaged tissues and dead neutrophils and the release of growth factors such as PDGF and TGF-β (Beanes *et al*., 2003). Damaged extracellular matrix is degraded by the action of macrophage-derived proteolytic enzymes such as metalloproteases. Macrophages stimulate re-epithelialisation by releasing insulin-like growth factor-1 (IGF-1), keratinocyte growth factor (KGF) and EGF (Chen *et al*., 2004) and angiogenesis via the secretion of VEGF (Shaw *et al*., 1990). During wound clearance, macrophages experience a balanced switch from pro-inflammatory (classically activated) M1 macrophages to anti-inflammatory (alternately activated) M2 macrophages (Mosser and Edwards, 2008).

Adaptive immunity plays a major role in regulating inflammation and improving wound healing (Falanga, 2005). The adaptive immune response is late, but specific, compared to the innate immune response. It has the capacity to memorise invaders/pathogens and induce prompt reactions to later immunological tasks. The key cells linked with adaptive immune responses are T cells and B cells (Jameson *et al*., 2002). They are initiated in reaction to antigens or cells presenting antigens. When activated, B cells produce specific antibodies that label pathogens for destruction, activate the complement system, ingest bacteria, and activate toxins. T cells are induced by the major histocompatibility complex (MHC) molecule that presents antigens. Antigens presented by MHC class I are detected by CD8+ cytotoxic T cells, whereas antigens presented by MHC class II are recognised by CD4+ helper T lymphocytes. CD8+ cells are more specialised in targeting cells than

CD4+ cells, which are more involved in cytokine production and paracrine signalling to adjacent cells. T cells are suggested to play a role in tissue repair (Jameson *et al*., 2002). A specific group of T cells known as gamma delta (γδ) T cells, or dendritic epidermal T cells, are cells that are able to recognize antigens expressed by damaged and/or stressed keratinocytes (Jameson *et al*., 2002). Research demonstrated that (γδ) T cells enhance re-epithelialisation via producing growth factors such as Keratinocyte growth factor-7 (KGF-7) and keratinocyte growth factor-10 (KGF-10). This has been reported in an animal study where γδT cell deficiency led to a delay in wound repair (Jameson *et al*., 2002). CD4+ and CD8+ cells have also been shown to have an impact on the infiltration of neutrophils and macrophages to the wound site, which has an evident impact on the regulation of the inflammatory phase in wound healing. Furthermore, an induced deficit of CD4+ cells is linked with an increase in the levels of interferon gamma (IFN-γ), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), interleukin-17 (IL-17) and a diminution in interleukin-4 (IL-4). However, an induced deficit of CD8+ cells has the opposite effect on these inflammatory factors (Chen *et al*., 2004). A balanced innate/adaptive immune reaction is essential for an appropriate inflammatory phase and wound healing.

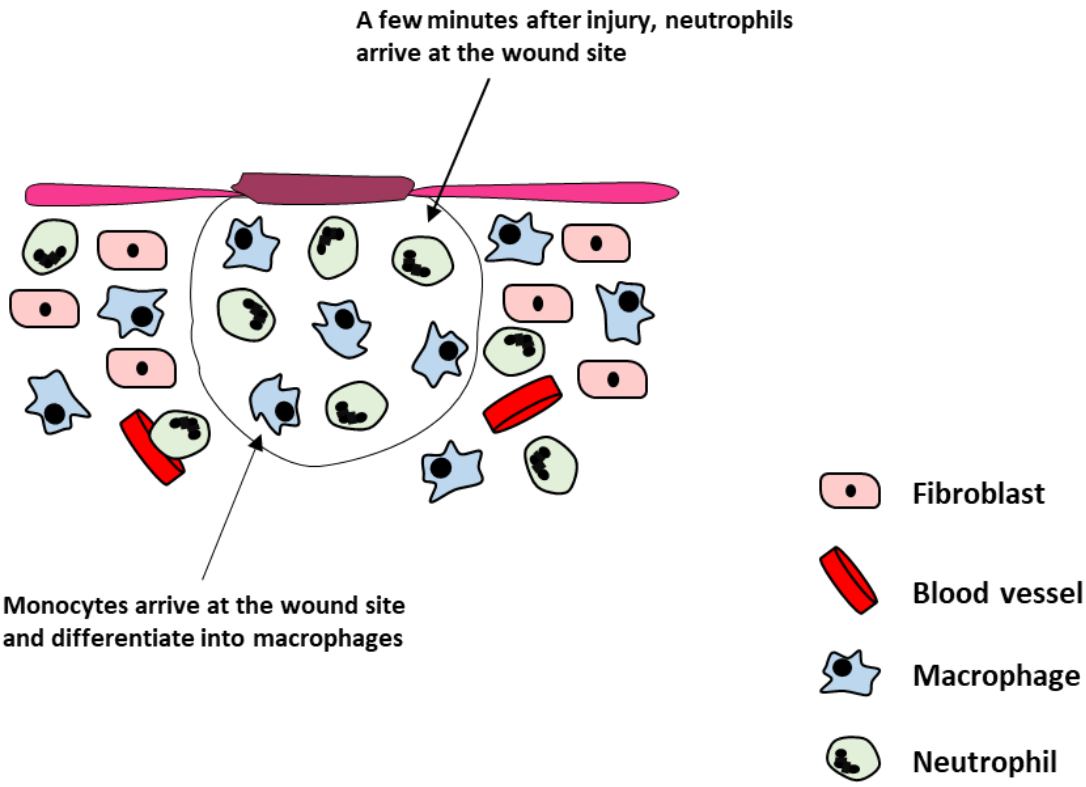

Figure 1.3. Representation of the inflammatory phase of acute wound healing. Cytokines released by platelets initially attract neutrophils and then monocytes to the wound site. Monocytes subsequently differentiate into tissue macrophages. Figure drawn based on information in Singer and Clark (1999) and Guo and DiPietro (2010).

1.1.3 Proliferative Phase

The third phase of the acute wound healing is called the proliferative phase. The major events during this phase are ECM formation, re-epithelialisation and angiogenesis (Kirsner and Eaglstein, 1993).

1.1.3.1 ECM Formation

Granulation tissue formation occurs approximately between 3 and 10 days post-injury. The temporary matrix produced during haemostasis is replaced with a more defined granulation tissue, consisting of newly formed blood vessels and various cell types including fibroblasts, macrophages and granulocytes within loose connective tissue (El Ghalbzouri *et al.*, 2004; El Ghalbzouri and Ponec, 2004). Fibroblasts exert several functions during wound healing processes such as wound contraction and ECM deposition. Fibroblasts are activated by PDGF and FGF-2, and

are encouraged to proliferate by insulin-like growth factor 1 (IGF-1) and TGF-β1 (Singer and Clark, 1999). TGFβ1, released by macrophages during inflammation and induced by mechanical pressure in the ECM, directs fibroblasts to differentiate into contractile myofibroblasts (Gabbiani, 1999; Serini and Gabbiani, 1999). Fibroblasts attach together and to the extracellular matrix, producing stress fibres of contractile actin bundles that close the wound (Hinz, 2007). Fibroblasts synthesise new ECM through the generation of collagen, fibronectin, elastin, glycosaminoglycans, proteoglycans and hyaluronic acid (Hinz, 2007).

1.1.3.2 Re-epithelialisation

Re-epithelialisation is the process of restoring an intact epidermis after cutaneous injury, and it involves an orderly series of events including the detachment, migration and proliferation of adjacent epidermal keratinocytes across the wound, the differentiation of the neo-epithelium into a stratified epidermis, and the restoration of an intact epidermal barrier function (Li *et al*., 2007). Keratinocytes at the wound borders proliferate to replace the cells that migrate across the temporary wound matrix (Li *et al*., 2007). The main factors inducing keratinocyte migration are EGF, keratinocyte growth factor (KGF), Insulin-like growth factor 1 (IGF-1) and nerve-growth factor (NGF) (Marikovsky *et al*., 1993; Tokumaru *et al*., 2000). Growth factors that induce re-epithelialisation are released by activated platelets during haemostasis and by inflammatory cells, mainly macrophages, during inflammation (Li *et al*., 2007). Keratinocytes upregulate the production of proteolytic enzymes including matrix metalloproteinases (MMPs) that contribute to create a path between the scab and tissue below. Migration of cells then stops causing a rearrangement of the actin cytoskeleton (Jacinto *et al*., 2001; Mayor and Carmona-Fontaine, 2010).

1.1.3.3 Angiogenesis

Angiogenesis is the revascularisation procedure that re-establishes a blood supply to regenerate tissues following wounding. Angiogenesis is induced by growth factors including members of the TGF-β family, VEGF, FGF-2 and PDGF, which are released during haemostasis, and by inflammatory cells in response to injury

(Adams and Alitalo, 2007). Recruited endothelial cells release proteolytic enzymes that facilitate their entrance to the wound site, where they multiply and assemble into tubular canals, and secrete MMPs, leading to lysis of adjacent tissue. Endothelial cells increase in number and reinforce their vessel walls by recruiting smooth muscle cells and pericytes, reinstating blood flow to the injured tissue (Clark *et al*., 1982).

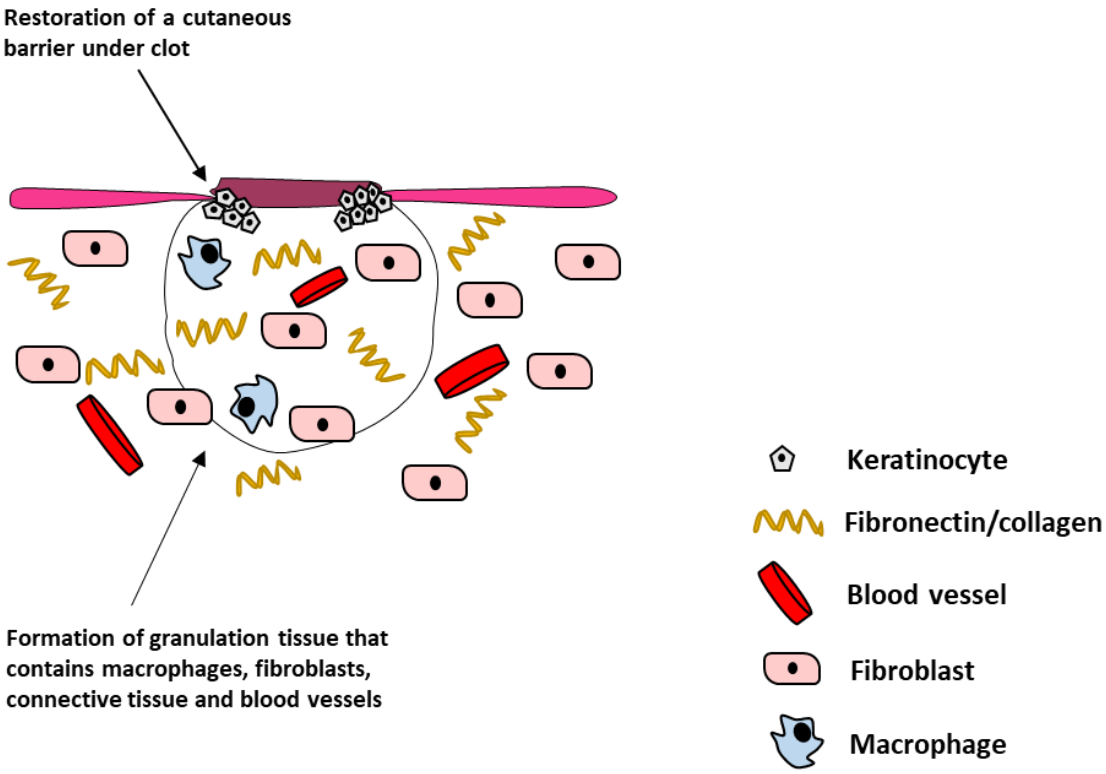

Figure 1.4. Schematic illustration of the proliferative phase in wound repair. As the inflammatory phase resolves, the overlapping proliferative phase includes the synthesis of granulation tissue that is characterised by blood vessel formation and contains recruited fibroblasts and macrophages. Keratinocytes at the wound edge migrate over the wound to restore the epidermis. Figure drawn based on information in Singer and Clark (1999) and Guo and DiPietro (2010).

1.1.4 Remodelling Phase

Remodelling is the last phase of wound repair that can endure for many months after wounding. It is characterised by a termination of the inflammatory response and the continuous remodelling of the ECM. The production/release of cytokines stops and inflammatory cells are removed from the wound area by apoptosis and/or phagocytosis (Serhan *et al*., 2008). The new ECM is remodelled over several

months to generate a robust dermal structure in which collagen III is gradually replaced by collagen I leading to the formation of a mature tissue scar (Singer and Clark, 1999). Blood vessels also degenerate during the remodelling phase (Singer and Clark, 1999).

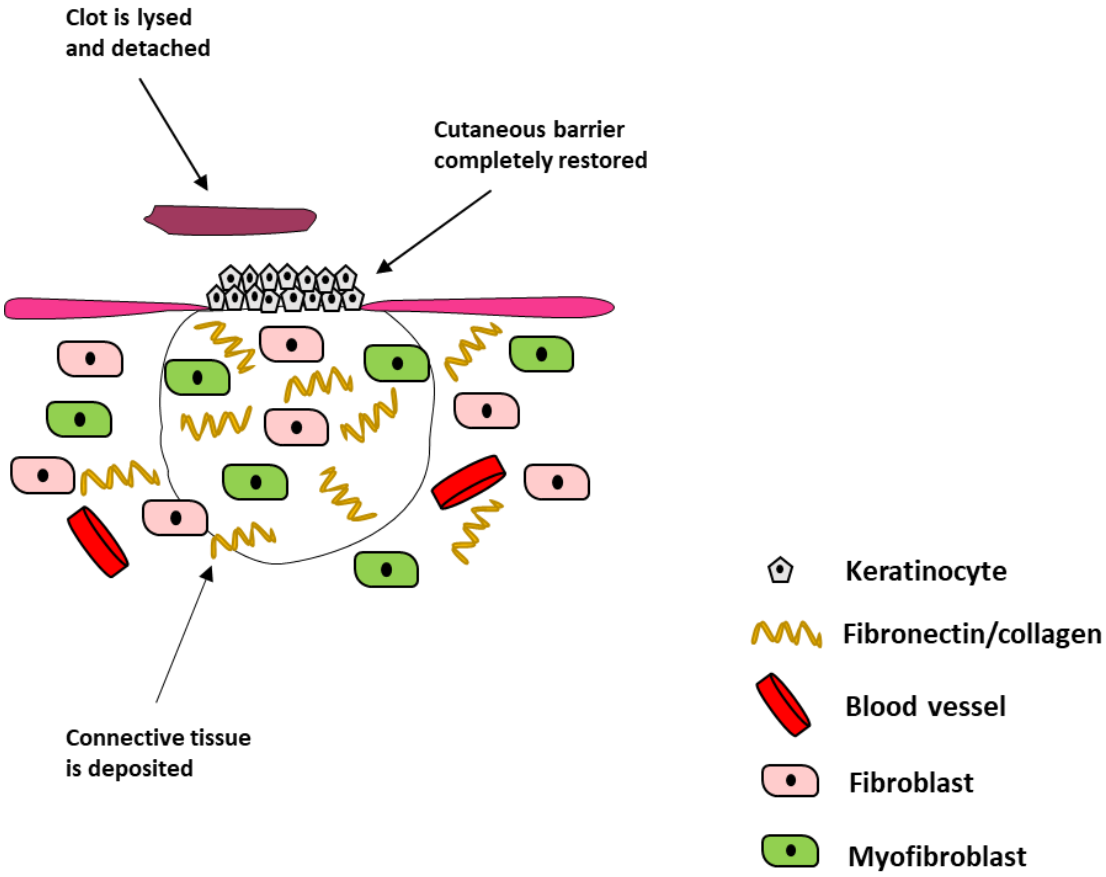

Figure 1.5. Schematic representation of the remodelling phase of wound healing. Blood vessels degenerate. The provisional extracellular matrix of fibronectin and fibrin is replaced with connective tissue, including collagen, and the formation of a mature scar. Figure drawn based on information in Singer and Clark (1999) and Guo and DiPietro (2010).

1.2 Abnormal Wound Healing

After an injury, wound healing is vital in re-establishing a new cutaneous barrier and preventing infection by invading foreign microorganisms. The speed and quality of wound repair are influenced by several factors, with age being a key factor that is known to markedly delay healing in the elderly (Ashcroft *et al*., 2002).

Pathological circumstances such as, diabetes, contribute directly to the development of chronic wounds, with foot ulcers being the most common cause of hospitalisation in diabetic patients. The treatments for chronic wounds are often

ineffective and place a substantial financial burden on the world's health organisations (Harding *et al*., 2002; Boulton *et al*., 2005).

1.2.1 Age-Related Impaired Healing

With increasing age, acute wound healing proceeds but becomes delayed**.** This detrimental change in acute wound healing in the elderly is called age-related impaired healing and is linked with an increase in skin fragility, reduced immune responses and cellular aging (Thomas, 2001). Delayed wound healing in the elderly is associated with delayed haemostasis (Ashcroft *et al*., 1999b), prolonged and excessive inflammatory response**,** delayed re-epithelialisation, impaired angiogenesis, and reduced matrix deposition (Ashcroft *et al*., 1997b; Ashcroft *et al*., 2002)**.** Although the inflammatory response becomes more pronounced with increasing age, the propensity for wound infections increases in the elderly (Ashcroft *et al*., 2002; Cooper *et al*., 2015), in part due to the delay in wound repair. Moreover, despite the pronounced inflammatory response, evidence suggests that increasing age may result in an impaired ability of inflammatory cells to eliminate bacteria from the wound site (Emori *et al*., 1991; Thomas, 2001). It is suggested that an excessive presence of macrophages lead to an excessive matrix demolition due to the production of great amounts of nitric oxide (NO) and ROS which can delay wound healing and cause damage to the wound zone (Sen and Roy, 2008).

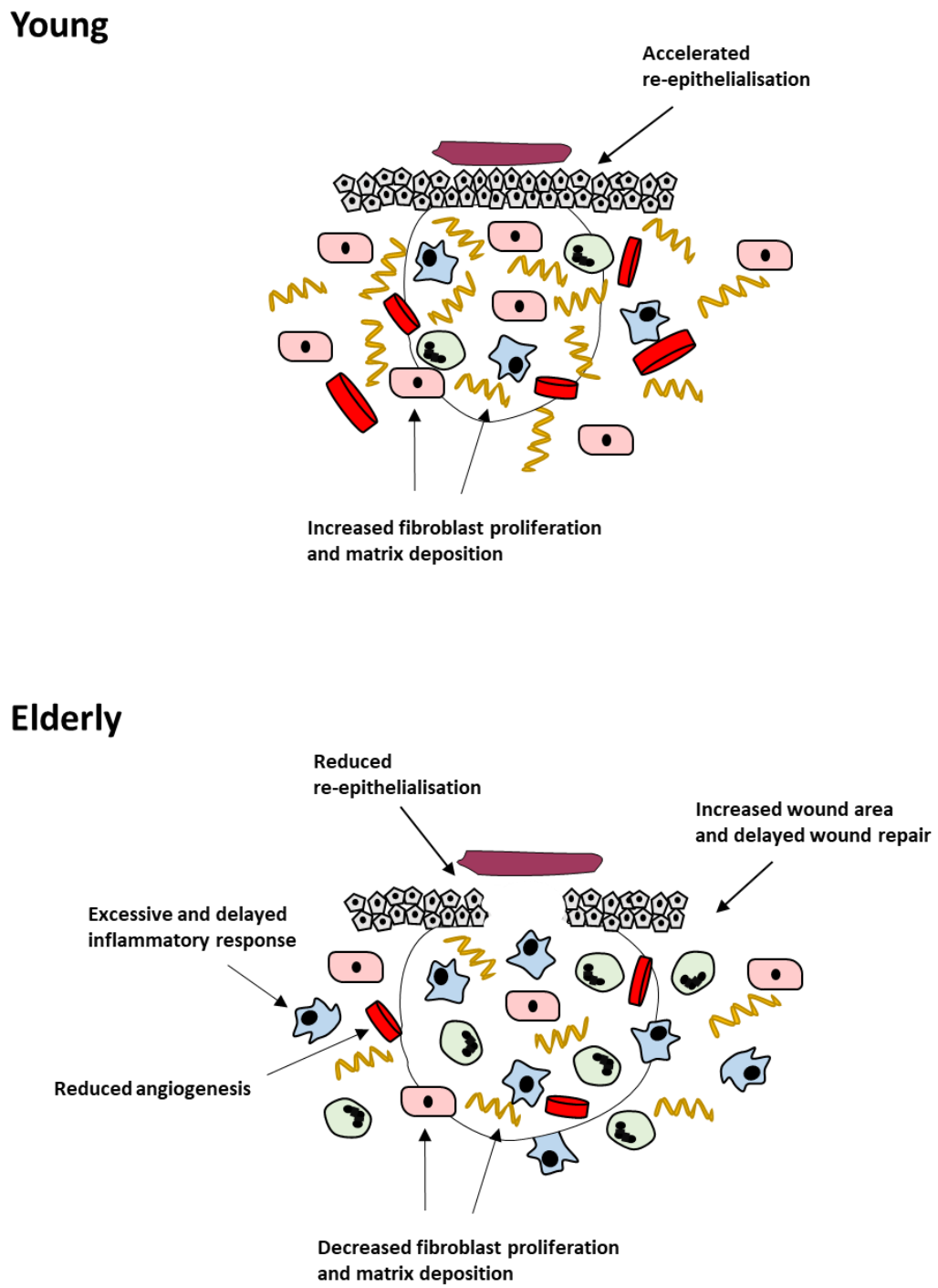

Figure 1.6. Schematic representation of the effect of age on wound healing. Age-related impaired healing is linked with delayed haemostasis, delayed and excessive inflammatory response and enhanced pro-inflammatory cytokine production, impaired platelet function and delayed re-epithelialisation, in addition to decreased fibroblast proliferation and matrix deposition. Figure drawn based on information in Gosain and DiPietro (2004).

1.2.2 Chronic Wounds

A chronic wound develops when a wound fails to proceed through an orderly set of wound healing phases within an expected timeframe. Chronic wounds can take

several months or even years to heal (if they heal at all) properly. Wounds are considered chronic if they do not heal within three months (Mustoe, 2005; Adeyi *et al*., 2009). Chronic wounds are mainly linked to an underlying disease that is common in the elderly (over 65 years of age). Chronic wounds represent a major clinical issue that causes an enormous burden to healthcare services, demanding huge medical efforts and a substantial amount of healthcare funds (Harding *et al*., 2002; Boulton *et al*., 2005). Chronic wound treatment costs the UK National Health Service (NHS) about £5 billion per annum (Guest *et al*., 2015). At present, effective therapies/treatments for chronic wounds are somewhat limited, making this an area of research that needs urgent attention. Chronic wounds are typically trapped within the inflammatory phase of wound repair and are characterised by an excessive, unabated inflammatory response that leads to tissue breakdown (Snyder, 2005; Taylor *et al*., 2005; Fazli *et al*., 2009). They are also characterised by a loss in the specific balance between the formation and degradation of the extracellular matrix (ECM), particularly collagen and elastin, with a gross shift to ECM destruction (Edwards *et al*., 2004; Schönfelder *et al*., 2005). Chronic wounds are characterised by increased pro-inflammatory cytokine production, decreased tissue growth factor secretion, reduced matrix production, delayed but excessive inflammation, decreased angiogenesis, delayed re-epithelialisation, bacterial infection and defective macrophage function (Hohn *et al*., 1976; Harding *et al*., 2002). There are several underlying pathologies associated with chronic wounds, with more than 90 % of chronic wounds being venous, diabetic or pressure ulcers (Boulton *et al*., 2005)**.** Moreover, it has been reported that diabetic foot ulcers and venous leg ulcers have an increased number of B cells and an inferior CD4+/CD8+ ratio in comparison with acute wounds, mainly due to a lower number of CD4+ cells (Moore *et al*., 1997).

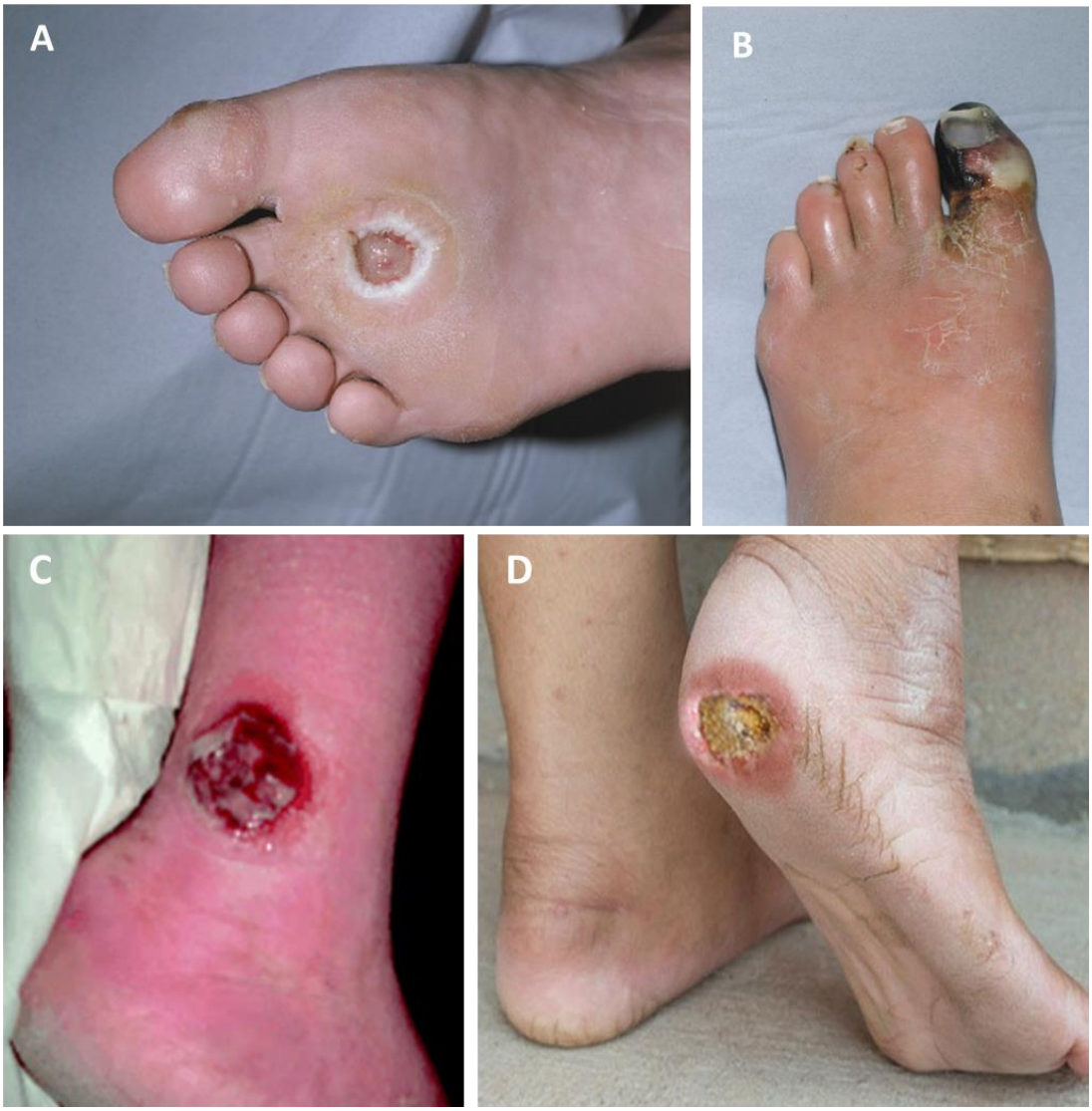

Figure 1.7. Patients with a chronic wound. A typical example of; (A) diabetic foot ulcer, (B) diabetic foot ulcer developing gangrene (Jeffcoate and Harding, 2003), (C) venous leg ulcer and (D) pressure foot sore (Grey *et al*., 2006).

1.2.2.1 Venous Ulcers

Venous ulcers (Figure 1.7c) are most frequently observed in the elderly and represent approximately 70 % of chronic wounds of the lower leg (Baker *et al*., 1991). Venous ulcers mainly develop in the gaiter area of the leg and signify a clinical manifestation of sustained venous hypertension, typically characterised by excessive vascularisation and unhealthy granulation tissue formation. At an advanced stage, venous ulcers can impair the function of the calf muscle (Cockett, 1955; Grey *et al*., 2006).

Research has suggested the delay in healing and development of venous ulcers is connected with an inappropriately excessive inflammatory response (White and Ryjewski, 2005). It is thought that leukocytes recruited during the inflammatory phase are trapped in the microcirculation of the wounded leg (Moyses *et al*., 1987) and release inflammatory mediators that lead to further recruitment and accumulation of leukocytes. Inflammatory cells release proteolytic enzymes that break down the dermal structure, leading to the development of an ulcer (Wysocki and Grinnell, 1990; Grinnell *et al*., 1992).

1.2.2.2 Diabetic Ulcers

It has been reported that 15 % of diabetic patients in the UK are at risk of developing a chronic diabetic foot ulcer (Guo and DiPietro, 2010), and around 85 % of diabetic ulcer patients will require an amputation of their lower leg due to the development of gangrene (Figure 1.7b) (Apelqvist and Larsson, 2000; Boulton, 2004; Clinton and Carter, 2015). Diabetes is characterised by an alteration of blood sugar levels which can cause nerve damage leading to autonomic neuropathy and vasculopathy (microcirculation dysfunction) (Boulton, 2004). This can generate local hypertension and hypoxia, resulting in an ulcer due to tissue necrosis. A diabetic ulcer (Figure 1.7a) typically develops on feet and ankle regions that are repetitively exposed to mechanical pressures. Diabetic ulcers occur frequently in patients who develop diabetic neuropathy, because they are incapable of sensing and relieving cutaneous pressure and pain (Boulton *et al*., 1983; Boulton, 2004). Like many chronic wounds, diabetic wounds exhibit impaired macrophage and neutrophil function, in turn increasing the risk of infection and prolonging healing time.

1.2.2.3 Pressure Ulcers

A pressure ulcer, also called a pressure sore (Figure 1.7d), is local damage of the skin and/or underlying tissues, resulting from pressure, or pressure combined with shear (Black *et al*., 2007). Pressure ulcers are mostly hospital-acquired chronic wounds, occurring predominantly in the elderly and particularly in patients who remain stationary for a long period of time, such as paralysed or disabled patients (Allman *et al*., 1995). Pressure ulcers are usually small ulcers caused by the

interruption of blood flow to the tissue by persistent pressure. The absence of oxygen at the pressure site results in necrosis of the tissue and the development of a pressure sore (Black *et al*., 2007).

1.3 Wounds and Bacteria

1.3.1 Wound Infection

It is believed that low levels of bacteria in a wound have a beneficial impact on acute wound healing by stimulating the inflammatory response and improving granulation tissue formation (Robson, 1997). However, chronic wounds, that take months or years to heal, typically have microbes surviving and proliferating within the wound site. Research has indicated that all chronic wounds are subject to colonisation with a varied range of bacteria (Bowler and Davies, 1999). When the bacteria outweigh the host's immune system, the result is infection (Friedman and Su, 1984). It has been reported that the environment of chronic wounds is potentially favourable to bacterial infections, particularly when the wound tissue is ischaemic and dry (Rubinstein *et al*., 1983; Whiston *et al*., 1994).

1.3.2 Bacterial Species in Chronic Wounds

Numerous studies have demonstrated that the main bacterial species found in chronic wounds are *S. aureus*, *P. aeruginosa*, *S. epidermidis*, *Streptococcus spp*, *Enterococcus* spp. and coliform bacteria (Hansson *et al*., 1995; Bowler, 1999; Gjødsbøl *et al*., 2006; Kirketerp-Møller *et al*., 2008; Rybtke *et al*., 2015)**.** Research has identified bacterial species present in infected surgical wounds of 676 patients. The most dominant pathogen was *S. aureus* (28.2 %), followed by *P. aeruginosa* (25.2 %), *E. coli* (7.8 %), *S. epidermidis* (7.1 %) and *Enterococcus faecalis* (5.6 %) (Giacometti *et al*., 2000). The most frequent bacteria cultured from chronic wounds are illustrated in Table 1.1.

Table 1.1. Bacteria frequently associated with chronic wounds.

Information in table adapted from (Robson, 1997; Bowler and Davies, 1999).

Gram-positive		Gram-negative	
Aerobe	Anaerobe	Aerobe	Anaerobe
Staphylococcus	*Peptostreptococcus*	*Pseudomonas*	*Bacteriodes*
Streptococcus	*Clostridium*	*Escherichia*	*Prevotella*
Enterococcus		*Klebsiella*	*Fusobacterium*

It has been reported that chronic wounds are notably susceptible to *S. aureus* infections (Bowler and Davies, 1999; Beasley and Hirst, 2004). Along with anaerobic bacteria, *S. aureus* is known to be the most predominant bacteria found in chronic wounds, followed by *P. aeruginosa, Peptostreptococcus* spp*. and Bacteriodes* spp*.* (Louie *et al*., 1976; Lookingbill *et al*., 1978; Stephens *et al*., 2003; Davies *et al*., 2004).

Staphylococcus spp. and *Pseudomonas* spp. are the aerobic species most frequently detected in all wound types, with a great predominance in chronic wounds (Davies *et al*., 2004).

1.3.2.1 *Staphylococcus aureus*

S. aureus is a facultative anaerobic Gram-positive cocci, typically 1 µm in diameter. It is commonly found on the skin, nose and in the respiratory tract (Stryjewski and Corey, 2014; Chambers, 2001). In normal healthy and immunocompetent people, *S. aureus* colonisation of the skin, intestinal tract, or nasopharynx is not pathogenic. However, it is considered the classic opportunist since it is frequently associated with wound infections and abscesses (Chambers, 2001; Missiakas and Schneewind, 2016).

Subpopulations of *S. aureus* comprise antibiotic resistant strains, such as methicillin-resistant *S. aureus* (MRSA). Penicillin was the antibiotic of choice to treat infections with *S. aureus*. After the acquisition of a resistance to penicillin, methicillin was introduced in 1959 as a treatment for penicillin-resistant *S. aureus* (Jevons, 1961; Jessen *et al.,* 1969*)*. However, reports from the UK indicated the first case of MRSA just two years later (in 1961) and, during the period between the 1960s and the 1990s, hospital-acquired MRSA cases became increasingly common,

leading to a serious worldwide health problem (Enright *et al*., 2002). MRSA is now recognised as a nosocomial pathogen (hospital acquired-MRSA) throughout the world (Diekema *et al*., 2001). However, cases of MRSA in healthy, community-resident individuals have been recorded towards the end of the 1990s, with such infections referred to as community-acquired MRSA (Naimi *et al*., 2001). MRSA has the ability to live and survive undetected in a host without provoking any symptoms (Stryjewski and Corey, 2014). Nevertheless, the pathogen can cause endocarditis, sepsis, and meningitis if it is introduced into the bloodstream, or if the host is incapable of developing a normal immune response (i.e. immunocompromised). Vancomycin was the only antibiotic used as a treatment for MRSA. However, resistance to this antibiotic has also been reported in recent years (Hiramatsu *et al*., 1997; Cardona and Wilson, 2015).

MRSA is frequently linked with chronic wounds. Giacometti *et al*. (2000) reported that 54.4 % of *S. aureus* isolated from surgical wounds were discovered to be methicillin-resistant. The treatment of chronic wounds infected with MRSA represents a big challenge for the modern healthcare organisations (Beasley and Hirst, 2004). When chronic wounds are heavily infected with MRSA, or other hospital-acquired pathogens, treatment may necessitate aggressive medication with last line of defence antibacterial therapies (Beasley and Hirst, 2004).

1.3.2.2 *Pseudomonas aeruginosa*

P. aeruginosa is a Gram-negative bacillus, typically 3 µm × 0.5 µm in diameter. It is an opportunistic pathogen and a causative agent of a wide range of diseases in both immunocompromised and otherwise healthy patients (Passador *et al*., 1993; Ryan and Ray, 2004). It is a typical-multidrug-resistant (MDR) organism known for its ubiquity, its fundamentally advanced antibiotic resistance mechanisms, and its link with serious diseases, particularly nosocomial infections such as sepsis (Cross *et al*., 1983). It is also considered to be a serious danger to patients with impaired host defences, such as patients with burn wounds and cystic fibrosis (CF) (Hachem *et al*., 2007).

P. aeruginosa has the ability to produce a biofilm in several environments (O'Toole and Kolter, 1998; Costerton *et al*., 1999; Yoon *et al*., 2002). Biofilms are surface-attached bacterial communities in which microbial aggregates are enclosed in a self-formed, extracellular polymeric matrix (Whitchurch *et al*., 2002; Matsukawa and Greenberg, 2004; Fazli *et al*., 2009). Biofilms have a specific complex architecture and structure with biochemical characteristics that lead to increased bacterial resistance against host immune mechanisms and antibiotics (O'Toole and Kolter, 1998; Costerton *et al*., 1999; Mah *et al*., 2003). Thus, it is usually difficult to treat such biofilms effectively with antibiotics since the biofilm structure appears to protect the bacteria from diverse environmental factors (Stover *et al.*, 2000). Biofilms of *P. aeruginosa* can be the cause of many chronic opportunistic infections in immunocompromised and elderly patients. It is the main cause of mortality and morbidity in CF patients, since it adapts, persists and produces biofilms in anaerobic CF environments, as opposed to the aerobic biofilms typically formed under laboratory conditions (Oliver *et al*., 2000; Singh *et al*., 2000).

P. aeruginosa are resistant to a wide range of antibiotics and has the ability to acquire additional resistance after failed treatments. Resistance of *P. aeruginosa* to many common first-line antibiotics, such as carbapenems, polymyxins, and more lately tigecycline, has been demonstrated (Strateva and Yordanov, 2009). However, such antibiotics are still in use in cases where resistance has not yet been determined. The antibiotic fluoroquinolone is one of the few antibiotics that are efficient against *P. aeruginosa*. In some hospitals, treatment with this drug is severely controlled to avoid the progress of resistant strains (Köhler *et al*., 1997; Pesci *et al*., 1999). Although *S. aureus* has been established as a main participant in chronic wounds, recent evidence has demonstrated that *P. aeruginosa* infects the deeper layers of the chronic wounds (Fazli *et al*., 2011; Rybtke *et al*., 2015).

1.3.3 Chronic Wounds and Biofilms

The growth of microorganisms in chronic wounds has been shown to take the form of irregularly shaped micro-colonies that can comprise hundreds of bacteria (Bjarnsholt *et al*., 2008; Rybtke *et al*., 2015).

It is becoming increasingly apparent that biofilms play an important role in the production and maintenance of the chronic wound environment (Davis *et al*., 2008; James *et al*., 2008a; Kirketerp-Møller *et al*., 2008; Fazli *et al*., 2009). Nevertheless, the role of biofilms in chronic wounds is still not entirely understood, but it is supposed that their presence could be one of many causes of non-healing wounds (Bjarnsholt *et al*., 2005; Jensen *et al*., 2007; Fazli *et al*., 2009). Chronic impaired wounds are frequently colonised with biofilms of multiple bacterial species including *S. aureus* and *P. aeruginosa* (Clinton and Carter, 2015). Treatment of biofilm-infected wounds is extremely difficult due to the polymicrobial nature of these biofilms, and their high resistance to traditional antibiotic combinations (Clinton and Carter, 2015). Compared to normal wounds, chronic wounds are highly susceptible to colonisation with biofilms (James *et al*., 2008b). This makes wound infection one of the main factors that delay wound healing in chronic wounds (James *et al*., 2008b).

Antimicrobial genes of biofilms, composed of one or multiple bacterial species, can interact with each other leading to an increased virulence of the overall biofilm (James *et al*., 2008b). Moreover, the metabolism of these multi-species biofilms can contribute to the development of antibacterial resistance, leading to auto-regeneration of the biofilm after treatment with antibiotics (Fauvart *et al*., 2011). When established, biofilms become difficult to eradicate leading to a higher persistence of chronic wounds (James *et al*., 2008b).

1.3.4 Bacterial Invasion of Host Tissues

Invading bacteria adhere to host cells, generally epithelial cells, and start colonising host tissues. Bacteria develop complex strategies to successfully invade and survive inside the host (Cossart and Sansonetti, 2004). The key mechanisms by which bacteria invade the host include adhesion to host cells, release of toxins causing tissue damage, and development of resistance against host antimicrobial defences, thus assuring effective growth and survival (Finlay and Falkow, 1989; Galan, 1994; Ofek and Doyle, 2012). Such resistance mechanisms are particularly important in establishing a persistent, chronic infection.

Bacteria bind to host cells using filamentous proteins named adhesins, such as pili and fimbriae. Lipopolysaccharide (LPS), M proteins, capsule, glycocalyx and lipoteichoic acids all act directly or indirectly as adhesins (Ofek and Doyle, 2012). It is known that the membrane surface of host cells and bacteria are negatively charged, leading them to repel each other (Gottenbos *et al*., 2001). However, when bacteria use adhesins, their hydrophobicity changes, which is a key feature that allows them to adhere to host cells (Peschel, 2002). Bacteria release a range of molecules, such as enzymes and endotoxins, which cause host tissue damage. Endotoxins are produced by both Gram-positive and Gram-negative bacteria and act mainly to disturb host cell functions. Endotoxins, produced by Gram-negative bacteria including, *P. aeruginosa,* are normally expressed on their cell wall and are typically released in huge quantities after bacterial lysis (Shenep and Mogan, 1984). LPS is a component of the Gram-negative bacterial membrane and is the most common endotoxin (Ulevitch and Tobias, 1999). The over-reaction to LPS can cause severe inflammatory diseases such as sepsis (Peyssonnaux *et al*., 2007)

1.3.4.1 Host-Pathogen Interactions

Research has largely described the immune system response to free-living (not in a biofilm arrangement) pathogenic infection. It is essential to understand the immune system's response to bacteria and apply that understanding to acute and chronic wound infections (Wolcott *et al*., 2008; Mahla *et al*., 2013).

The host has highly developed approaches to recognise and identify bacteria and other microorganisms. Innate immunity (Figure 1.8) is initiated by pathogen-associated molecular patterns (PAMPs), which are molecules expressed on the cell membrane of bacteria and microorganisms. PAMPs are recognised by pattern recognition receptors (PRRs) expressed on the membrane of immune cells (Hoffmann *et al*., 1999; Janeway and Medzhitov, 2002; Broz and Monack, 2013; Fukata and Arditi, 2013). PAMPs, such as LPS, bind to PRRs including Toll-like receptors (TLRs), and initiate a signalling cascade normally, via activation of nuclear transcription factor kappa beta (NF-κB) (Aliprantis *et al*., 1999; Kopp and Medzhitov, 1999). This event initiates the immune system to release a variety of

pro-inflammatory cytokines, such as granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), interferon-gamma (IFN-γ), tumour necrosis factor alpha (TNF-α), interleukin-1 (IL-1), interleukin-8 (IL-8) and interleukin-6 (IL-6) (Wolcott *et al*., 2008). These cytokines, particularly IL-8, are responsible for regulating the recruitment and the activation of neutrophils, and upregulating antibacterial activity in macrophages and dendritic cells. As the innate immune response progresses, pathogenic invaders are labelled with complement, allowing neutrophils and macrophages to identify, bind, engulf and finally digest them (Shepherd, 1986). Neutrophils are then digested by tissue macrophages to avoid host tissue destruction that could result from excessive secretion of neutrophil-derived proteases.

The PRRs are distributed into four functional families: Toll-like receptors (TLRs), nucleotide-binding oligomerisation domain-like receptors (NLRs), C-type lectin receptors (CLRs), and RIG-1 like receptors (RLRs) (Kopp and Medzhitov, 1999). Several TLRs respond to constituents of Gram-positive and Gram-negative bacteria and stimulate the immune inflammatory reaction (Hoffmann *et al*., 1999).

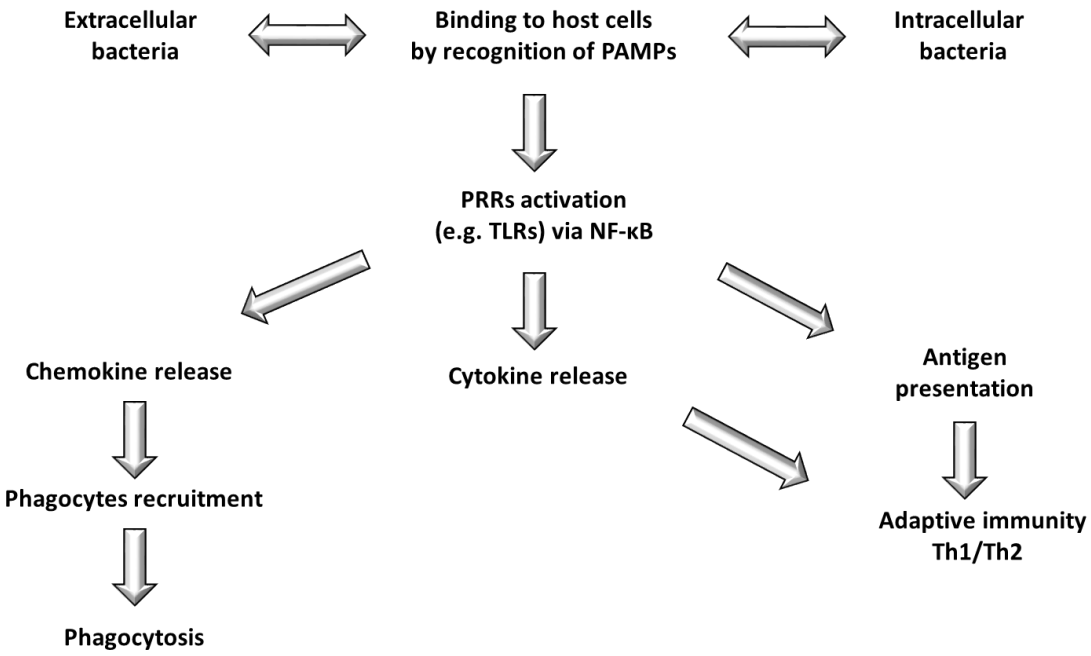

Figure 1.8. Innate immunity in host-pathogen interaction. When TLRs are activated, the innate immune response is initiated through the release of chemokines and cytokines. The acquired immune response is initiated by cytokines of Th1 and Th2, resulting in the production of antibodies. Figure drawn based on information in Basset *et al*. (2003).

The activated complement system (Figure 1.9) consists of three main pathways: classical pathway, alternative pathway and lectin pathway. Complement is a key player of the innate immune system, providing a quick, robust response against foreign invaders, but it also has a very significant impact on the acquired immune system involving T and B cells to remove invaders (Dunkelberger and Song, 2010). Thirty proteins are involved in the complement pathways (Sarma and Ward, 2011). One of their main roles is to lyse microorganisms and to encourage the production of inflammatory mediators that attract phagocytes and initiate the inflammatory response (Sarma and Ward, 2011). The main complement components involved in host cell recruitment at the infection site are C3a, C4a and C5a. Even though the complement system comprises several proteins, they all finally converge in the production of C3, which is the most abundant complement protein in the blood. C3 is converted into various active forms including C3a, C3b, C5a and the membrane attack complex (C5b-9) (Sarma and Ward, 2011).

The alternative pathway is an antibody independent pathway and represents a key element of innate immunity. It is initiated by molecules such as carbohydrates and

endotoxins found on foreign invaders (Qu *et al*., 2009). The classical pathway depends on the production of antibodies, and is a key element of the adaptive specific immune system (Sarma and Ward, 2011). The lectin pathway is activated by the binding of either mannose binding lectin (MBL) or ficolin to carbohydrates present on the membrane of invaders, including bacteria, viruses and parasites (Sarma and Ward, 2011).

During bacterial infection, C3 is cleaved to opsonin (C3b), which binds to the pathogen to enable the immune phagocytic cells to remove the foreign invader (Walport, 2001; Sarma and Ward, 2011). Key cells of the innate immunity are epithelial, mast and dendritic cells in addition to phagocytic cells such as neutrophils, monocytes and macrophages.

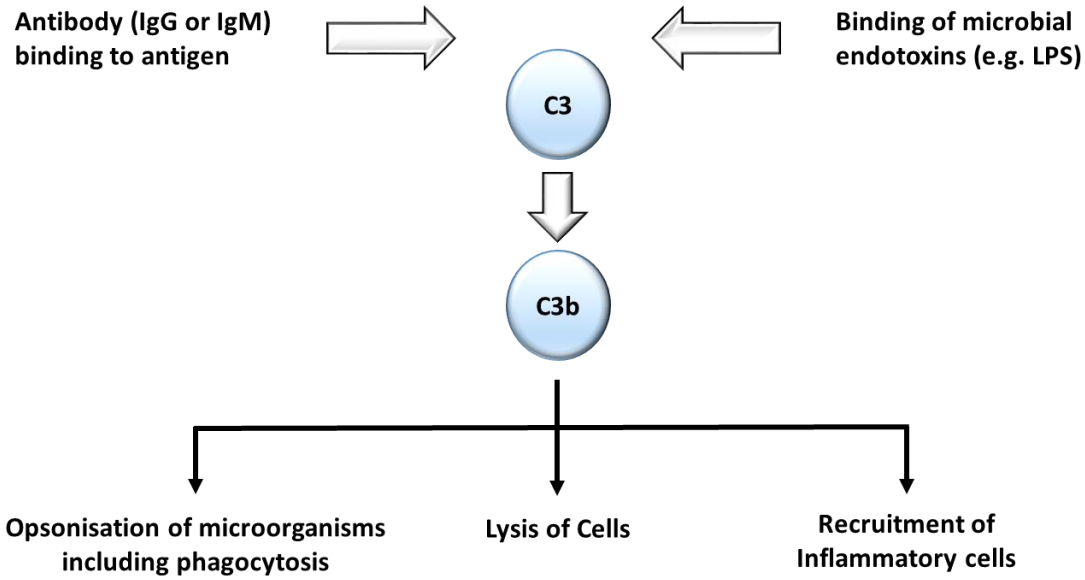

Figure 1.9. Representation of the complement system in host-pathogen interactions. Proteins of the complement system all converge at C3, which is then cleaved into C3b. C3b binds to the pathogen, resulting in its recognition and removal by immune cells, mainly via phagocytosis. Figure drawn based on information in Dumitru *et al*. (2000).

1.3.4.2 The Phagocytosis Process

Phagocytosis is a complex process whereby a phagocyte eliminates or engulfs large cargo 0.5> µm by an actin-dependent mechanism (Mukherjee *et al*., 1997; Aderem and Underhill, 1999). The term "professional phagocytes" was first introduced by Rabinovitch (1995). It includes a specialised subset of host cells such as monocytes, macrophages, neutrophils and dendritic cells (Ginhoux and Guilliams, 2016).

Phagocytosis serves as a system to clear away the cellular and non-cellular materials from the extracellular space. It also removes microorganisms, such as bacteria, via opsonisation (antibody or complement) binding or pathogen-specific receptors (Arandjelovic and Ravichandran, 2015).

Phagocytes are attracted to an infection site via chemotaxis in response to chemokines, cytokines such as IL-8, and bacterial elements such as LPS (Aliprantis *et al*., 1999). The process of phagocytosis begins with recognition of foreign invaders by a phagocytic cell. Microorganisms, such as bacteria, associate with the surface of the phagocyte resulting in actin polymerisation (Freeman and Grinstein, 2016; Garcia-Gomez *et al*., 2016), arrangement of the actin cytoskeleton followed by engulfment, phagosome and phagolysosome formation. Bacterial destruction is mediated by lysosomal enzymes and ROS (Pauwels *et al*., 2017).

Many receptors contribute to phagocytosis with many particles being detected by several receptors such as antibody receptors (e.g. Fc receptor) and/or complement receptors (CR), in addition to the dual function of some receptors in internalisation and adhesion (Freeman and Grinstein, 2014; Freeman and Grinstein, 2016). It is important that immune cells can distinguish harmful invaders from self (Randow *et al*., 2013), which is achieved through recognition of pathogen-associated particulates by phagocytic receptors (Santoni *et al*., 2015; Silva-Gomes *et al*., 2016). These particulates are recognised by pattern recognition receptors (PRRs) on the membrane of phagocytes (Broz and Monack, 2013; Fukata and Arditi, 2013). Pathogen-associated molecular patterns (PAMPs) that are recognised by PRRs include lipopolysaccharides (LPS) from Gram-negative bacteria, lipoteichoic acid (LTA) from Gram-positive bacteria, mannans from yeast and formulated bacterial peptides (Bæk *et al*., 2016; Galinari *et al*., 2017; Lam *et al*., 2017; Pauwels *et al*., 2017).

When the innate system is functioning appropriately, the eradication of bacteria and pathogens occurs quickly but when the system is compromised by host comorbidities, bacteria can become difficult to eradicate. After eliminating an invading pathogen, the immune system memorises the pathogen and develops

long-term defences against it, known as adaptive immunity (acquired immune response) (discussed in 1.1.2) (Wolcott *et al*., 2008).

1.3.4.3 Monocytes/Macrophages

Monocytes are cells that circulate in the blood, spleen, and bone marrow, and represent around 10% of the entire population of human leukocytes (Geissmann *et al*., 2010). They are the biggest white blood cells, and have distinctive morphological structures, such as irregular cell shape, a folded or kidney-shaped nucleus, and cytoplasmic vesicles. Monocytes remain circulating in the bloodstream for about 1 to 3 days before moving into tissues, where they differentiate into macrophages or dendritic cells. If they are not recruited into tissues by a stimulus, they die and are removed by other immune cells. Monocytes are created in the bone marrow from hematopoietic stem cells (HSCs) and mature over successive differentiation phases: the common myeloid progenitor (CMP) (Akashi *et al*., 2000), the granulocyte-macrophage progenitor (GMP) (Akashi *et al*., 2000), the macrophage and dendritic cell precursors (MDP) (Fogg *et al*., 2006), and the committed monocyte progenitor (cMoP) (Hettinger *et al*., 2013; Italiani and Boraschi, 2014). Monocytes are considered to be the main reservoir of myeloid precursors for the regeneration of tissue macrophages and dendritic cells. However, some subpopulations of macrophages and dendritic cells (for instance skin Langerhans cells (LC), lymphoid organ dendritic cells and brain microglia) are generated originally from the MDP instead of monocytes (Liu *et al*., 2009; Boltjes and Van Wijk, 2014), and in some circumstances, they can be directly generated from the bone marrow (Ajami *et al*., 2007).

Human peripheral blood monocytes are a heterogeneous population of cells (Passlick *et al*., 1989; Italiani and Boraschi, 2014). Recent studies have indicated that human monocytes are divided into three functional subsets (Italiani and Boraschi, 2014). The characterisation of these subsets is under investigation since it remains largely unclear, as do the exact functions they play in inflammation *in vivo*, in comparison with activated macrophages (see below).

Macrophages are heterogeneous phagocytic cells that reside in lymphoid and non-lymphoid tissues of all adult humans and animals. When they are inactive (under normal healthy conditions), they represent up to 15 % of the entire number of immune cells. However, this percentage becomes higher in reaction to inflammatory stimuli. Depending on their tissue location, macrophages have various designations such as alveolar macrophages (lung), osteoclasts (bone), Küpffer cells (liver), histiocytes (connective tissue), and skin Langerhans cells (skin) (Gautier *et al*., 2012). Macrophages exert the same functions in all tissues. They are crucial in the immune response to pathogens (by producing and directing the inflammatory response), in tissue development, in monitoring tissue changes, and in the maintenance of steady-state tissue homeostasis (by elimination of apoptotic cells, by remodelling and repairing tissues, and by the production of growth factors). Macrophages are equipped with a broad range of pathogen-recognition receptors that make them efficient at phagocytosis, and they induce the production of inflammatory cytokines (Lin *et al*., 2008).

Macrophages are characterised by the ability to engulf and digest cellular debris, microbes, cancer cells, and any other substance that does not express on its surface the normal, healthy-cell specific proteins, via a process known as phagocytosis (Duque and Descoteaux, 2015). In addition to phagocytosis, macrophages play a crucial role in both innate (non-specific) and adaptive (specific) immunity as they have the capacity to present antigens to other immune cells, such as T cells, to release cytokine (pro-inflammatory/anti-inflammatory mediators) and to move in response to chemokines in a process called chemotaxis (Duque and Descoteaux, 2015). Macrophages are also crucial for the secretion of growth factors and cytokines. Growth factors and cytokines cause the attraction and division of cells involved in tissue repair (Werner and Grose, 2003), leading to angiogenesis, re-epithelialisation of wounds, and the formation of new extracellular matrix (Greenhalgh, 1998; Stashak *et al*., 2004).

Evidence has shown that there are numerous, activated forms of macrophages (Mosser and Edwards, 2010). There are two subtypes of macrophages, designated as M1 and M2. M1 macrophages encourage inflammation through the secretion of

pro-inflammatory cytokines whereas M2 macrophages reduce inflammation and encourage tissue repair (Mills, 2012). Lipopolysaccharide (LPS) and interferon-gamma (IFN-γ) are typically present in infected wounds and lead to the generation of M1 macrophages. In contrast, M2 macrophages that stimulate tissue formation (e.g. during wound healing), are promoted by factors such as by interleukin-4 (IL-4) (Biswas and Mantovani, 2010; Sica and Mantovani, 2012).

Table 1-2. Main functions of tissue resident macrophages.

Apart from tissue-specific roles, tissue macrophages exert a series of common roles including removal of cellular debris, immune surveillance, wound repair, defence against microorganisms including bacteria, and the origination and resolution of inflammation. Figure adapted from Italiani and Boraschi (2014).

Tissue Macrophages	Tissue location	Functions
Heart macrophages	Heart	Surveillance.
Osteoclasts	Bone	Bone modelling and remodelling, support to haematopoiesis (Pollard, 2009; Blin-Wakkach *et al*., 2014).
Microglia	Brain	Brain progress, immune surveillance, synaptic remodelling (Paolicelli *et al*., 2011; London *et al*., 2013).
Alveolar macrophages	Lung	Clearance of surfactant and inhaled pathogens (Maus *et al*., 2002).
Intestinal macrophages	Gut	Tolerance to microbiota, defence against pathogens, intestinal haemostasis (Zigmond and Jung, 2013).
Bone marrow macrophages	Bone marrow	Reservoir of monocytes, waste removal (Davies *et al*., 2013).
Langerhans cells	Skin	Immune surveillance.
Küpffer cells	Liver	Toxin elimination, lipid metabolism, erythrocyte, microbes' clearance, and cell debris clearance from blood (Kle (Klein *et al*., 2007; Ganz, 2012).
Adipose tissue associated macrophages	Adipose tissue	Metabolism, adipogenesis, adaptive thermogenesis (Nguyen *et al*., 2011).
Marginal zone, red pulp macrophages	Spleen	Erythrocyte clearance, iron recycling, uptake of microorganisms from blood (Den Haan and Kraal, 2012).
Inflammatory macrophages	All tissues	Protection against pathogens and harmful stimuli (Labonte *et al*., 2014).
Healing macrophages	All tissues	Angiogenesis, branched morphology (Mantovani *et al*., 2013).

U937 monocytes are an eternal human cell line commonly used to generate macrophage-like cells for *in vitro* studies. U937 cells exhibit characteristics of immature monocytes and, after conversion into macrophages, these cells display similar features and characteristics to human tissue macrophages (Minafra *et al*., 2011). Phorbol 12-Myristate 13-Acetate (PMA), also known as TPA, is a phorbol ester used to transform monocytes into macrophage-like cells *in vitro*. PMA-treated U937 cells express high levels of CD11b, CD11c and protein kinase-C (PKC) (Sintiprungrat *et al*., 2010). When treated with PMA, U937 monocytes upregulate their cell adhesion molecules, such us beta-2 integrins (CD11a, CD11c, CD18 and CD11b) allowing the cells to attach to cell culture flask surfaces. PMA triggers calcium and phospholipid-dependent isoforms of PKC and encourages cyclic AMP metabolism, initiating maturation into a macrophage-like morphology (Luscinskas *et al*., 1994).

1.4 Estrogen

Estrogens are a group of sex steroid hormones which were first discovered by Edgar Allen and Edward Doisy in 1923 (MacCorquodale *et al*., 1936). In humans, three main forms of estrogen are described; estradiol, estrone and estriol with 17-β-estradiol being the most potent and biologically active form. Estrogen is involved in several physiological roles such as the regulation of ovulation and fertility, development of female secondary sexual characteristics, synthesis of lipoproteins, regulation of insulin sensitivity, maintenance of bone mass, and conservation of cell growth and cognitive function (Cagnacci *et al*., 1992). Estrogen is also important in males as it is involved in spermatogenesis (Gladen *et al*., 1996; Miura *et al*., 1999; O'donnell *et al*., 2001), maintaining the density of bone (Stěpán *et al*., 1989; Daniell, 1997) and cardiovascular health (Group, 1973; Eriksson *et al*., 1989). Evidence has suggested that estrogen deficit is a key regulator of delayed wound healing in the elderly (Ashcroft *et al*., 1997b; Ashcroft and Ashworth, 2003). Estrogen has protective anti-inflammatory proprieties in several tissues, such as the brain (Straub, 2007). Despite the anti-inflammatory effect of estrogen on the skin, mediated via macrophage migration inhibitory factor (MIF) (Hardman *et al*., 2005), estrogen has been reported to stimulate wound repair independently from its anti-

inflammatory effects in both genders. Systemic and peripheral levels of estrogen decline with increasing age in both males and females (with this diminution highly noticeable in post-menopausal females) (Ashcroft *et al*., 1997b). HRT-treated post-menopausal females heal acute wounds faster than their age-matched control counterparts, who take no estrogen supplementation (Ashcroft *et al*., 1997b). Another study indicated that topical estrogen supplementation enhanced wound healing in elderly male and female patients, connected with a reduced inflammatory response (Ashcroft *et al*., 1997b; Ashcroft *et al*., 1999b). Accordingly, these findings collectively indicate that estrogen has notably beneficial effects on wound healing.

1.4.1 Estrogen Biosynthesis

Estrogens are produced from cholesterol (Figure 1.10). Cholesterol is converted by several enzymes to androgens, such as testosterone and androstenedione, which are then converted to estrogens through the action of the P450 enzyme aromatase, in the endoplasmic reticulum of estrogen-producing cells (Payne and Hales, 2004). In adipose tissues, androstendione is converted to estrone whilst in ovarian granulosa cells testosterone is converted into estradiol. Aromatase is found in many peripheral tissues such as bone (Nawata *et al*., 1995), adipose tissue (Simpson, 2000), brain (Azcoitia *et al*., 2001) and vascular smooth muscle (Ling *et al*., 2004). In females at the age of reproduction, systemic estrogen is produced mainly by the ovary. It is fundamentally biosynthesised in the granulose cells of the ovarian follicles and the corpora lutea. In males, the gonad produces the largest quantity of systemic estrogen. However, a substantial amount of estrogen is also produced locally in peripheral tissues, acting in an autocrine and paracrine manner (Labrie *et al*., 1998). A substantial amount of inactive steroid precursors including dehydroepiandrosterone (DHEA), its sulphate (DHEA-S), and androstenedione (4-dione) are produced by the adrenals and converted into active steroid hormones in peripheral tissues (Labrie *et al*., 1998). Several peripheral human tissues, such as adipose tissue, bone and skin can produce active estrogens and androgens locally from conversion of adrenal-derived precursors (Nelson and Bulun, 2001). The reduction in the formation of DHEA-S by the adrenals during aging results in a

dramatic fall in the formation of androgens and estrogens in peripheral target tissues, a situation which could be associated with age-related diseases (Labrie *et al*., 1998).

Estrogen is synthesised locally in both young and old females and becomes progressively more important after postmenopause, when systemic levels are lost. After the menopause, estrogen biosynthesis changes from a systemic source produced by the ovaries, to a local source, synthesised in peripheral tissues (Picard *et al*., 2000). However, the rapid decline in the local production of estrogens with increasing age means peripheral estrogen is still insufficient to compensate for the loss in systemic estrogen levels.

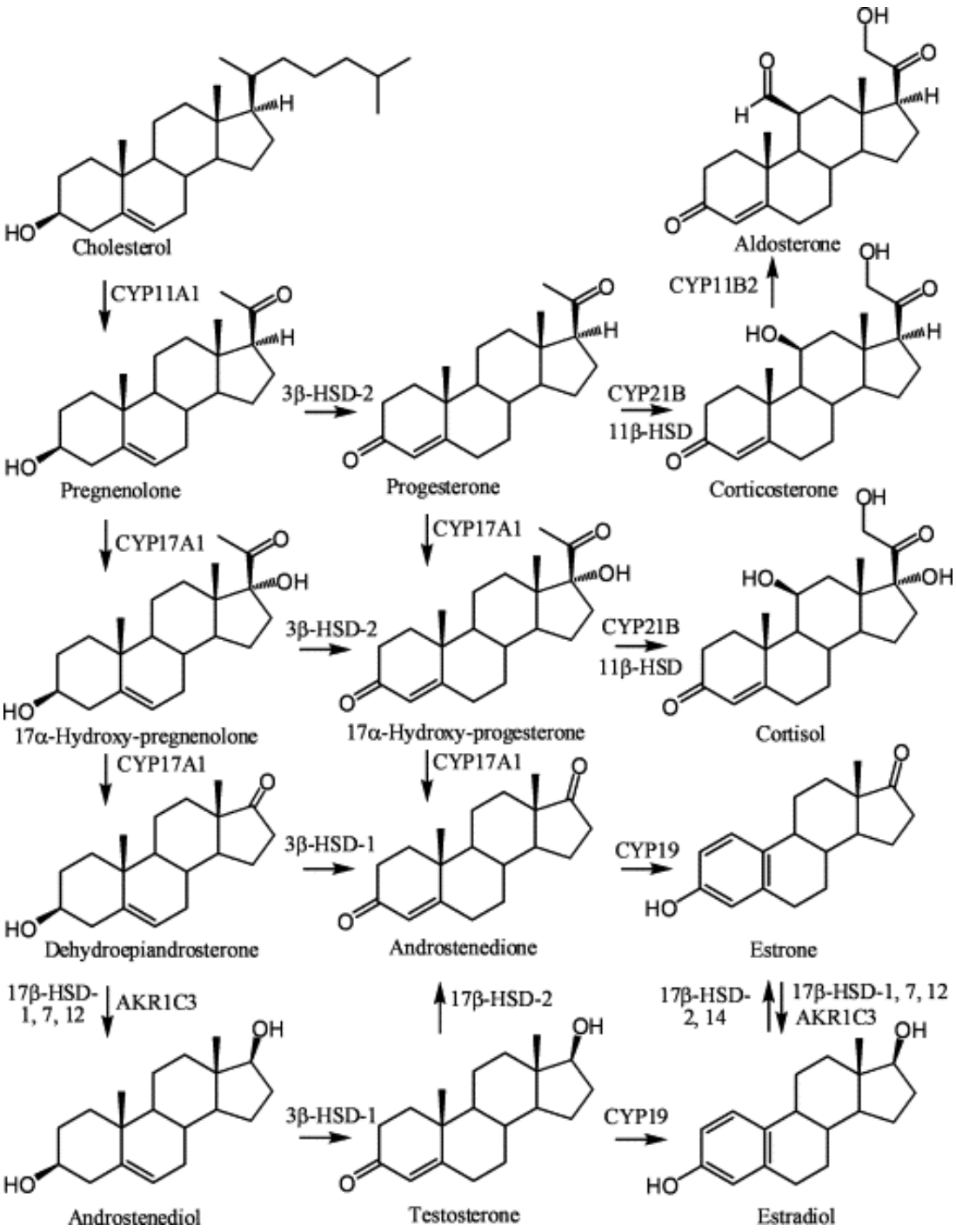

Figure 1.10. Estrogen biosynthesis from cholesterol and androgen precursors. CYP = Cytochrome P450, HSD = Hydroxysteroid dehydrogenase and AKR = Aldo-keto reductase enable hormone synthesis. Figure adapted from Blair (2010).

1.4.2 Estrogen Receptors

Estrogen has multiple functions, such as the regulation of haemostasis, inflammation, cell growth and differentiation. Over the past decades, the existence of two nuclear and membrane-bound estrogen receptor (ER) proteins have been

identified: ER-alpha (ER-α) and ER-beta (ER-β). ER-α was first discovered in 1958 (Jensen and Jacobson, 1960), whereas ER-β was first identified in rat prostate and ovary in 1996 (Mosselman *et al*., 1996). The biological effects of estrogens are mediated by the binding of estrogen to ER homodimers or heterodimers (Matthews and Gustafsson, 2003), and subsequent activation or repression of gene transcription and signalling pathways, including ligand-dependent/independent pathways (Paige *et al*., 1999), estrogen response element (ERE)-independent pathways and non-genomic pathways. The ligand-dependent pathway is the central pathway. Interestingly, estrogen signalling can directly target specific ER genes and upregulate the expression of mRNA (Gruber *et al*., 2002; Ascenzi *et al*., 2006). Recent research also suggests estrogen could have a direct influence on inflammatory cells, such as monocytes and macrophages, and other cells in the human skin such as keratinocytes, due to the nuclear and membrane-bound localisation of ER proteins. (Weusten *et al*., 1986; Stimson, 1988; Cocchiara *et al*., 1990). The response of these particular inflammatory cells depends on the local levels of estrogen and the maturity (stage of differentiation) of the cells (Ashcroft and Ashworth, 2003).

1.4.3 Effect of Estrogen on Skin

It is commonly accepted that the age-related reduction in estrogen levels is linked with skin degeneration. Estrogen deficiency results in detrimental effects on skin's appearance (dry fragile skin, sagging, wrinkling) with decreased thickness and collagen(Ashcroft *et al*., 1999b; Shah and Maibach, 2001)**.** Estrogen has been shown to have an opposite effect, improving collagen quality, improving skin thickness and augmenting vascularisation. The link between menopause and wound healing has been investigated where it was reported that systemic estrogen supplementation results in an intensification of collagen deposition in post-menopausal women (Savvas *et al*., 1993; Ashcroft *et al*., 1997b), whereas topical estrogen increases collagen I and III deposition in the skin of postmenopausal women, conserving skin thickness (Savvas *et al*., 1993; Sauerbronn *et al*., 2000). Another study on post-menopausal women reported that there was a reduction in collagen type I and mainly type III in the skin of post-menopausal women compared to pre-menopausal

women, and a decrease in type III/type I ratio within the dermis. This alteration is predominantly associated with estrogen deficiency (Affinito *et al*., 1999; Horng *et al*., 2017). It has also been reported that estrogen replacement therapy can improve skin elasticity by 5% per year (Brincat *et al*., 1987). When locally applied on the skin of post-menopausal women, estradiol significantly increased the production of hydroxyproline and collagen fibres (Albright *et al*., 1941; Affinito *et al*., 1999; Sator *et al*., 2001; Horng *et al*., 2017)**.**

Additionally, topical estrogen improves the fibres elasticity in the dermis (Albright *et al*., 1941; Sator *et al*., 2001). Estrogen also augments the deposition of glycosaminoglycans in the ECM, enhances skin turgor and water retention, stimulates keratinocytes and inhibits matrix metalloproteinase (MMP) synthesis (Brincat, 2000).

1.4.4 Estrogen and Wound Healing

Recent research has demonstrated the key role of sex-steroid hormones in inflammation and wound healing process (Guo and DiPietro, 2010; Gilliver *et al*., 2007). This is particularly observed in age-related impaired wound healing research where there is an important impact of these hormones on the inflammatory response *in vivo*.

The impact of estrogen on wound healing was first studied in animals in 1947 (Sjövall, 1947; Horng *et al*., 2017) and then in humans in 1953 (Sjöstedt, 1953; Horng *et al*., 2017). Afterwards, there has been an accumulating body of evidence supporting that estrogen has a key impact on wound healing (Brincat *et al*., 1987; Varila *et al*., 1995) (Affinito *et al*., 1999; Sauerbronn *et al*., 2000; Mills *et al*., 2005; Hardman and Ashcroft, 2008; Brufani *et al*., 2009; W.-L. Lee *et al*., 2013; Midgley *et al*., 2016; Mukai *et al*., 2016; Chenu *et al*., 2017; Leblanc *et al*., 2017; Horng *et al*., 2017; Pepe *et al*., 2017; Wilkinson and Hardman, 2017). It has been indicated that estrogen deficiency contributes to cutaneous aging and delayed and/or impaired wound healing (Mukai *et al*., 2016; Wilkinson and Hardman, 2017). It has also been reported that estrogen replacement therapy could improve the skin elasticity by 5% in one year (Brincat *et al*., 1987). When locally applied on the skin of

postmenopausal women, estradiol significantly increases the production of hydroxyproline and collagen fibres (Affinito *et al*., 1999; Horng *et al*., 2017). Similar effects on the skin and collagen fibres were observed with systemic oral estrogen replacement therapy (Sauerbronn *et al*., 2000).

Variance in the human immune system between male and female subjects have been identified in some epidemiological and medical studies (McGowan *et al*., 1975; Bone, 1992), with evidence indicating women have an improved immune system compared to men (Gulshan *et al*., 1990; Wichmann *et al*., 1996). This enhanced immune system in women is attributed to the hormonal regulation of the immune system. Other experiments have indicated that estrogen has an immune-improving impact during stress and by contributing resistance to several pathogenic infections (Yamamoto, 1999). Ashcroft *et al*. (1997b) have reported that both systemic and topical estrogen treatments enhance wound healing in elderly men and women. Estrogen has been shown to accelerate re-epithelialisation, encourage angiogenesis, promote matrix deposition and wound contraction, dampen the inflammatory response and the inhibit expression of pro-inflammatory cytokines and proteolytic mediators (Ashcroft and Ashworth, 2003).

1.4.4.1 Effect of Estrogen on the Inflammatory Phase of Wound healing

It is commonly known that age-related impaired healing is associated with an excessive and prolonged inflammatory response, linked with increased inflammatory cell migration and adhesion, and increased pro-inflammatory cytokines such as TNFα (Ashcroft and Ashworth, 2003). Recent research has indicated that chronic wounds are associated with elevated levels of elastase and MMPs, which are released by neutrophils/macrophages and linked with extreme tissue destruction (Gosain and DiPietro, 2004). Estrogen controls and dampens the early inflammatory response during acute wound healing by inhibiting neutrophil infiltration to the wound by reducing the expression of neutrophil adhesion molecules (Ashcroft *et al*., 1999b). Estrogen causes a decrease in tissue-damaging proteases levels, such as elastase, and an increase in the content of collagen and fibronectin in the dermis (Ashcroft *et al*., 1999b). Furthermore, estrogen increases

the oxidative metabolism of neutrophils, suggesting estrogen deprivation should lead to diminished phagocytic capability of neutrophils, an increased risk of infection and a postponement in healing (Ashcroft and Ashworth, 2003). Estrogen is also believed to have a direct influence on monocytes and macrophages, due to their possession of nuclear and membrane-bound ER (Weusten *et al*., 1986; Suenaga *et al*., 1996; Suenaga *et al*., 1998). Macrophage migration inhibitory factor (MIF) is a pro-inflammatory cytokine released by monocytes, macrophages, neutrophils, endothelial cells and keratinocytes. This cytokine is known to be a major regulator of the effects of estrogen on wound healing (Gilliver *et al*., 2010). Ashcroft *et al*. (2003) reported that mice with estrogen deficiency have higher MIF levels, resulting in an elevated inflammatory response and delayed wound healing, whereas MIF null-mice displayed enhanced wound healing, with lower inflammation and greater matrix formation. Estrogen causes a down-regulation of MIF expression leading to a decline in inflammation, enhanced matrix deposition, increased re-epithelialisation and an overall accelerated wound repair (Ashcroft and Ashworth, 2003; Ashcroft *et al*., 2003).

1.4.4.2 Effect of Estrogen on the Proliferative Phase of Wound Healing

Estrogen improves the proliferative phase of wound healing by enhancing re-epithelisation, wound contraction, granulation tissue construction and angiogenesis. Estrogen enhances the mitogenesis of keratinocytes and increases the speed of re-epithelialisation and wound contraction in post-menopausal women (Ashcroft *et al*., 1997b). The rate of wound re-epithelialisation of post-menopausal women treated with HRT for more than 3 months was similar to levels of re-epithelialisation in pre-menopausal females, whereas a non-HRT post-menopausal group showed diminished re-epithelialisation. One feature of this improved re-epithelialisation following estrogen supplementation is due to increased proliferation of epidermal keratinocytes (Raja *et al*., 2007). Estrogen promotes PDGF expression by monocytes and macrophages (Mendelsohn and Karas, 1999), leading to mitogenesis and chemotaxis of fibroblasts and a subsequent increase in wound contraction and ECM deposition (Seppä *et al*., 1982). Estrogen also enhances fibrosis by increasing wound TGF-β1 levels (Ashcroft *et al*.,

1997b; Ashcroft *et al*., 1999b), resulting in enhanced formation of ECM, particularly collagen deposition (Ashcroft and Ashworth, 2003). Estrogen promotes angiogenesis, leading to increased granulation tissue (Iyer *et al*., 2012) through a direct stimulation of endothelial cells (Rubanyi *et al*., 2002). Estrogen increases endothelial cell attachment to laminin, collagen I and IV, laminin, and fibronectin. In addition, estrogen enhances the creation of capillary-like structures by endothelial cells, when positioned on a reconstructed basement membrane (Morales *et al*., 1995).

1.4.4.3 Effect of Estrogen on the Remodelling Phase of Acute Wound Healing

Estrogen improves wound remodelling by controlling the degradation of collagen through wound proteases. Topical estrogen supplementation increases the deposition of collagen in during the remodelling phase of wound repair in elderly patients (Ashcroft *et al*., 1997b; Ashcroft *et al*., 1999b). Estrogen also acts by stimulating the expression of TGF-β1 to improve collagen deposition in the dermis (Ashcroft *et al*., 1997b; Ashcroft *et al*., 1999b). Matrix collagen deposition at 7 and 84 days post-wounding was decreased in post-menopausal women lacking HRT treatment. In contrast, post-menopausal females who took HRT for more than 3 months had similar levels of matrix collagen deposition and wound remodelling as younger pre-menopausal females (Ashcroft *et al*., 1997b; Ashcroft *et al*., 1999b).

1.4.5 Estrogen and Chronic Wounds

Since systemic estrogens decline with age, it has been suggested that estrogen deprivation in the elderly could possibly be linked with pathological wound healing. Margolis *et al*. (2002) performed a case-cohort study to investigate the protective effects estrogen on chronic wounds. Patients aged oved 65 years receiving HRT treatment were shown to be 30-40% less likely to develop a venous leg ulcer than age-matched patients lacking HRT supplementation (Margolis *et al*., 2002). Chronic wounds are characterised by an excessive and chronic prolonged inflammation. High levels of inflammatory mediators, including TNFα, TGFβ, IL-1β, IL-6, IGF-1 and MMPs, present in chronic wound exudate (Ashcroft *et al*., 1997b; Ashcroft *et al*.,

1999b) are downregulated via the action of estrogen (Straub, 2007; Wira *et al*., 2015).

1.5 Aim and Objectives

Despite its anti-inflammatory activity in wound healing, the effect of estrogen on bacterial clearance from acute or chronic wounds remains largely unknown. Moreover, it is not known whether estrogen deprivation in the elderly exacerbates chronic wound infections. However, there is a vital need to develop novel therapeutic strategies to treat bacterial wound infections in the elderly and one exciting approach is to enhance innate host responses so the immune system is better able to eradicate potential pathogens. Thus, the aim of this study was to determine the effects of estrogen on the phagocytic function of macrophages using *in vitro* and *ex vivo* host-pathogen models of the inflammatory phase of age-related impaired healing.

Specific objectives of this study were to:

- Generate an *in vitro* model of HPBM and U937 monocyte differentiation into M0-like, M1-like and M2-like macrophages.

- Determine the effect of estrogen on the phagocytosis of Gram-positive (MRSA) and Gram-negative (*P. aeruginosa*) wound pathogens by U937- and HPBM-derived macrophages.

- Compare the effect of age-related changes in estrogen levels on the phagocytic activity of M0-like, M1-like and M2-like macrophages against Gram-positive and Gram-negative bacteria.

- Explore the role of ER-α and ER-β in mediating the effects of estrogen on the phagocytosis of bacteria by pro-inflammatory M1-like macrophages.

- Investigate the effect of estrogen on downstream mediators of phagocytosis including regulating protein levels of FAK, Rac1, RhoA, Cdc42 and RhoG in pro-inflammatory M1-like macrophages.

Chapter 2: Materials and Methods

2.1 Materials

Alexa Fluor 647-conjugated anti-human MMR/CD206 (BioLegend, UK)

Bicinchoninic acid assay reagent (Sigma-Aldrich, UK)

Bovine serum albumin (Fisher Scientific, UK)

Brain heart infusion agar (Oxoid, UK)

Brain heart infusion broth (Oxoid, UK)

Chloramphenicol (Sigma-Aldrich, UK)

Diarylpropionitrile (Sigma-Aldrich, UK)

Dimethylsulphoxide (Sigma-Aldrich, UK)

Dulbecco's phosphate-buffered saline (Sigma-Aldrich, UK)

Estrogen (Sigma-Aldrich, UK)

Ethanol (Fisher Scientific, UK)

Ethylenediaminetetraacetic acid (Fisher Scientific, UK)

Ethylene glycol-bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid (Fisher Scientific, UK)

EZ-ECL chemiluminescence detection kit (Biological Industries IBH Ltd, USA)

Fluorescein isothiocyanate (FITC)-conjugated anti-human CD11c antibody (Clone Bu15; BioLegend, UK)

Fluorescein isothiocyanate (FITC)-conjugated anti-human CD197 (CCR7) antibody (BioLegend, UK)

Foetal bovine serum (Lonza, UK)

Fulvestrant (Sigma-Aldrich, UK)

Gentamicin (Sigma-Aldrich, UK)

Glutaraldehyde (Sigma-Aldrich, UK)

Green fluorescent protein (GFP)-*S. aureus* strain SH1000 (Kind gift from Paul Williams, Professor of Molecular Microbiology, Centre for Biomolecular Sciences, Faculty of Medicine & Health Sciences, University of Nottingham, UK)

Human blood cell culture medium (Cell Applications Inc, San Diego, USA)

Human peripheral blood $CD14^+$ monocytes (Cell Applications Inc, San Diego, USA)

Hydrogen chloride (Thermo Fisher Scientific, UK)

Interferon gamma (Sigma-Aldrich, UK)

Interleukin-4 (Sigma-Aldrich, UK)

Interleukin-13 (Sigma-Aldrich, UK)

Lipopolysaccharide from the membrane of *Escherichia coli* (Sigma-Aldrich, UK)

MCherry- *P. aeruginosa* strain PAO1 (Kind gift from Paul Williams, Professor of Molecular Microbiology, Centre for Biomolecular Sciences, Faculty of Medicine & Health Sciences, University of Nottingham, UK)

Methanol (Thermo Fisher Scientific, UK)

Methicillin Resistant *Staphylococcus aureus* strain 11 (Hospital isolates, Manchester, UK)

Mouse primary antibody to Cdc42 (Abcam, UK)

Mouse primary antibody to Cyclophilin (Abcam, UK)

Mouse primary antibody to Fak (Abcam, UK)

Mouse primary antibody to Rac1 (Abcam, UK)

Mouse primary antibody to RhoA (Abcam, UK)

Mouse primary antibody to β-actin (Abcam, UK)

Mouse primary antibody to β-tubulin (Abcam, UK)

Nitrocellulose membrane (GE Healthcare Life Sciences, UK)

Nutrient agar (Oxoid, UK)

Nutrient broth (Oxoid, UK)

Paraformaldehyde (Sigma-Aldrich, UK)

Penicillin – streptomycin (Lonza, UK)

Peroxidase-HRP anti-mouse IgG (produced in A9044 rabbit) (Sigma-Aldrich, UK)

Peroxidase-HRP anti-rabbit IgG (produced in goat) (Sigma-Aldrich, UK)

pH meter (Hanna Instruments, UK)

Phalloidin–tetramethylrhodamine B isothiocyanate conjugate from *Amanita phalloides* (Fluka, Germany)

Phorbol 12-myristate 13-acetate (Applichem, Germany)

Propylpyrazoletriol (Sigma-Aldrich, UK)

Protease inhibitor cocktails (Sigma-Aldrich, UK)

Pseudomonas aeruginosa strain PAO1 (Hospital isolates, Manchester, UK)

Pyrazole triol (Sigma-Aldrich, UK)

Rabbit primary antibody to RhoG (Abcam, UK)

Roswell Park Memorial Institute Medium (RPMI) (Lonza, UK)

Saline tablets (Sigma-Aldrich, UK)

Skimmed milk powder (Premier Foods Group, UK)

Sodium chloride (Thermo Fisher Scientific, UK)

Sodium deoxycholate, phenylmethylsulfonyl fluoride (Thermo Fisher Scientific, UK)

Sodium dodecyl sulfate (Sigma-Aldrich, UK)

Sodium hydroxide (Fisher Scientific, UK)

Tamoxifen (Sigma-Aldrich, UK)

Tetracycline (Sigma-Aldrich, UK)

Theophylline, 8-[(benzylthio)methyl]-(7CI,8CI) (Sigma-Aldrich, UK)

Trypan blue (Lonza, UK)

Trypsin EDTA (Lonza, Belgium)

Tris base (Fisher Scientific, UK)

Tris hydrochloride (Fisher Scientific, UK)

Tris (hydroxymethyl) aminomethane (THAM) hydrochloride (Fisher Scientific, UK)

Triton X-100 (Lonza, Belgium)

Tween-20 (Fisher Scientific, UK)

U937 Human monocytic cell line (Health Protection Agency Culture Collections, UK)

2.2 Methods

2.2.1 Preparation of RPMI-1640 Complete Medium

Foetal bovine serum (FBS) was heat-inactivated in a water bath maintained at 56 °C for 30 minutes. Roswell Park Memorial Institute (RPMI)-1640 (L-Glutamine, 25 mM HEPES) supplemented with 10% FBS and 100 I.U./mL penicillin-streptomycin was prepared under aseptic conditions and referred to as RPMI-1640 complete medium throughout the study.

2.2.2 Estrogen Treatment Preparation

Estrogen (17β-estradiol) was prepared to final concentrations of 1 x 10^{-7} M, 1 x 10^{-8} M, 1 x 10^{-9} M and zero M in antibiotic-free RPMI-1640 complete medium or human blood cell culture medium (HBCCM). Concentrations of estrogen were chosen according the previously published models of ageing to represent physiological levels of estrogen (1 x 10^{-8} M) typical of young human adults, supraphysiological levels of estrogen (1 x 10^{-7} M) to model estrogen supplemention, estrogen deprivation (1 x 10^{-9} M) to represent declining levels found in elderly humans and an absolute absence of estrogen (zero M) (Ashcroft and Ashworth, 2003; Hardman *et al*., 2005; Sproston *et al*., 2018).

2.2.3 Cell Culture

U937 monocytes were cultured under aseptic conditions at 37 °C and 5% CO_2 using RPMI-1640 complete medium. $CD14^+$ human peripheral blood monocytes (HPBM) were cultured in HBCCM under aseptic conditions at 37 °C and 5% CO_2. The U937 and HPBM cell suspensions were centrifuged at 500 *g* for 7 minutes before the medium was aspirated. U937 monocytes and HPBM were maintained at 0.5 x 10^6 cells/mL and 1 x 10^6 cells/mL respectively by resuspension in fresh medium every other day. Sterile filtered 0.4% trypan blue dye was used to check cell viability according to published methodologies (Mir *et al*., 1991) using a 1:1 ratio of cell suspension to trypan blue and counting the number of non-viable cells that took up

the blue dye using a TC10 automated cell counter (Bio-Rad, USA). The viability of the cells was above 80% for all experiments.

2.2.4 Differentiation of Monocytes into M0 Macrophages

HPBM and U937 monocytes at a concentration of 1 x 10^6 viable cells/mL were differentiated into macrophage-like cells by incubating with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) in RPMI complete medium for 24 hours at 37 °C and 5% CO_2. Cells were washed twice with complete medium and then incubated for a further 48 hours in PMA-free complete medium to obtain resting macrophages known as M0 macrophages (Daigneault *et al*., 2010; Rios de la Rosa *et al*., 2017).

2.2.5 Generation of M1 and M2 Macrophages

U937 monocytes and HPBM were cultured as described in 2.2.3. Cells were differentiated into resting M0 macrophages as described in 2.2.4. The medium was aspirated and the cells washed twice with complete medium. M1 and M2 macrophages were generated using the method adapted from Rios de la Rosa *et al*. (2017). For M1 differentiation/polarisation, M0 macrophages were incubated with 100 ng/mL lipopolysaccharide (LPS) and 20 ng/mL interferon gamma (IFN-γ) in PMA-free complete medium for 24 hours at 37 °C and 5% CO_2. The adherent cells were washed twice with LPS/IFN-γ-free complete medium and then incubated with complete cell culture medium for 4 days at 37 °C and 5% CO_2, with medium changes every 48 hours to obtain classically activated resting M1 macrophages. For M2 differentiation/polarization, M0 macrophages were incubated for 24 hours with 20 ng/mL interleukin-4 (IL-4) and 20 ng/mL interleukin-13 (IL-13) in complete medium. The adherent cells were washed twice with IL-4/IL-13-free complete medium prior to incubation in complete medium for 4 days, with medium changes every 48 hours to obtain alternatively activated resting M2 macrophages.

2.2.6 Flow Cytometry

2.2.6.1 Confirming the Differentiation of Monocytes into M0 Macrophages

Differentiation of U937 and HPBM monocytes into M0 macrophages was confirmed by flow cytometry via analysis of $CD11c^+$ surface marker expression. Monocytes and M0 macrophages were seeded in 1 mL of RPMI/HBCCM complete medium at a density of 1×10^6 cells/mL in 12-well plates according to methods described in 2.2.3 and 2.2.4 respectively. Adherent macrophages were detached from six replicate wells by removing the supernatant and adding 250 µL trypsin EDTA for 3 minutes at 37 °C and 5% CO_2. The trypsin EDTA was neutralised by adding 250 µL RPMI/HBCCM complete cell culture medium before being washed (centrifugation of well constituents at 500 *g* for 7 minutes and resuspension of the cell pellet) in 1 mL complete medium. The monocytes (6 replicate wells) and detached macrophages (6 replicate wells) were then centrifuged at 500 *g* for 7 minutes and supernatants discarded prior to fixing pelleted cells with 200 µL 4% paraformaldehyde in Dulbecco's phosphate-buffered saline (DPBS) for 10 minutes at room temperature (RT). Cells were washed twice in 200 µL DPBS. Half the fixed macrophage samples (n = 3) and half the fixed monocyte samples (n=3) were stained for 30 minutes at RT with FITC-conjugated anti-human CD11c antibody diluted 1:40 with 10% FBS in DPBS. The remaining fixed monocyte (n = 3) and fixed macrophage (n = 3) samples were prepared as unstained negative controls by incubating at RT for 30 minutes with 10% FBS in DPBS lacking antibody. Unbound antibody was removed from samples by two sequential wash steps in DPBS, before resuspension in 500 µL DPBS. CD11c surface marker expression was assessed on 10,000 events (live, individual cells) with a BD Accuri C6F1 cytometer (BD Biosciences, USA). Data were analysed with BD Accuri C6 Software after gating events in the forward scattered channel (FSC)/side scattered channel (SSC) and fluorescence parameter 1 (FL1-A) windows. The average percentage $CD11c^+$ cells (%) and median fluorescence intensity (MFI) were determined relative to unstained negative control U937/HPBM monocytes.

2.2.6.2 Polarisation of M0 Macrophages into M1/M2 Macrophages

Polarisation of U937 and HPBM M0 macrophages into M1/M2 macrophages was confirmed by flow cytometry via analysis of $CD197^+$ and $CD206^+$ surface marker expression respectively. M0 macrophages were seeded in 1 mL of RPMI/HBCCM complete medium at a density of 1×10^6 cells/mL in 12 well plates according to methods described in 2.2.4. M0 macrophages were polarised into M1 (n = 6 replicate wells) and M2 (n = 6 replicate wells) macrophages as described in 2.2.5. Adherent M0/M1/M2 macrophages (n = 6 replicate wells) were detached using trypsin, fixed with 4% paraformaldehyde and washed twice with DPBS as described in 2.2.6.1. Supernatants were discarded and half the M0/M1/M2 samples (n = 3 replicate wells) were stained for 30 minutes at RT with FITC-conjugated anti-human CD197 (CCR7) antibody diluted 1:50 with 10% FBS in DPBS, washed twice with 500 µL DPBS before being incubated with Alexa Fluor 647-conjugated anti-human MMR/CD206 antibody (1:50 with 10% FBS in DPBS) for 30 minutes at RT. The remaining M0/M1/M2 samples (n = 3 replicate wells) were incubated at RT for 1 hour with 10% FBS in DPBS lacking any antibodies (unstained negative controls). Two sequential wash steps were performed in DPBS prior to resuspension in 500 µL DPBS as described in 2.2.6.1. Samples were analysed as described in 2.2.6.1 after gating events in the FSC, SSC, FL1-A and fluorescence parameter 2 (FL2-A) windows. The average percentage $CD197^+/CD206^+$ cells (%) and median fluorescence intensity (MFI) were determined relative to the unstained M0 macrophages.

2.2.7 Bacterial Culture Preparation

Methicillin-resistant *Staphylococcus aureus* (MRSA) strain 11, *Pseudomonas aeruginosa* strain PAO1, green fluorescent protein (GFP) labelled-*S. aureus* strain SH1000WT and mCherry-*P. aeruginosa* strain PAO1 were used in this project. When required, cultures of MRS11 and PAO1 were inoculated onto nutrient agar (NA) plates and incubated at 37 °C for 24 hours. Cultures of GFP-*S. aureus* were inoculated onto brain heart infusion (BHI) agar plates supplemented with 10 µg/mL chloramphenicol and incubated at 37 °C for 24 hours. Cultures of mCherry- *P. aeruginosa* strain PA01 were inoculated onto NA plates supplemented with 1.25

µg/mL tetracycline and incubated at 37 °C for 24 hours. The bacterial inoculated agar plates were stored at 4 °C and replaced every four weeks.

When required for experimental assays, MRSA 11 and *P. aeruginosa* PAO1 were cultured in nutrient broth (NB) overnight at 37 °C on an orbital shaker (New Brunswick Scientific, USA). Ten-fold serial dilutions of each broth were prepared in saline and 100 µL aliquots were inoculated onto duplicate NA plates with subsequent incubation at 37 °C overnight, whilst the broths were refrigerated overnight at 4 °C to inhibit further bacterial growth. The colony-forming units (CFU) counted on the NA plates were used to calculate the density of bacteria (CFU/mL) in each NB, taking into account the dilution factor. The neat broths were centrifuged at 3500 rpm for 10 minutes and the bacterial cell pellets washed twice with saline prior to re-suspension at 2 x 10^6, 2 x 10^5, and 2 x 10^4 CFU/mL in saline.

GFP-*S. aureus* strain SH1000WT was cultured in BHI broth supplemented with 10 µg/mL chloramphenicol and incubated at 37 °C overnight on an orbital shaker. Ten-fold serial dilutions of the broth were prepared in saline and 100 µL aliquots of each dilution were inoculated onto duplicate BHI agar plates supplemented with 10 µg/mL chloramphenicol with subsequent incubation at 37 °C overnight. The neat broth was refrigerated overnight at 4 °C to inhibit further bacterial growth. The CFU counted on the agar plates were used to calculate the density of bacteria (CFU/mL) in the neat broth, taking into account the dilution factor. The bacterial broth was centrifuged at 3500 rpm for 10 minutes and the cell pellet washed twice with saline prior to re-suspending the bacteria at 2 x 10^5 CFU/mL in saline.

Red mCherry-*P. aeruginosa* strain PAO1 was cultured in NB supplemented with 1.25 µg/mL tetracycline and incubated at 37 °C overnight on an orbital shaker. Ten-fold serial dilutions of the broth were prepared in saline and 100 µL aliquots of each dilution were inoculated onto duplicate NA plates supplemented with 1.25 µg/mL tetracycline with consequent incubation at 37 °C overnight. The neat broth was refrigerated overnight at 4 °C to inhibit further bacterial growth. The CFU counted on the agar plates were used to calculate the density of bacteria (CFU/mL) in the neat broth, taking into account the dilution factor. The bacterial broth was

centrifuged at 3500 rpm for 10 minutes and the cell pellet washed twice with saline prior to re-suspending the bacteria at 2 x 10^5 CFU/mL in saline.

2.2.8 *In vitro* and *Ex vivo* Host-Pathogen Interaction Assays

2.2.8.1 Interaction of M0 Macrophages with MRSA and PAO1

M0 resting macrophages generated from HPBM and U937 monocytes were prepared in 24-well plates as described in 2.2.4. The medium was aspirated and replicate wells (n = 6) were treated with different concentrations (1 x 10^{-7} M, 1 x 10^{-8} M, 1 x 10^{-9} M and zero M) of estrogen in corresponding antibiotic-free medium (HBCCM or RPMI-1640 for macrophages derived from HPBM and U937 respectively) for 24 hours at 37 °C and 5% CO_2. In addition, replicate (n = 6) blank control wells consisting of antibiotic-free HBCCM or RPMI-1640 medium, but lacking macrophages, were prepared and incubated for 24 hours at 37 °C and 5% CO_2. Replicate negative control wells (n = 6) were prepared by treating macrophage-containing wells with 1 x 10^{-7} M bovine serum albumin (BSA) in corresponding antibiotic-free medium and incubating for 24 hours at 37 °C and 5% CO_2. The supernatant was removed from all wells prior to treating with 1 µg/mL LPS and 100 ng/mL IFN-γ in corresponding antibiotic-free medium for 2 hours at 37 °C and 5% CO_2.

The supernatant was removed from all wells before inoculating the macrophages with 1 x 10^4 CFU of bacteria in a total volume of 100 µL antibiotic-free medium containing 1 µg/mL LPS and 100 ng/mL IFN-γ (1:1). Plates were incubated for 3 hours at 37 °C and 5% CO_2 to enable host-pathogen interactions (phagocytosis) to occur.

Following the 3 hours host-pathogen interaction period, the supernatant of each well was collected. M0 macrophages were released from each well by adding 450 µL trypsin EDTA and incubating for 3 minutes at 37 °C and 5% CO_2. The trypsin EDTA was neutralised by adding 450 µL antibiotic-free medium to each well. The constituents of each well (900 µL) were collected and added to the previously collected supernatant (100 µL) and thoroughly mixed. Aliquots (100 µL) of each

sample were then inoculated onto NA plates in duplicate and incubated at 37 °C overnight. The number of CFU formed on the agar plates were counted to calculate the bacterial recovery in each sample following the period of host-pathogen interaction.

2.2.8.2 Interaction of M1/M2 Macrophages with MRSA and PAO1

Classically activated resting M1 macrophages and alternatively activated resting M2 macrophages were generated from HPBM and U937 macrophages as described in 2.2.5. The medium was aspirated and 3-hour host-pathogen interaction assays were conducted as described in 2.2.8.1.

2.2.9 Interaction of M0 Macrophages with GFP- *S. aureus*

M0 U937 macrophages were prepared in 50 mL cell culture flasks as described in 2.2.4. The medium was discarded and the cells washed with complete RPMI medium. Flasks were treated with different concentrations (1 x 10^{-7} M, 1 x 10^{-8} M, 1 x 10^{-9} M and zero M) of estrogen in antibiotic-free RPMI medium for 24 hours at 37 °C and 5% CO_2. A negative control flask was prepared by treating with 1 x 10^{-7} M BSA and incubating at 37 °C and 5% CO_2 for 24 hours. The supernatants in all flasks were discarded before treating all flasks with 1 µg/mL LPS and 100 ng/mL IFN-γ (all in antibiotic-free RPMI medium) for 2 hours at 37 °C and 5% CO_2.

The supernatants were discarded and each flask was then inoculated with 1 x 10^5 CFU of GFP-*S. aureus* in a total volume of 10 mL antibiotic-free RPMI medium containing 1 µg/mL LPS and 100 ng/mL IFN-γ. All flasks were incubated for a 3-hour period of host-pathogen interaction at 37 °C and 5% CO_2 prior to collecting the supernatant of each flask. Adherent M0 macrophages were released from each flask by adding 5 mL trypsin EDTA and incubating for 3 minutes at 37 °C and 5% CO_2 with gentle detachment using a sterile cell scraper. A volume of 5 mL antibiotic-free RPMI medium was added to each flask to neutralise the EDTA and the contents of each flask (10 mL) was combined with the previously collected supernatant (10 mL) and thoroughly mixed. Each sample (20 mL) was centrifuged at 500 *g* for 7 minutes and the cell pellet re-suspended in 5 mL antibiotic-free RPMI medium.

A countess II FL cell counter (Life technologies, USA) was used to detect the GFP signals and capture images (n = 10) of bacterial internalisation within macrophages from each flask. The phagocyte density, number of internalised bacteria, total number of macrophages and total bacterial counts were determined from images in order to calculate the total number of phagocytes/mL and the ratio of bacterial internalisation/recovery.

2.2.10 Gentamicin Protection Assay

U937 monocytes (1 x 10^6 cell/well) were seeded in a 24-well plate and differentiated into resting M0 macrophages as previously described in 2.2.6.1. Cells were treated with estrogen (1 x 10^{-7} M, 1 x 10^{-8} M, 1 x 10^{-9} M and zero M) or 1 x 10^{-7} M BSA and stimulated with LPS (1 µg/mL) and IFN-γ (100 ng/mL) before being incubated with 1 x 10^4 CFU of MRSA 11 and *P. aeruginosa* PAO1 for 3 hours as described in 2.2.8.1. Protection gentamicin assay was performed according to methods detailed by Hockenberry *et al*. (2016). After a three-hour incubation period, the supernatant (100 µL) was collected and the wells were treated with 0.1% Triton X-100 (100 µL) in DPBS for 5 minutes at 37 °C and 5% CO_2 to release the membrane-adherent bacteria. The supernatant was collected and the cells were washed 10 times with DPBS before being incubated with 50 µg/mL gentamicin in antibiotic-free RPMI medium for 1 hour to eradicate extracellular and membrane-bound gentamicin-sensitive bacteria. The supernatant was discarded and the cells were washed 10 times with DPBS. The supernatant was discarded and cells were detached after incubation with 400 µL Trypsin EDTA at 37 °C and 5% CO_2 for 3 minutes. A volume of 400 µL antibiotic-free RPMI medium was added to each well to neutralise the trypsin EDTA. The constituents of each well (800 µL) were then collected and added to the previously collected supernatants (200 µL) and thoroughly mixed. CFU/mL were counted after plating 100 µL aliquots on duplicate NA plates and incubating at 37 °C overnight.

2.2.11 Visualising Host-Pathogen Interactions

2.2.11.1 Fluorescence Microscopy

Silicon wafers (Montco Technologies, USA) were cut at a diameter of 1 cm^2 and sterilised with 70% ethanol for 30 minutes before being rinsed with dH_2O and washed with DPBS for 10 minutes. U937 monocytes (0.5 x 10^6 cell/well) were seeded and differentiated into adherent M0 macrophages on sterile silicon wafers inside a 12- well plate. Cells were treated with/without estrogen (1 x 10^{-7} M) or 1 x 10^{-7} M BSA, stimulated with LPS (1 μg/mL) and IFN-γ (100 ng/mL), and incubated with GFP *S. aureus* or mCherry *P. aeruginosa* (both at 1 x 10^5 CFU) using methods described in 2.2.8.1. The supernatant from each well was removed and the cells were fixed with 4% paraformaldehyde in DPBS for 10 minutes at RT. The supernatants were discarded and the cells washed three times with DPBS before being incubated with Triton X-100 in DPBS (0.1%) for 5 minutes at RT. The cells were washed three times with DPBS and treated with a blocking buffer (3% BSA in DPBS) for 1h at RT. The supernatants were aspirated and the cells were immediately treated with phalloidin–tetramethylrhodamine B isothiocyanate conjugate from *Amanita phalloides* (1:200 in blocking buffer) and incubated in the dark at RT overnight. The supernatant was removed and the cells were washed three times with DPBS. The silicon wafers with attached cells were removed using sterile tweezers and attached to a microscope slide using double-sided tape. Samples were observed at 100X on a Nikon E600 epifluorescence microscope (Nikon, Japan) and at 63X under a Leica TSC SPE1000 confocal microscope (Leica Microsystems, UK). Z-stack analysis was performed on samples to localise the bacterial internalisation by macrophages.

2.2.11.2 Scanning Electron Microscopy

Sterile silicon wafers of 1 cm^2 diameter were washed with DPBS for 10 minutes before being placed in 12-well plates. Adherent U937 M0 macrophages were generated on the silicon wafers. Cells were treated with/without estrogen (1 x 10^{-7} M) or 1 x 10^{-7} M BSA, stimulated with LPS (1 μg/mL) and IFN-γ (100 ng/mL), and incubated with 1 x 10^5 CFU of MRSA 11 or PAO1 for 1 hour at 37 °C and 5% CO_2

using methods described in 2.2.8.1. Following the 3-hour period of host-pathogen interaction, the silicon wafers from each well were collected and fixed with 2.5% glutaraldehyde in DPBS. Samples were incubated at 4 °C overnight. The silicon wafers were washed twice in DPBS, then soaked in 20% methanol/ethanol for 30 minutes, 40% methanol/ethanol for 30 minutes, 60% methanol/ethanol for 30 minutes, 80% methanol/ethanol for 30 minutes, and finally in 100% methanol/ethanol for 30 minutes twice before being dried overnight in a vacuum-assisted desiccator (Sigma-Aldrich, UK). A Supra 40VP scanning electron microscope (Zeiss, Germany) was used to capture scanning electron microscopy (SEM) images using SmartSEM software (Carl Zeiss Ltd, Germany) to compare the internalisation of MRSA strain 11 or *P. aeruginosa* strain PAO1 by M0 macrophages with/without presence of estrogen.

2.2.12 Estrogen Receptor Stimulation/Blockade

HPBM and U937 monocytes were differentiated into M1 macrophages as described in 2.2.5. Replicate wells (n = 6) were treated with 1 x 10^{-7} M BSA, 1 x 10^{-7} M estrogen, zero M estrogen, 1 x 10^{-6} M tamoxifen, 1 x 10^{-6} M propyl pyrazole triol (PPT), and 1 x 10^{-6} M diarylpropionitrile (DPN) (all prepared in complete RPMI medium) and incubated at 37 °C and 5% CO_2 for 24 hours. Treatments were aspirated and the cells washed with DPBS. A volume of 500 µL of complete RPMI medium was added to each well and incubated for 24 hours at 37 °C and 5% CO_2. Further replicate wells (n = 6) containing M1 macrophages were treated with 1 x 10^{-6} M fulvestrant and 1 x 10^{-6} M theophylline, 8-[(benzylthio)methyl] (TPBM) in complete RPMI medium for 24 hours. Treatments were aspirated and the cells washed with DPBS. Cells were treated with 1 x 10^{-7} M estrogen and incubated at 37 °C and 5% CO_2 for 24 hours.

Host-pathogen interaction assays were then performed for 3 hours in all wells as described in 2.2.8 and bacterial (MRSA11 and PAO1) recovery determined.

2.2.13 Investigating Mediators of Host-Pathogen Interactions by Immunoblotting

2.2.13.1 Preparation of Buffers

TBS-Tween buffer, adjusted to pH 7.4

- 10 mM Tris-base
- 140 mM Sodium chloride (NaCl)
- 0.1% Tween-20
- Distilled water (dH_2O)

Blocking buffer, adjusted to pH 7.4

- 1% BSA in TBS-Tween

RIPA buffer

- 140 mM Sodium chloride (NaCl)
- 25 mM Tris-hydrochloride (Tris-HCl)
- 1 mM Ethylenediaminetetraacetic acid (EDTA)
- 0.5 mM Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA)
- 0.5% Sodium deoxycholate, phenylmethylsulfonyl fluoride (PMSF)
- 1% Triton X-100
- 0.1% Sodium dodecyl sulfate (SDS)
- 0.1% Protease inhibitors cocktail
- Ultrapure water

2.2.13.2 Protein Extraction and Quantification

U937 monocytes were differentiated into M1 macrophages in three T25 flasks (1 x 10^7 cells/flask) using methods described in 2.2.5. The medium in all flasks was discarded and adherent M1 macrophages were treated with 10 mL of 1 x 10^{-7} M BSA (negative control), zero M estrogen or 1 x 10^{-7} M estrogen in RPMI complete medium in a single flask and incubated at 37 °C and 5% CO_2 for 24 hours. The treatments were aspirated and cells washed with DPBS before incubation with 3 mL

trypsin EDTA for 3 minutes at 37 °C and 5% CO_2 to initiate cell detachment. A volume of 3 mL complete RPMI medium was added to the flasks to neutralise the trypsin EDTA. Cells were transferred into 50 mL centrifuge tubes and centrifuged at 500 *g* for 7 minutes. Supernatants were discarded and 200 µL of ice-cold radio immunoprecipitation assay (RIPA) buffer containing protease inhibitor cocktail (1:1000) was added the cell pellets in order to lyse the cells. Samples were transferred to −80 °C for 30 minutes. Cells were defrosted at 37 °C and sonicated 10 times for 10 seconds, mixing well between each period of sonication. Samples were then centrifuged for 5 minutes at 12000 *g* and the lysates were collected on ice. The total protein content of each sample was quantified using the bicinchoninic acid (BCA) protein quantification assay according to the manufacturer's instructions. Briefly, a 1 mg/mL stock solution of BSA in dH_2O was used to provide a set of protein standards (0.8, 0.6, 0.4 and 0.2 mg/mL). A volume of 25 µL of each BSA standard was added to a 96-well plate with dH_2O (zero mg/mL BSA) used as a blank. A working BCA solution, consisting of reagents A and B (1:50) was prepared as described in the BCA kit. A volume of 200 µL of this solution was added to all the BSA standards and incubated at 37 °C for 30 minutes before measuring the absorbance at 562 nm. A standard curve of absorbance against protein concentration was generated from the protein standards and used to determine the concentration of total protein in cell lysates via interpolation. Protein samples were normalised to 20 mg/mL prior to storage at -80 °C for later use.

2.2.13.3 Immunoblotting

A volume of 5 µL of each of the protein samples (n = 6) was blotted onto nitrocellulose membranes. BSA at a concentration of 1 mg/mL (n = 6) was used as a negative control. The membranes were allowed to dry before being treated with the blocking buffer and then incubated at RT on a rocking shaker (Cole-Parmer, UK) for one hour with rocking at 50 rpm. The buffer was discarded and the membranes were treated with primary antibodies specific for the proteins Rac1, Fak, RhoG, RhoA, Cdc42 and β-tubulin (Table 2.1) overnight at 4 °C with rocking at 30 rpm. The primary antibodies were recovered (stored at 4 °C for further use) and the membranes washed five times for 5 minutes each with TBS-Tween buffer. The

membranes were treated with the horseradish peroxidase (HRP) anti-mouse IgG (produced in the A9044 rabbit) or HRP anti-rabbit IgG (produced in goat) secondary antibodies as appropriate, made in blocking buffer (1:1000), and incubated at RT for 1 hour with rocking at 50 rpm. When the primary antibody was raised in mouse, a rabbit anti-mouse secondary antibody was used. When the primary antibody was raised in rabbit, a goat ant-rabbit secondary was used (Table 2.1). The secondary antibodies were removed and membranes were washed five times for 5 minutes with TBS-Tween buffer. A working EZ-ECL chemiluminescence detection solution, consisting of reagents A and B (1:1) was prepared in the dark according to the manufacturer's instructions provided in the EZ-ECL kit. The membranes were covered with EZ-ECL working solution and incubated in the dark for 2 minutes at RT. Excess solution was aspirated and the membranes were visualised with a Chemidoc Touch Transilluminator (Bio-Rad, USA) using the Image Lab^{TM} Touch Software at 1-minute exposure times. Dot blots were measured and analysed using ImageJ Software (Version 1.48).

Table 2-1. Primary and secondary antibodies used for immunoblotting.

Primary antibody	Working concentration	Host primary species	Secondary antibody	Host secondary species
β-tubulin	1:5000	Mouse	HRP anti-mouse IgG	Rabbit
Rac1	2 µg/mL	Mouse	HRP anti-mouse IgG	Rabbit
Fak	0.5 µg/mL	Mouse	HRP anti-mouse IgG	Rabbit
RhoG	1:500	Rabbit	HRP anti-rabbit IgG	Goat
RhoA	2 µg/mL	Mouse	HRP anti-mouse IgG	Rabbit
Cdc42	1:500	Mouse	HRP anti-mouse IgG	Rabbit

Chapter 3: Effect of 17β-estradiol on the Phagocytosis of MRSA and *P. aeruginosa* by Human M0 Macrophages

3.1 Introduction

3.1.1 Estrogen Promotes Wound Healing

Active estrogens are secreted by the gonads into the bloodstream, with smaller amounts of active hormones produced by the adrenals. However, a substantial amount of inactive steroid precursors, including DHEA, its sulphate DHEA-S and androstenedione (4-dione) are produced by the adrenals and converted locally into active estrogens (such as 17β-estradiol) in peripheral tissues such as adipose tissue, bone and skin (Labrie *et al*., 1998; Nelson and Bulun, 2001). The reduction in the formation of DHEA-S by the adrenals during ageing results in a dramatic fall in the formation of active estrogens in peripheral target tissues, a situation which is strongly associated with age-related processes such as age-related impaired healing (Labrie *et al.*, 1995).

Estrogen declines significantly in postmenopausal women, due to a rapid decline in ovarian secretion of estrogens and a loss of both adrenal-derived estrogen and its precursors with increasing age. In men, estrogen declines rapidly with increasing age, largely due to a loss of adrenal-derived estrogen and its precursors. It is believed that the decline in estrogen in both sexes with increasing age results in detrimental effects on skin appearance (dry skin, sagging, wrinkling) and wound healing (Hardman *et al*., 2007). However, the detrimental effect of hormone-driven ageing (estrogen deprivation) on the development and progression of wound infections remains largely unknown.

Ageing is known to be a key risk factor for impaired wound healing, with the declining production of estrogen in the elderly being the main key regulator of age-related delayed wound healing (Hardman and Ashcroft, 2008). The reduction in estrogen that occurs with increasing age has a pronounced effect on cutaneous healing in both genders. Ashcroft *et al*. (1997a) and Ashcroft *et al*. (1999a) reported that estrogen deficiency causes a significant delay in wound healing, with topical and systemic estrogen replacement reversing this delay. Moreover, estrogen supplementation promoted age-related wound healing in both elderly males and females (Ashcroft *et al*., 1999a; Guo and DiPietro, 2010).

Estrogen has been shown to accelerate re-epithelialisation, promote angiogenesis, enhance matrix deposition and wound contraction, dampen the inflammatory response (Ashcroft and Ashworth., 2003). In particular, the key role of estrogen in regulating inflammation has been extensively reviewed (Guo and DiPietro, 2010; Gilliver *et al.*, 2007), with estrogen supplementation decreasing the inflammatory response in elderly subjects of both genders (Ashcroft et al., 1997b; Ashcroft et al., 1999a). Delayed wound healing in the elderly is associated with a delayed but prolonged and excessive inflammatory response, with dysregulated expression of pro-inflammatory cytokines and proteolytic mediators. However, despite the pronounced inflammatory response, evidence suggests increasing age may result in an impaired ability of inflammatory cells to eliminate bacteria from the wounds (Emori *et al*., 1991; Thomas, 2001). Indeed, the propensity for wound infections increases in the elderly, in part due to the delay in wound repair (Cooper *et al*., 2015).

Impaired wounds often become colonised with a variety of microbial species, such as *S. aureus, P. aeruginosa, Proteus* species and anaerobic bacteria (Gjødsbøl *et al*., 2006; Kirketerp-Møller *et al*., 2008; Rybtke *et al*., 2015). When innate immunity functions appropriately, the eradication of wound bacteria is generally successful without intervention. However, if the host immune system becomes compromised by comorbidities, particularly in the elderly, clearance of wound bacteria can fail in the absence of effective treatments. In particular, if wounds become heavily infected with MRSA, *P. aeruginosa* or other hospital-acquired pathogens, treatment might necessitate aggressive medication with last line of defence antibacterial therapies (Beasley and Hirst, 2004). Indeed, the treatment of wounds infected with MRSA or *P. aeruginosa* represent a big challenge for the modern healthcare organisations (Beasley and Hirst, 2004).

This study used *in vitro* and *ex vivo* assays to investigate the effects and potential mechanisms by which estrogen can affect the clearance of MRSA and/or *P. aeruginosa*. Previous work suggests the sex steroid hormones, particularly estrogen and testosterone, play a role in regulating host responses to infection. Findings indicate testosterone acts as an immunosupressor, whereas estrogen activates the

immune system (Cutolo *et al*., 2004). These effects are linked with sexual dimorphism found in bacterial infections, where males (human and animal) are more vulnerable to bacterial infections than females (Klein and Roberts, 2010). In addition, changes in estrogen levels during menstrual cycles and pregnancy can influence bacterial infections (García-Gómez *et al*., 2012). Administration of sex steroid hormones, such as estrogen, in models of ovariectomized mice can control the progression of bacterial infections, acting as a supplement to antibiotic therapies. In line with this, some bacteria have developed potential mechanisms to degrade sex steroid hormones or reduce their effectiveness(García-Gómez *et al*., 2012).

3.1.2 Macrophages and Wound Healing

Macrophages are amongst the main inflammatory cell types present in age-related impaired wounds where inflammation is typically pronounced and prolonged. The phenotype of macrophages changes during the phases of wound healing (Mosser and Edwards, 2010). Following injury, pro-inflammatory macrophages, known as "M1" macrophages, arrive at the wound site in order to remove foreign invaders such us bacteria, debris and dead cells. During acute wound healing, as the tissue starts to heal, M1 macrophages undergo a process of conversion into macrophages that promote anti-inflammatory effects, referred to as "M2" macrophages, to resolve inflammation (Koh and DiPietro, 2011). Macrophages also have key roles in vascularisation, arranging themselves nearby restored blood vessels in order to support their fusion and stability (Ogle *et al*., 2016). Macrophages release matrix metalloproteinases (MMPs) to breakdown the provisional extracellular matrix prior to undergoing apoptosis in order to bring about the deposition of mature matrix in the dermis as the wound progresses through the proliferative phase of wound repair (Vannella and Wynn, 2017). However, in non-healing wounds, macrophages persist in a pro-inflammatory state and the transition to an anti-inflammatory phenotype is blocked (Hesketh *et al*., 2017).

Macrophages in the skin are either derived from a resident tissue-macrophage population established before birth or from circulating bone marrow-derived

monocytes that are recruited to the site of injury and differentiate into macrophages (Malissen *et al*., 2014; Vannella and Wynn, 2017). When the wound site is infiltrated by microorganisms such as bacteria, monocyte-derived macrophages are systemically recruited within 24 hours post-injury in order to carry out phagocytosis (Minutti *et al*., 2017).

Tissue-resident macrophages serve as early indicators of an injury or foreign invading pathogens (Malissen *et al*., 2014) by recognizing PAMPs such as LPS via binding to TLRs. Responses to PAMPs include initial recruitment of neutrophils to the wound area within minutes of the injury to eliminate foreign invaders (Malissen *et al*., 2014). Monocytes are subsequently recruited to the wound area to intensify the inflammatory response and become the predominant inflammatory cell type at around 3-7 days post-injury (Davies *et al*., 2013). Dermal tissue-resident macrophages can be identified by numerous surface markers, such as CD64, CD11c, CD14, CD16, CD68, CD71 and CCR5 (Malissen *et al*., 2014). Close to the resolution of injury, dermal tissue macrophages self-renew, and eliminate apoptotic cells as the tissue returns to homeostasis (Davies *et al*., 2013).

Dendritic cells in the skin can also be derived from monocytes (e.g. Langerhans cells). Langerhans cells and macrophages share many similarities such as expressing similar surface markers (e.g. F4/80, CD14, and IL-10), which makes it difficult to distinguish between these two types of cell (Malissen *et al*., 2014). Many researchers consider Langerhans cells as a type of tissue-resident macrophage, as they also have a very similar gene expression profile (Davies *et al*., 2013; Doebel *et al*., 2017; Minutti *et al*., 2017). Interestingly, it has been reported that there is a link between healing diabetic foot ulcers and augmented numbers of Langerhans cells (Stojadinovic *et al*., 2013). However, the exact function of Langerhans cells in wound repair (particularly impaired wound healing) has not been clarified yet (Stojadinovic *et al*., 2013).

3.1.3 Effect of Estrogen on Macrophages

Estrogen is believed to be a key player in regulating immune responses against bacterial infections (Cutolo *et al*., 2004). Known for its anti-inflammatory

proprieties, estrogen is reported to resolve excessive inflammation by directly effecting inflammatory responses of monocytes and macrophages (Ashcroft *et al*., 2003; Guo and DiPietro, 2010). Previous research reported that ovariectomized mice expressed high numbers of macrophages in injured tissues compared to normal healthy mice during the wound healing process (Ashcroft *et al*., 2003).

Macrophage migration inhibitory factor (MIF) is a pro-inflammatory cytokine, produced by many cell types (Calandra and Roger, 2003). MIF induces the production of other cytokines and growth factors by inflammatory cells such as TNF-α, IL-1 and IL-6 (Ashcroft *et al*., 2003; Hardman *et al*., 2005). MIF is a key pro-inflammatory mediator inhibited by estrogen during wound healing (Ashcroft *et al*., 2003; Hardman *et al*., 2005). MIF is upregulated in human and murine impaired wounds *in vivo* (Ashcroft *et al*., 2003; Hardman *et al*., 2005; Emmerson *et al*., 2009), whereas MIF is decreased in wounds following exposure to estrogen (Ashcroft *et al*., 2003). Recent research indicated that estrogen inhibits MIF production by tissue macrophages (Ashcroft *et al*., 2003; Hardman *et al*., 2005).

TNF-α is a key pro-inflammatory cytokine released by activated macrophages and is associated with chronic wounds in the elderly (Emmerson *et al*., 2009). After a pathogenic infection, bacterial clearance is reliant on effective regulation of TNF-α, and abnormal expression of the cytokine may cause substantial morbidity and mortality (Billiau and Vandekerckhove, 1991). Uncontrolled TNF-α production is also associated with the progression of several autoimmune diseases, such as rheumatoid arthritis, which are more dominant in females than males (Zandman-Goddard *et al*., 2007). TNF-α wound levels are reduced in healthy premenopausal females and young males in comparison with postmenopausal females and elderly males (Vural *et al*., 2006). Research suggests that estradiol suppresses the production of TNF-α in murine macrophages (Deshpande *et al*., 1997; Srivastava *et al*., 1999).

Long-time exposure of *ex vivo* macrophages to estrogen augments the expression of iNOS and the production of numerous cytokines, particularly IL-1β, IL-12 and IL-6 following TLR4 activation by LPS. Other studies have reported anti-inflammatory effects of estrogens on macrophages *in vitro* (Deshpande *et al*., 1997; Ghisletti *et*

al., 2005). For example, a 2-hour estrogen treatment of macrophages (RAW 264.7 cell line) *in vitro* caused a substantial reduction in the expression of the inflammatory mediators IL-1α, IL-6, and TNF-α by splenic macrophages following TLR4 activation (Deshpande *et al*., 1997; Ghisletti *et al*., 2005).

In summary, estrogen accelerates wound healing and is associated with a reduced inflammatory response. Estrogen inhibits the recruitment of monocytes to the wound and reduces the production of macrophage-derived pro-inflammatory mediators. However, the precise role estrogen plays in macrophage-mediated bacterial clearance has not been elucidated to date. Thus, this study used *in vitro* and *ex vivo* models of host-pathogen interaction to investigate estrogen-mediated phagocytosis of bacteria by human U937- and HPBM-derived macrophages. Macrophage-mediated phagocytosis of two major wound pathogens, MRSA and *P. aeruginosa*, were assessed under *in vitro* and *ex vivo* conditions that model estrogen levels in the elderly (estrogen deprivation), physiological concentrations in young adults and following exogenous estrogen supplementation. U937 and HPBM-derived macrophages are routinely used as flexible models of macrophages to study numerous aspects of the inflammatory response; including chemotaxis, phagocytosis, apoptosis and cytokine production (Hall, 2017).

3.2 Aim and Objectives

3.2.1 Aim

To investigate the effects of age-related changes in estrogen levels on the phagocytic function of M0 macrophages using *in vitro* and *ex vivo* models of host-pathogen interactions.

3.2.2 Objectives

- To compare the phagocytic function of M0 U937- and HPBM-derived macrophages under *in vitro* and *ex vivo* conditions of physiological (typical of youth) and supraphysiological levels of estrogen, estrogen deprivation and the absolute absence of estrogen.

- To determine the effect of estrogen on the phagocytosis of Gram positive (MRSA) and Gram negative (*P. aeruginosa*) wound pathogens by U937- and HPBM-derived M0 macrophages.
- To visualise estrogen-mediated internalisation of Gram positive (MRSA) and Gram negative (*P. aeruginosa*) bacteria by U937- and HPBM-derived M0 macrophages.

3.3 Methods

3.3.1 Generation of U937- and HPBM-Derived M0 Macrophages

The differentiation of U937 and HPBM monocytes into macrophages was confirmed by flow cytometry as described in Section 2.2.6.1. U937 and HPBM monocytes were cultured as described in Sections 2.2.3. Cells were treated with/without 50 ng/mL PMA as described in Section 2.2.4 to induce differentiation of monocytes into macrophages. Cells were then stained with FITC-conjugated anti-CD11c antibody as described in 2.2.6.1. The expression of CD11c (relative MFI and % fluorescence) on untreated control monocytes was then compared with PMA-treated M0 macrophages by flow cytometry as described in Section 2.2.6.1.

3.3.2 Effect of Estrogen on Bacterial Growth

Before conducting host-pathogen assays to test the effect of estrogen on the macrophage-mediated phagocytosis of MRSA and PAO1, it was important to investigate its direct effect on the growth of both pathogens.

MRSA 11 and PAO1 were cultured in NB overnight as described in Section 2.2.7. Neat broths were centrifuged at 3500 rpm for 10 minutes and the bacterial cell pellets washed twice with saline before re-suspension at 1 x 10^4 CFU/mL (see Section 2.2.7) in 1 mL saline alone (zero M estrogen) or 1mL saline containing either 1 x 10^{-8} M estrogen or 1 x 10^{-7} M BSA (negative control). Bacterial suspensions were then incubated at 37 °C on an orbital shaker for 3 hours. Aliquots (50 µL) of each sample were then inoculated onto NA plates in duplicate and incubated at 37 °C overnight. The number of CFU formed on NA plates were counted in order to calculate the growth of bacteria in each sample.

3.3.3 Effect of Estrogen on the Clearance of MRSA/PAO1 by M0 Macrophages

3.3.3.1 Phenol Red-Free Medium Controls

In order to determine whether the phenol red component of cell culture medium had an effect on estrogen-mediated phagocytosis of MRSA/PAO1 by M0

macrophages, *in vitro* host-pathogen assays were conducted using RPMI medium containing/lacking phenol red. U937 monocytes were cultured in phenol red-free RPMI medium or RPMI complete medium using methods described in Section 2.2.3. M0 macrophages generated in Section 3.3.1 were then treated with physiological estrogen (1 x 10^{-8} M), prepared in either phenol-free RPMI medium or RPMI complete medium, for 24 hours and then incubated with MRSA or PAO1 for 3 h. Recovered bacterial colonies were then determined as described in Section 2.2.8.1.

3.3.3.2 Multiplicity of Infection (MOI)

The effect of estrogen on phagocytosis over a range of starting bacterial inoculations was assessed for both MRSA or PAO1. U937 M0 macrophages generated in Section 3.3.1 were treated with 1 x 10^{-8} M estrogen for 24 hours prior to incubation for 3 hours with different concentrations (1 x 10^3 CFU, 1 x 10^4 CFU, 1 x 10^5 CFU and 1 x 10^6 CFU) of MRSA or PAO1 to give initial bacteria: macrophage ratios of 0.001, 0.01, 0.1 and 1. Recovered bacterial colonies were then determined as described in Section 2.2.8.1.

3.3.3.3 *In vitro* and *Ex Vivo* Host-Pathogen Interaction Assays

U937 and HPBM M0 macrophages generated in Section 3.3.1 were treated in wells with different concentrations (1 x 10^{-7} M, 1 x 10^{-8} M, 1 x 10^{-9} M and zero M) of estrogen for 24 hours as described in Sections 2.2.2 and 2.2.8.1. Cells were incubated with MRSA or PAO1 (1 x 10^4 CFU) for 3 hours and bacterial recovery was determined as described in Section 2.2.8.1.

3.3.4 Interaction of Estrogen-Treated U937 M0 Macrophages with GFP- *S.aureus*

U937 M0 macrophages generated in Section 3.3.1 were incubated in flasks with different concentrations (1 x 10^{-7} M, 1 x 10^{-8} M, 1 x 10^{-9} M and zero M) of estrogen for 24 hours as described in Sections 2.2.2 and 2.2.8.1. Cells were then incubated with GFP- *S. aureus* (1 x 10^5 CFU/mL) for 3 hours prior to detection of GFP signal as described in Section 2.2.9.

3.3.5 Gentamicin Protection Assay

In order to confirm that internalisation of MRSA and PAO1 by U937 and HPBM M0 macrophages was leading to bacterial killing rather than simply bacterial sequestration, a gentamicin protection assay was conducted to determine bacterial survival within macrophages using methodologies described in Sections 2.2.10 and 3.3.1.

The gentamicin protection assay is commonly used in host-pathogen research due to the incapability of gentamicin to penetrate the membrane of eukaryotic cells (Elsinghorst, 1994). The gentamycin protection assay was performed to investigate the effect of estrogen on the killing of intracellular MRSA and PAO1 as gentamicin was previously described to eradicate extracellular bacteria, but not intracellular bacteria due to its poor ability to permeate the macrophage membrane (Hamad *et al*., 2010; Hockenberry *et al*., 2016). Moreover, incubating cells with gentamicin kills bacteria that fail to penetrate cells, and any bacterial recovery obtained following lysis of the macrophages must be due to bacterial survival within the macrophages. The gentamicin protection assay is a very sensitive technique that can allow the detection of even single colonies of bacteria in cells (Elsinghorst, 1994).

3.3.6 Visualising Host-Pathogen Interactions

Internalisation of GFP *S. aureus* or mCherry- *P. aeruginosa* by U937 and HPBM M0 macrophages, with/without estrogen treatment, was confirmed by fluorescence microscopy as described in Section 2.2.11.1. Host-pathogen interactions between U937 or HPBM M0 macrophages and MRSA/PAO1, with/without estrogen treatment, were visualised by SEM as described in Section 2.2.11.2.

3.4 Results

3.4.1 Differentiation of Monocytes into M0 Macrophages

According to previous research, monocytes (including U937 monocytes) can differentiate into macrophage-like cells via stimulation with PMA through PKC activation (Gidlund *et al*., 1981; Koeffler, 1983; Kiley and Parker, 1995).

Figure 3.1 illustrates alterations in the morphology of U937 cells after treatment with PMA at 50 ng/mL for 24 hours. Round floating monocytes were transformed into adherent cells characterised by increased adhesion, formation of clumps and ruffled cell membranes.

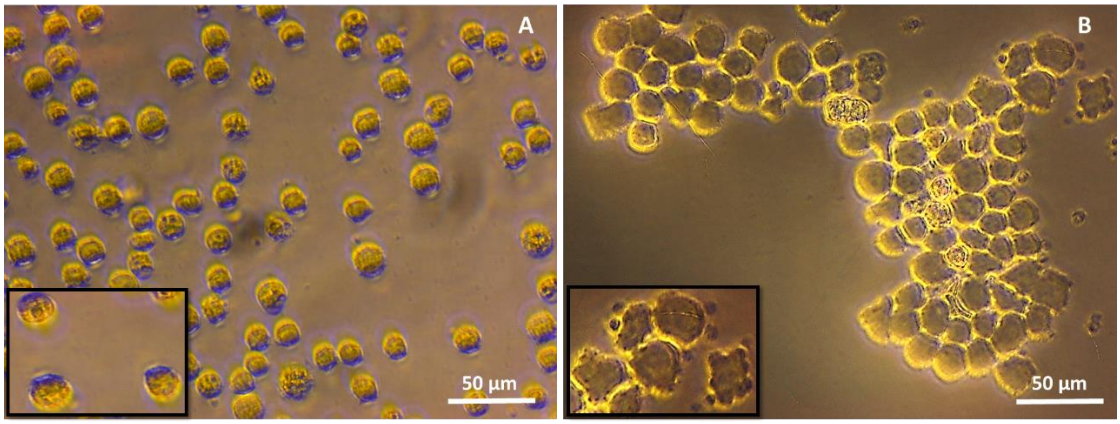

Figure 3.1. Morphological changes of U937 monocytic cells after treatment with 50 ng/mL PMA for 24 hours. Round floating monocytes (A) were differentiated into adherent cells (B) that formed clumps and had ruffled cell membranes. Images were taken using an Axiovert 40C inverted phase contrast microscope. Inserts show enlargements of highlighted cells.

The conversion of U937 and HPBM monocytes into a distinct population of macrophages was confirmed via detection of the FITC-conjugated anti-human CD11c surface marker (Rios de la Rosa *et al*., 2017) by flow cytometry (Section 2.2.6.1). U937 PMA-differentiated cells were almost exclusively (99.6%) $CD11c^+$ (Figure 3.2) whereas untreated control U937 monocytes predominantly lacked the CD11c surface marker (0.2% $CD11c^+$). The CD11c MFI was significantly ($P<0.001$) higher in PMA-treated U937 cells compared to untreated control U937 monocytes, confirming PMA transformed U937 monocytes into adherent U937 M0 macrophages.

There was high expression of CD11c by PMA-differentiated HPBM cells (76% $CD11c^+$), whereas the CD11c macrophage surface marker was almost absent (0.3% $CD11c^+$) in untreated control HPBM (Figure 3.3). The MFI of CD11c was significantly ($P<0.05$) greater in PMA-treated HPBM cells compared to untreated control HPBM, confirming the transformation of HPBM into adherent M0 macrophages had occurred following PMA treatment.

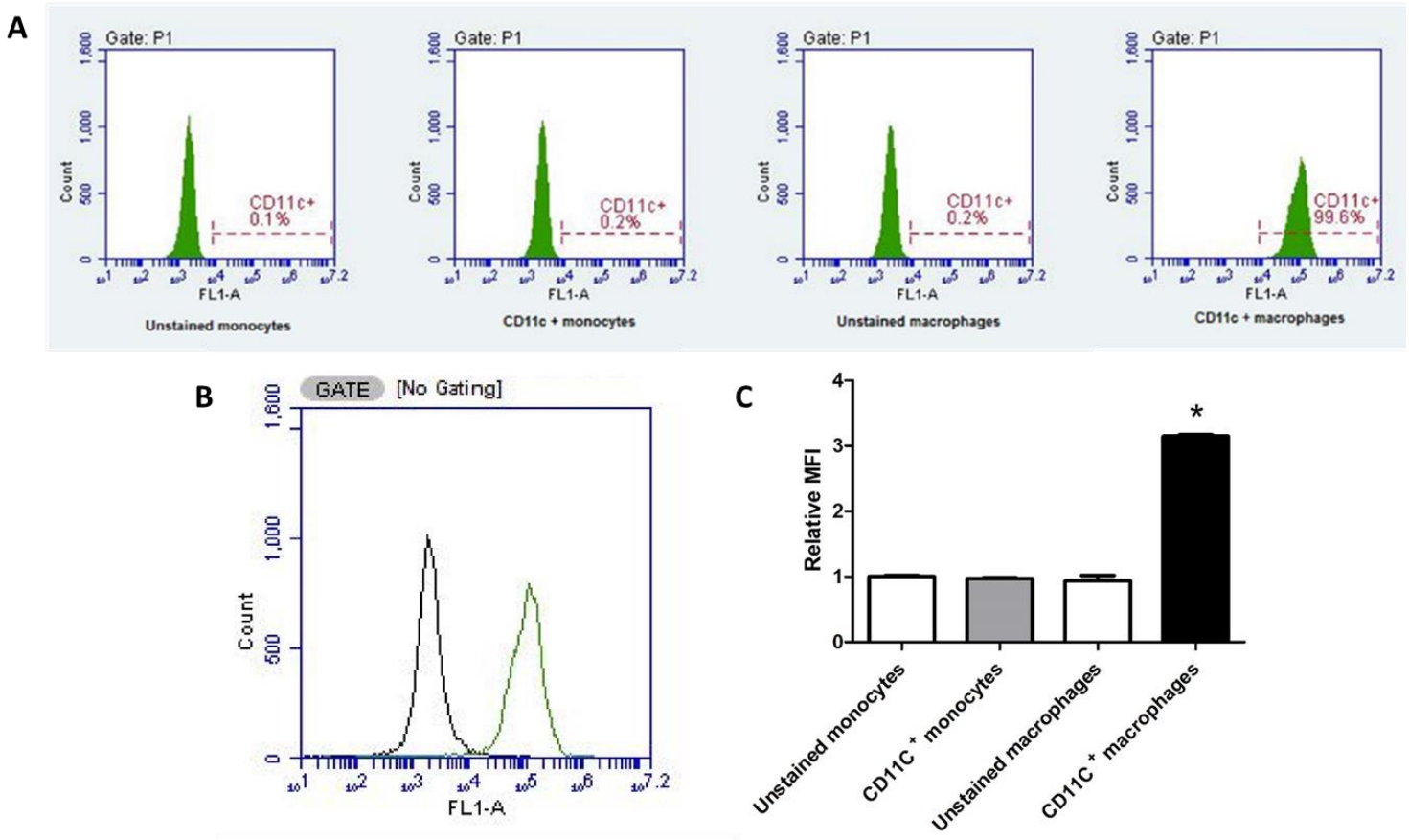

Figure 3.2. Flow cytometry confirming U937 monocytes differentiation into Mo macrophages. The conversion of U937 monocytic cells into macrophages was established via detection of the CD11c surface marker by flow cytometry. Phorbol 12-myristate 13-acetate (PMA)-differentiated cells almost exclusively expressed the CD11c macrophage marker, whereas monocytes predominantly lacked the CD11c surface marker (A). Two distinct populations of U937 cells were detected (B) with significantly (P<0.01) higher median fluorescence intensity (MFI) from U937 PMA-treated cells compared to undifferentiated U937 monocytes (C). Data are presented as average of n = 3 experiments. * Indicates significant difference (One-way ANOVA) in MFI (*P*<0.01). Error bars represent the standard error of the mean (StEM).

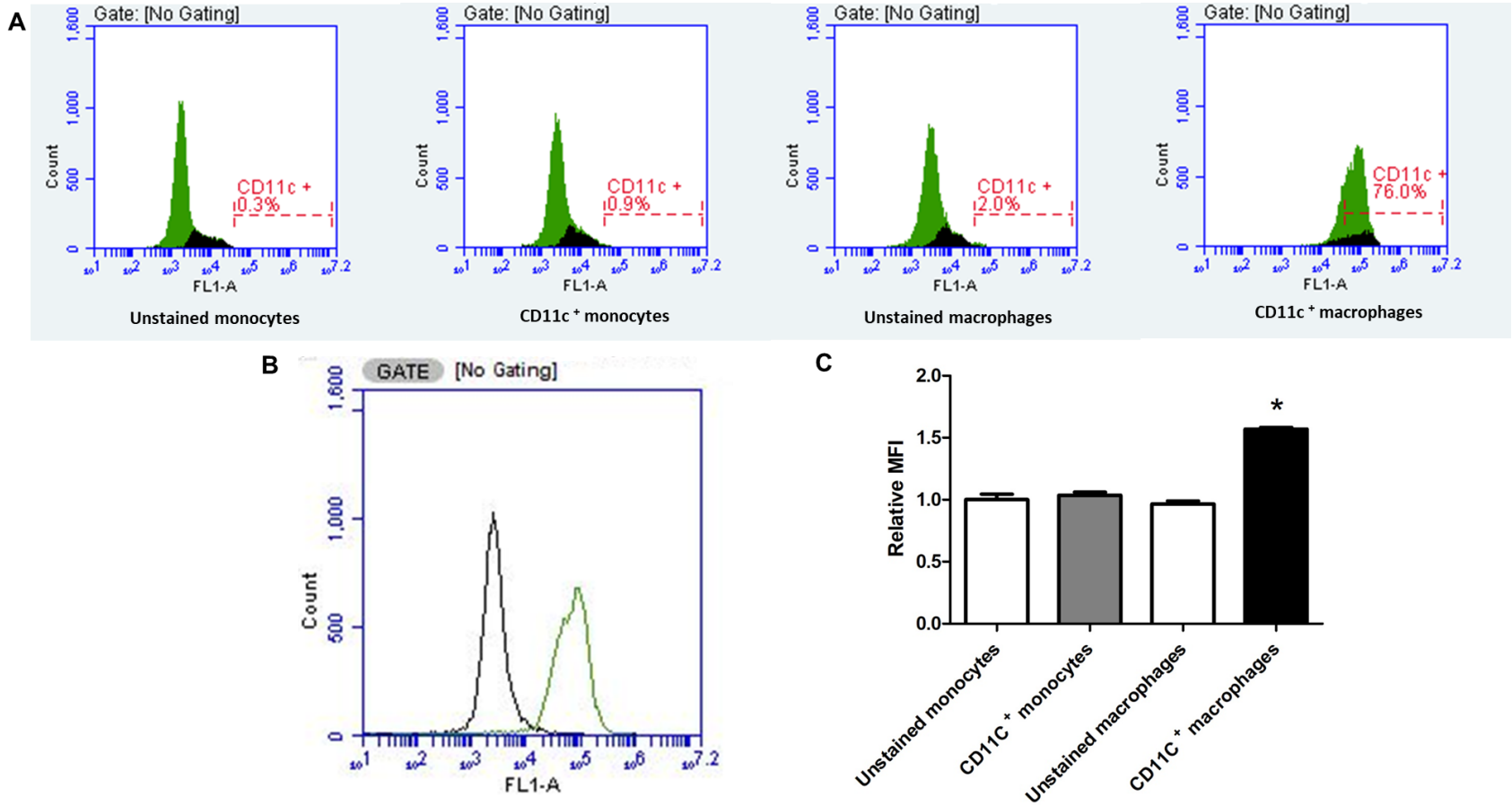

Figure 3.3Flow cytometry confirming HPBM differentiation into M0 macrophages. The differentiation of HPBM into macrophages was assessed via detection of the CD11c surface marker by flow cytometry. Phorbol 12-myristate 13-acetate (PMA)-differentiated cells highly expressed CD11c whereas monocytes predominantly lacked the CD11c surface marker (A). Two distinct cell populations were detected (B) with significantly (P<0.05) higher median fluorescence intensity (MFI) observed on HPBM PMA-treated cells compared to untreated monocytes (C). Data are presented as average of n = 3 experiments. * Indicates significant difference (One-way ANOVA) in MFI (P<0.05). Error bars represent the standard error of the mean (StEM).

3.4.2 Effect of Estrogen on the Growth of MRSA and PAO1

The effect of estrogen (1 x 10^{-7} M) on the growth of MRSA and PAO1 colonies after 3 hours incubation was investigated compared to a BSA (1 x 10^{-7} M) negative control and a untreated saline control. Estrogen had no significant effect on the MRSA or PA01 growth (*P*>0.05) (Figure 3.4A and Figure 3.4B) compared to the controls. These results indicated that estrogen had no direct effect on the growth of MRSA or PAO1 compared to the controls.

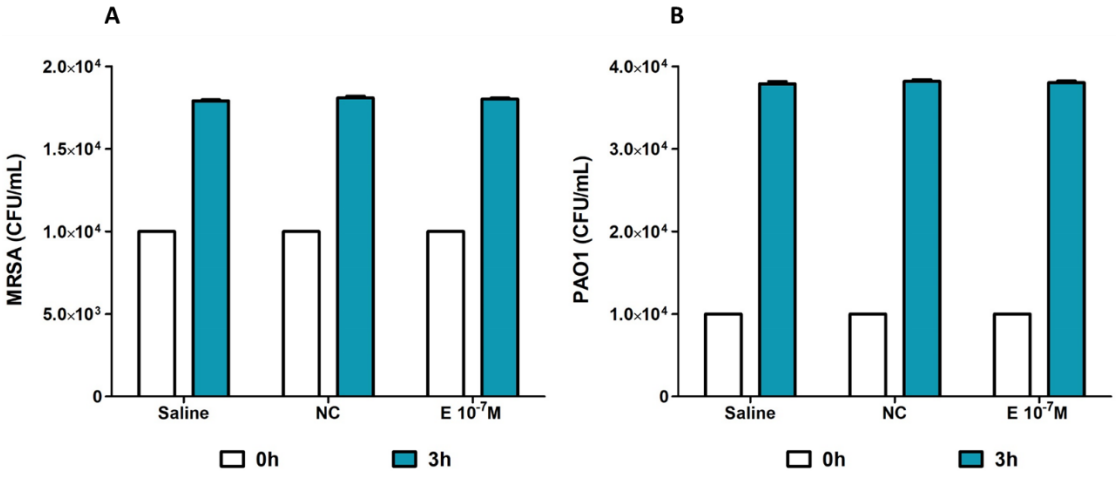

Figure 3.4. Effect of estrogen on MRSA and PAO1 growth. Estrogen (1 x 10^{-7} M) had no significant (*P*>0.05) effect on the growth of MRSA (A) or PA01 (B) compared to the 1 x 10^{-7} M bovine serum albumin (BSA) negative control (NC) or the untreated saline control. Data are presented as average of n = 24 experiments.

3.4.3 The Effect of Phenol Red on Bacterial Clearance

The effect of phenol red on the clearance of MRSA and PAO1 by U937 M0 macrophages with/without estrogen treatment was investigated. The findings confirmed that phenol red in the RPMI cell culture medium had no significant effect on the MRSA (A) and PAO1 (B) recovery compared to RPMI medium lacking phenol red (*P*>0.05), regardless of the presence of estrogen (Figure 3.5). Physiological (1 x 10^{-8} M) estrogen levels significantly decreased MRSA and PA01 recovery compared to the negative control (*P*<0.05), both in the presence or absence of phenol red.

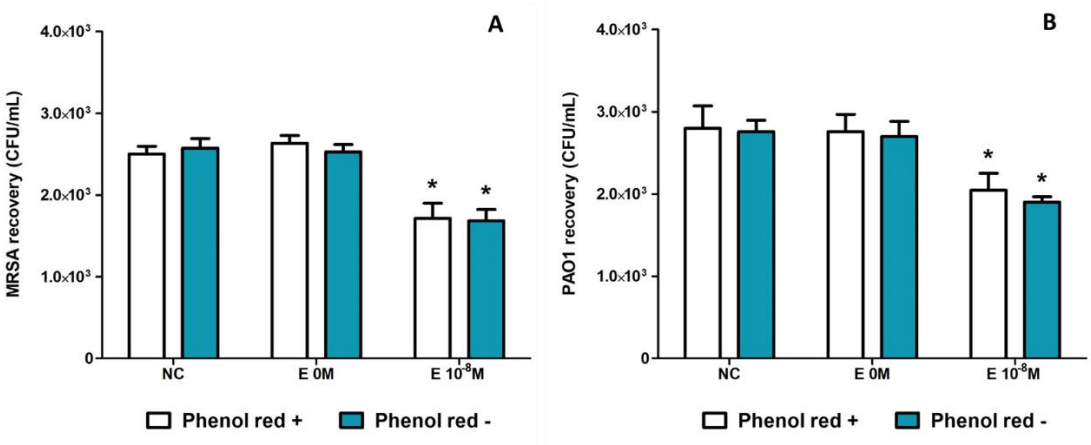

Figure 3.5. Effect of phenol red on bacterial clearance by M0 macrophages. Phenol red in cell culture medium had no significant effect on the recovery of MRSA (A) or PA01 (B) compared to the absence of phenol red (*P*>0.05), regardless of the presence/absence of estrogen (1 x 10^{-8} M). Physiological (1 x 10^{-8} M) estrogen levels significantly (*: *P*<0.05) decreased the MRSA (A) and PAO1 (B) recovery compared to the negative control (NC), with or without phenol red. Data are an average of n = 10 experimental repeats with error bars representing the standard error of the mean (StEM).

3.4.4 Multiplicity of Infection (MOI)

The effect of physiological estrogen (1 x 10^{-8} M) on the uptake of MRSA by M0 macrophages, *in vitro*, was investigated. Cells were incubated with a range of initial MRSA inoculations (1 x 10^3 CFU, 1 x 10^4 CFU, 1 x 10^5 CFU and 1 x 10^6 CFU) (Figure 3.6). The absence of estrogen had no significant effect (*P*>0.05) on the MRSA recovery compared to negative control. Physiological estrogen significantly decreased (*P*<0.05) the MRSA recovery, in comparison with the BSA control with all MOIs. The bacterial recovery of MRSA significantly increased (*P*<0.05) following estrogen (1 x 10^{-8} M) supplementation as the MOI [bacteria : macrophage ratio] increased from 0.001 to 1. However, the MRSA recovery followed a similar pattern throughout, regardless of the MOI ratio, with estrogen consistently increasing (*P*<0.05) MRSA clearance at all MOIs.

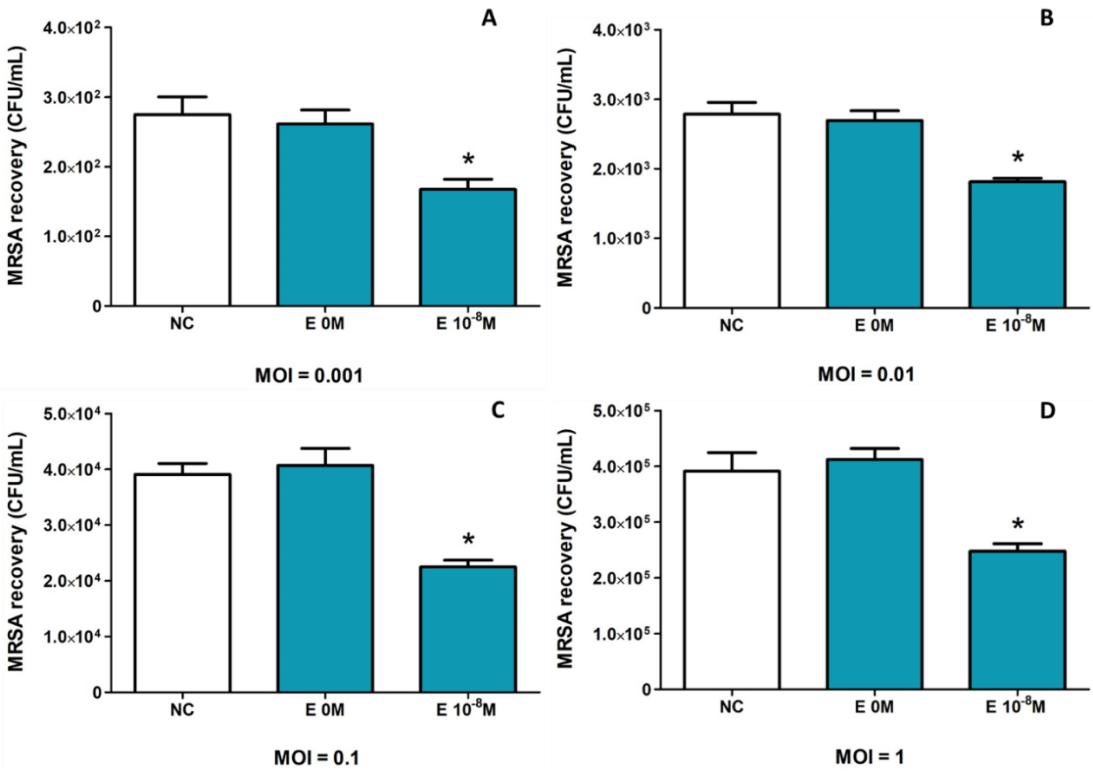

Figure 3.6. Effect of physiological estrogen on the recovery of MRSA by M0 macrophages *in vitro*. Cells were incubated with MRSA at MOIs of 0.001 (A), 0.01 (B), 0.1 (C) and 1 (D). Following treatment of macrophages with physiological estrogen (1 x 10^{-8} M), MRSA recovery significantly decreased (P<0.05), at all MOIs, in comparison with the absence (zero M) of estrogen and the negative control (NC). Data represent mean recovery (CFU/mL) ± standard error of the mean (StEM), n = 10. *: P<0.05, indicate significant differences in the MRSA/PAO1 recovery compared to the NC.

Figure 3.7 illustrates the effect of physiological estrogen (1 x 10^{-8} M) on the internalisation of PAO1 by macrophages. After incubating the macrophages with a varied range of initial PA01 colonies (1 x 10^3 CFU, 1 x 10^4 CFU, 1 x 10^5 CFU and 1 x 10^6 CFU), the absence of estrogen had no significant effect (P>0.05) on the recovery of in comparison with the BSA control. Interestingly, physiological estrogen significantly decreased (P<0.05) the PAO1 recovery, in comparison with the NC control at all MOIs. The PAO1 recovery significantly increased (P<0.05) following estrogen (1 x 10^{-8} M) supplementation as the MOI [bacteria : macrophage ratio] increased from 0.001 to 1. However, the PAO1 recovery followed a comparable pattern throughout, regardless of the MOI ratio, with estrogen consistently increasing (P<0.05) PAO1 clearance at all MOIs.

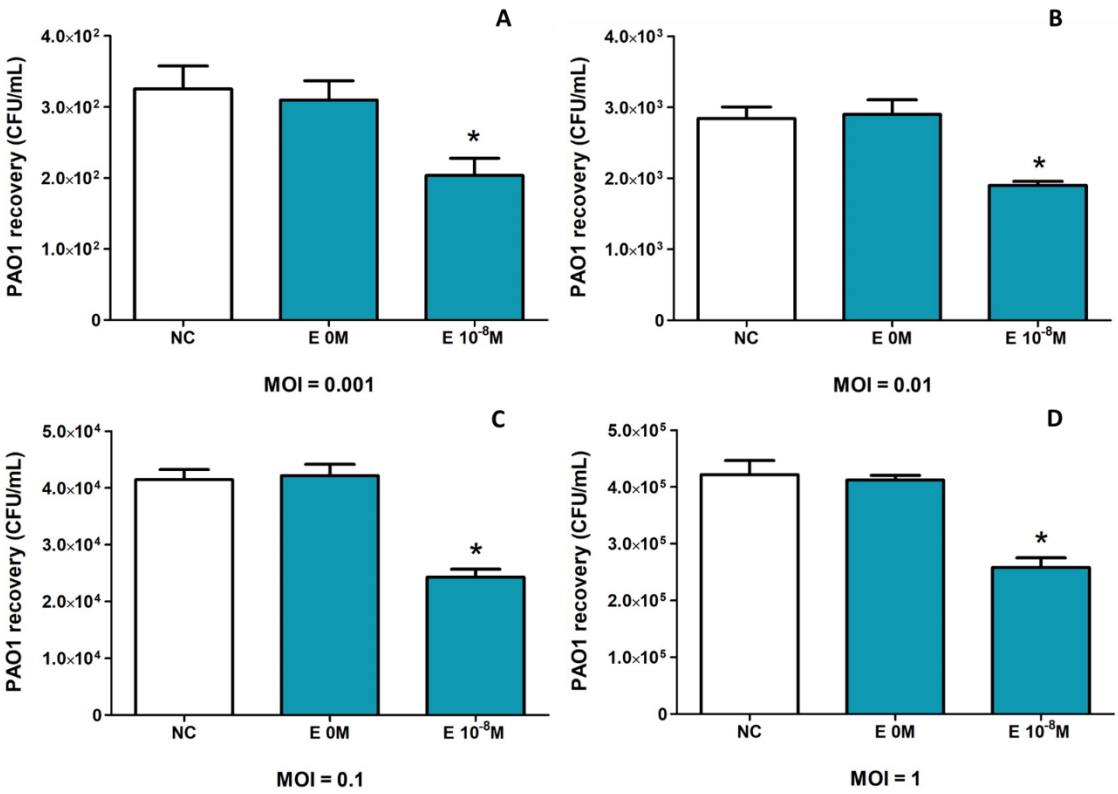

Figure 3.7. Physiological estrogen decreases the recovery of PAO1 by M0 macrophages *in vitro*. U937-derived M0 macrophages were incubated with PAO1 at MOIs of 0.001 (A), 0.01 (B), 0.1 (C) and 1 (D). Physiological estrogen (1 x 10^{-8} M) significantly decreased (P<0.05) the PAO1 recovery at all MOIs, in comparison with the absence (zero M) of estrogen and the negative control (NC). Data represent mean recovery (CFU/mL) ± standard error of the mean (StEM), n = 10. *: P<0.05, indicate significant differences in the MRSA/PAO1 recovery compared to the NC.

3.4.5 Effect of Estrogen on MRSA and PAO1 Clearance

3.4.5.1 Effect of Estrogen on MRSA and PAO1 Clearance by U937 Macrophages

The effect of estrogen on the clearance of MRSA and PAO1 by U937 M0 macrophages was investigated. The absolute absence of estrogen (zero M) showed no significant (P>0.05) change in the number of recovered MRSA or PA01 colonies (Figure 3.8A and Figure 3.8B) compared to the BSA negative control (NC).

Estrogen significantly (P<0.01) reduced the recovery of MRSA colonies compared to the negative control in a dose-dependent manner. The MRSA recovery (Figure 3.8A) was significantly (P<0.01) reduced from 1886 CFU/mL in the NC to 1759 CFU/mL, 1295 CFU/mL and 936 CFU/mL with estrogen at a concentrations of 1 x 10^{-9} M, 1 x 10^{-8} M and 1 x 10^{-7} M respectively. The MRSA recovery decreased significantly

(P<0.01) between increasing estrogen concentrations, with physiological estrogen concentrations (1 x 10^{-8} M) significantly reducing MRSA recovery compared to estrogen deprivation, and supraphysiological estrogen concentrations (1 x 10^{-7} M) significantly reducing MRSA recovery compared to physiological estrogen levels.

Physiological estrogen (1 x 10^{-8} M) and supraphysiological estrogen (1 x 10^{-7} M) significantly (P<0.05) reduced the PAO1 recovery (Figure 3.8.B) from 2410 CFU/mL in the NC to 2105 CFU and 1960 CFU respectively. The PAO1 recovery also decreased significantly (P<0.05) between physiological and supraphysiological estrogen levels.

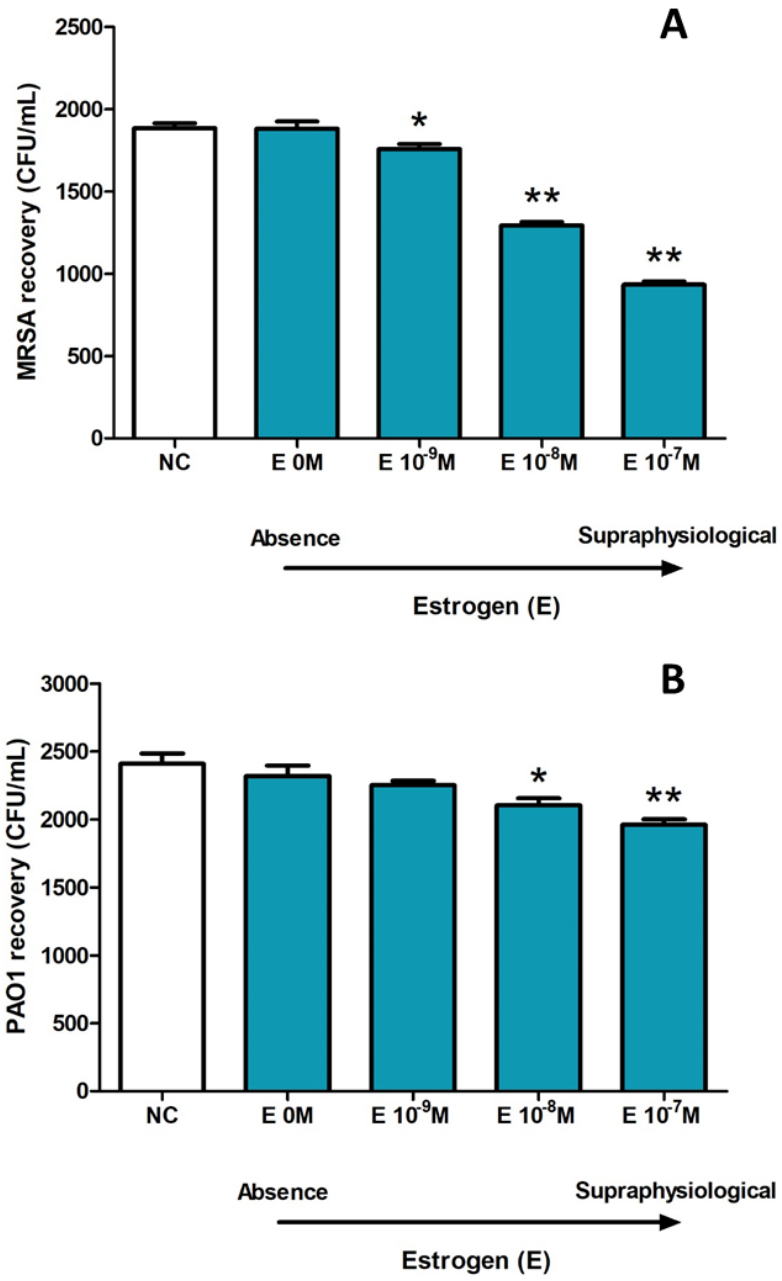

Figure 3.8. Estrogen enhances the uptake of MRSA11 (A) and PAO1 (B) by U937 M0 macrophages. Following treatment of U937 M0 macrophages with estrogen (1 x 10^{-7} M, 1 x 10^{-8} M) MRSA (A) and PAO1 (B) recovery significantly decreased in a concentration-dependant manner in comparison to the absence (zero M) of estrogen and the negative control (NC). Data represent mean recovery (CFU/mL) ± standard error of the mean (StEM), n = 48. *: *P*<0.05, **: *P*<0.01 indicate significant differences in the MRSA/PAO1 recovery compared to the NC.

3.4.5.2 Effect of Estrogen on MRSA and PAO1 Clearance by *Ex Vivo* M0 Macrophages

Host-pathogen assays were repeated with human peripheral blood monocytes (HPBM) in order to determine whether estrogen has similar effects on the MRSA

and PAO1 recovery with macrophages derived from primary human monocytes. In the absence of estrogen (zero M estrogen) there was no significant difference (P>0.05) in the clearance of MRSA or PA01 compared to the negative control (Figure 3.9.A and Figure 3.9.B). In contrast, estrogen treatment significantly reduced (P<0.05) MRSA recovery compared to the negative control in a dose-dependent manner. Physiological ($1x10^{-8}$ M) and particularly supraphysiological ($1x10^{-7}$ M) levels of estrogen were highly effective at increasing MRSA clearance, significantly decreasing (P<0.01) MRSA recovery (1775 CFU/mL, 1246 CFU/mL respectively) compared to the negative control (2692 CFU/mL). Estrogen had similar effects on the clearance of PAO1 when treated with physiological ($1x10^{-8}$ M) or supraphysiological ($1x10^{-7}$ M) levels, significantly (P<0.05) reducing PA01 recovery (2252 CFU/mL and 1817 CFU/mL respectively) compared to the negative control (2631 CFU/mL).

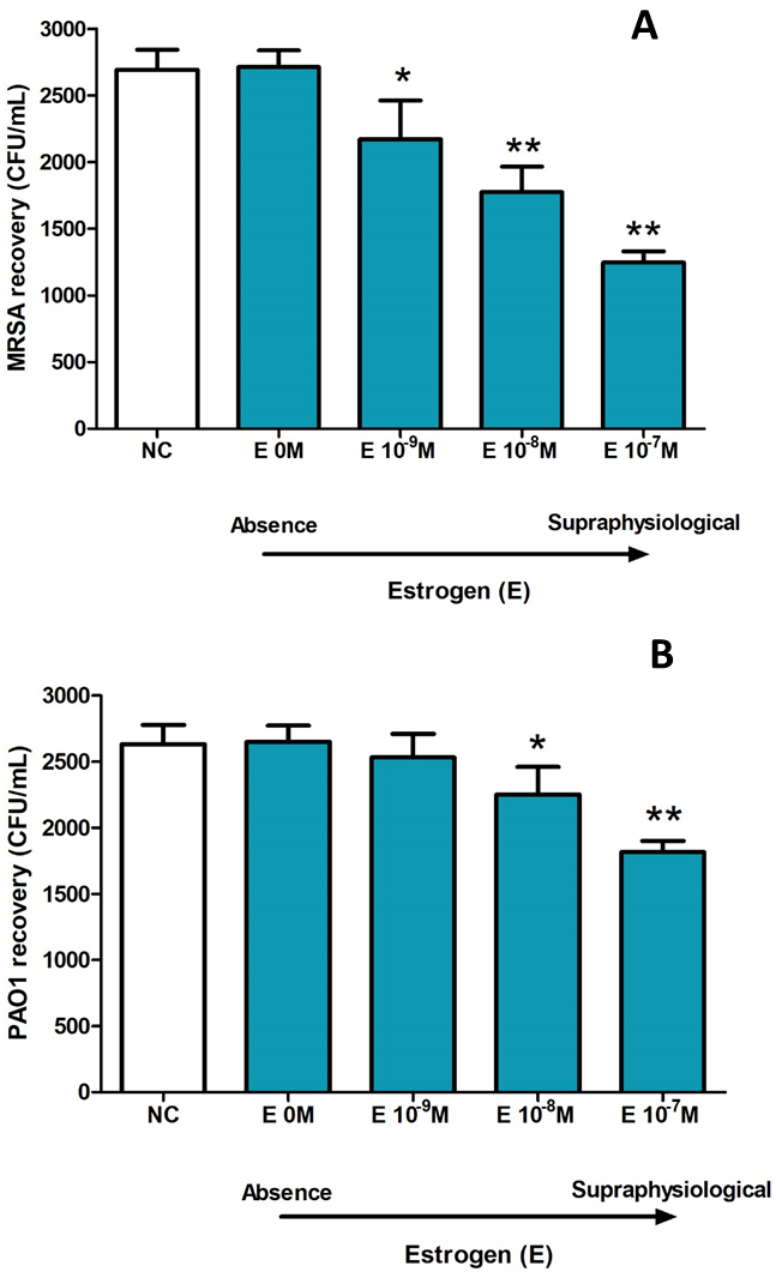

Figure 3.9. Estrogen enhances the uptake of MRSA11 (A) and PAO1 (B) by HPBM M0 macrophages. Following treatment of HPBM M0 macrophages wth estrogen (1 x 10^{-7} M, 1 x 10^{-8} M) the recovery of MRSA/PAO1 was significantly reduced in comparison to the negative control (NC). Error bars represent the mean ± standard error of the mean (SEM), n = 6. *: $P<0.05$, **: $P<0.01$ indicate significant differences in the MRSA/PAO1 recovery compared to the NC.

3.4.6 GFP *S. aureus* Host-Pathogen Assay

3.4.6.1 Effect of Estrogen on The Recovery of GFP- *S. aureus*

The effect of estrogen on the recovery of fluorescent GFP-labelled *S. aureus* colonies was investigated. U937-derived M0 Macrophages were incubated with

GFP-*S. aureus* for 3 hours. The bacterial colonies were tracked via detection of GFP signals inside and outside the macrophages using a countess II fluorescent cell counter. Images (n = 10) of bacterial internalisation within macrophages were captured. The phagocyte density, number of internalised bacteria, total number of macrophages and total bacterial counts were determined from images in order to calculate the bacterial recovery, total number of phagocytes/mL and the ratio of bacterial internalisation.

Consistent with previous results, physiological and supraphysiological levels of estrogen significantly reduced the *S. aureus* recovery ($P<0.05$) in a concentration-dependant manner in comparison with the absence of estrogen and BSA controls (Figure 3.10).

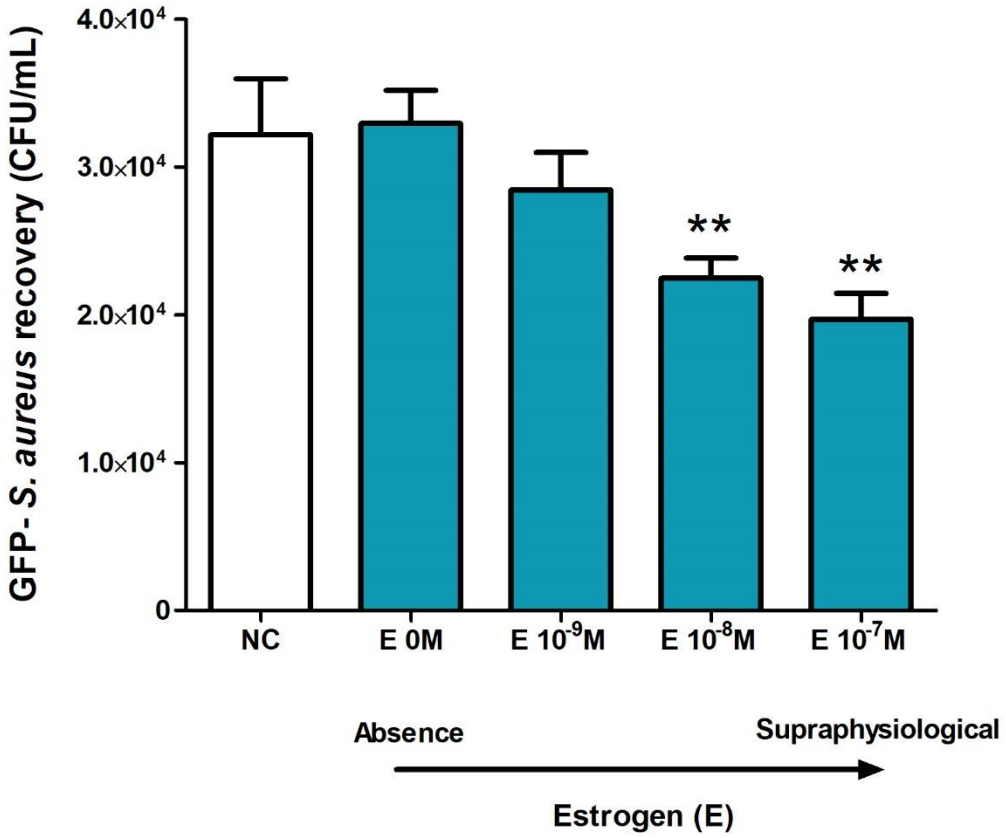

Figure 3.10. Estrogen increases the uptake of GFP-*S. aureus* by U937 M0 macrophages. Following treatment of U937 M0 macrophages with physiological and suppraphysiological estrogen (1 x 10^{-9} M, 1 x 10^{-7} M), the *S. aureus* recovery was significantly reduced in a concentration-dependant manner in comparison to the absence (zero M) of estrogen and the BSA control (NC). Data represent mean recovery (CFU/mL ± standard error of the mean (StEM), n = 10. **: $P<0.01$ indicate significant differences in the GFP-*S. aureus* recovery compared to the NC.

3.4.6.2 Effect of Estrogen on Phagocyte Density

The effect of estrogen on the number of active phagocytes after the 3-hour period of host-pathogen interaction was investigated (Figure 3.11). Stimulation of cells with 1 x 10^{-8} M or 1 x 10^{-9} M estrogen did not have any significant effect (P=0.16 and P=0.99 respectively) on phagocyte density (3.0 x 10^5 phagocytes/mL and 2.8 x 10^5 phagocytes/mL respectively) compared to the negative control (2.3 x10^5 phagocytes/mL) (Figure 3.11). However, supraphysiological (1 x 10^{-7} M) levels of estrogen significantly increased ($P < 0.01$) the number of active phagocytes (3.7 x 10^5 phagocytes/ mL) in comparison to the negative control (NC).

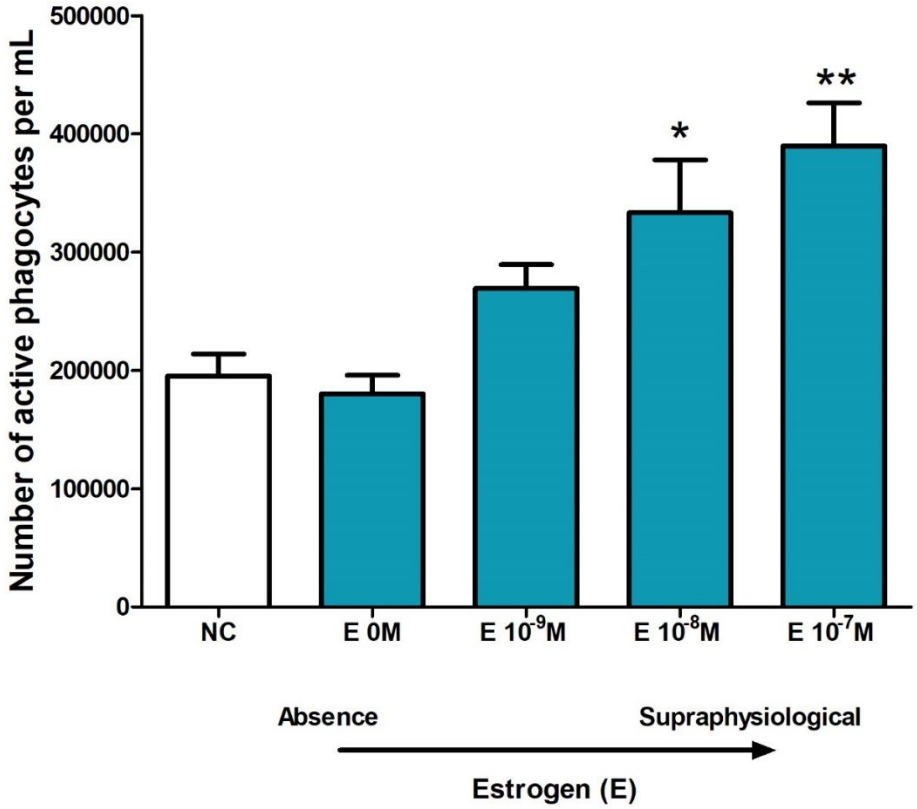

Figure 3.11. Estrogen Promotes an Increase in the Number of Active Phagocytes. Supraphysiological (1 x 10^{-7} M) levels of estrogen significantly (*: P<0.05, **: P<0.01) increased the detection of GFP signals inside macrophages compared to the negative control (NC). Data indicate mean number of active phagocytes per mL (n = 8) ± standard error of the mean (StEM).

3.4.6.3 Effect of Estrogen on the Proportion of Total GFP *S. aureus* Internalised by U937-Derived Macrophages

Figure 3.12 illustrates the proportion of total GFP- *S. aureus* internalised by U937-derived macrophages following treatment with 17β-estradiol. When the cells were treated with zero M and 1 x 10^{-9} M estrogen, there was no significant (P=0.89) effect on the proportion of GFP- *S. aureus* internalised (0.58) in comparison to the negative control (NC). However, when U937-derived macrophages were treated with physiological (1 x 10^{-8} M) or supraphysiological (1 x 10^{-7} M) levels of estrogen, there was a significant increase (P< 0.05) in the proportion of internalised bacteria (0.66 and 0.78 respectively) compared to the NC.

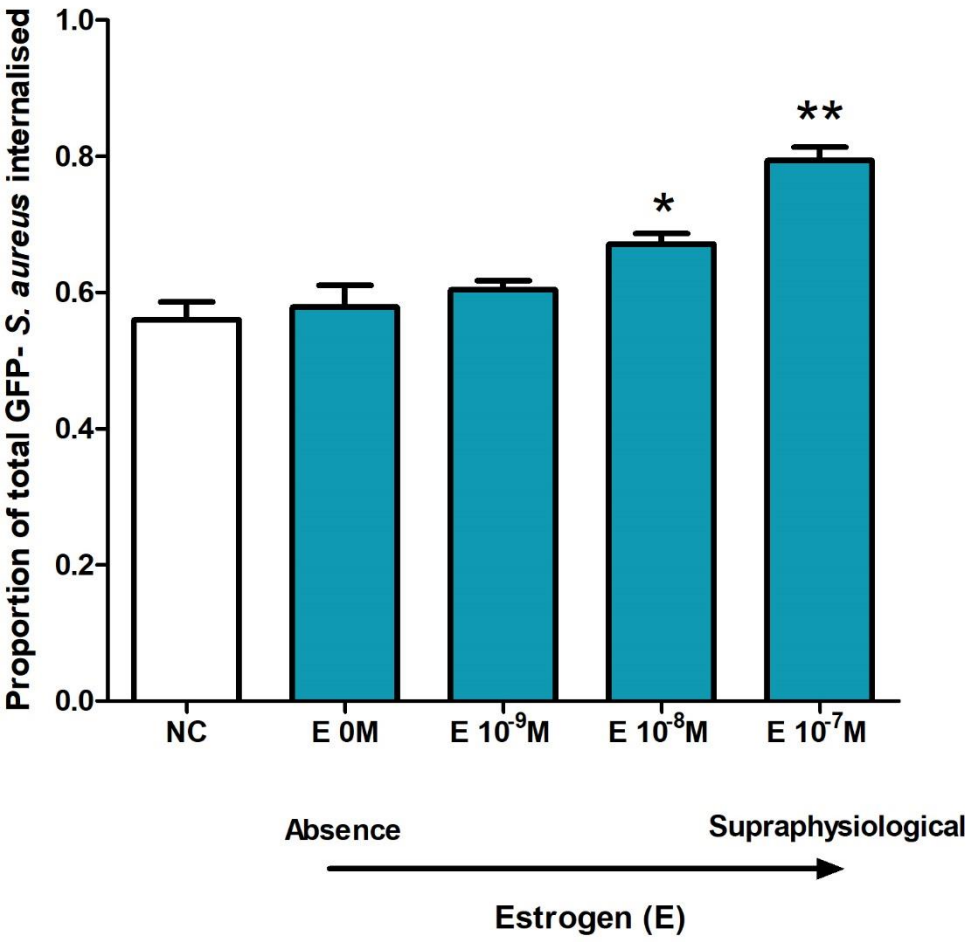

Figure 3.12. Effect of estrogen on the ratio of GFP- *S. aureus* internalisation. Data are presented as average of n = 8 experiments. * $P<0.05$, **: $P<0.01$ Indicate significant differences (One-way ANOVA) in bacterial recovery ($P<0.05$). Error bars represent the StEM.

3.4.7 Effect of Estrogen on Bacterial (MRSA and *P. aeruginosa*) Killing by U937-Derived Macrophages.

In order to determine the effect of estrogen on bacterial killing by U937-derived macrophages, a gentamicin protection assay was performed. The gentamycin protection assay is commonly used to confirm the killing of internalised bacteria by phagocytes, or recover viable bacteria from inside phagocytes when pathogens are able to evade host cell responses and grow within phagocytes (Elsinghorst, 1994). The assay utilised gentamycin to eliminate non-internalised (extracellular) bacteria after a 3-hour period of host-pathogen interaction, followed by the lysis of washed macrophages to recover internalised bacteria. Gentamycin is unable to pass across the host cell membrane so only bacteria that have been successfully internalised

(phagocytosed) by immune cells but remain viable within phagocytes at the end of the host-pathogen interaction are recovered by the assay (Elsinghorst, 1994., Waldbeser *et al*., 1994; Hess *et al*., 2004; Oelschlaeger, 2010).

Similar to previous assays, physiological (1 x 10^{-8} M) and supraphysiological (1 x 10^{-7} M) levels of estrogen significantly ($P<0.05$) decreased the recovery of viable internalised MRSA and PAO1 by U937 M0 macrophages in a dose-dependent manner (Figure 3.13) compared to the BSA (NC) and untreated controls. The data confirmed estrogen was not simply promoting internalisation of bacteria, but was also increasing the killing of phagocytosed MRSA and PAO1 by U937-derived macrophages. The absence of estrogen or estrogen deprivation (1 x 10^{-9} M) did not significantly ($P>0.05$) effect the killing of internalised MRSA and PAO1 by U937-derived macrophages compared to the NC.

Physiological (1 x 10^{-8} M) and supraphysiological estrogen (1 x 10^{-7} M) significantly ($P<0.05$) reduced the MRSA recovery (Figure 3.12.A) from 976 CFU/mL in the NC to 787 CFU and 692 CFU respectively. The PAO1 recovery also decreased significantly ($P<0.05$) from 1019 CFU/mL in the BSA control to 822 CFU/mL and 692 CFU/mL following treatment with physiological (typical of youth) and supraphysiological levels of estrogen respectively.

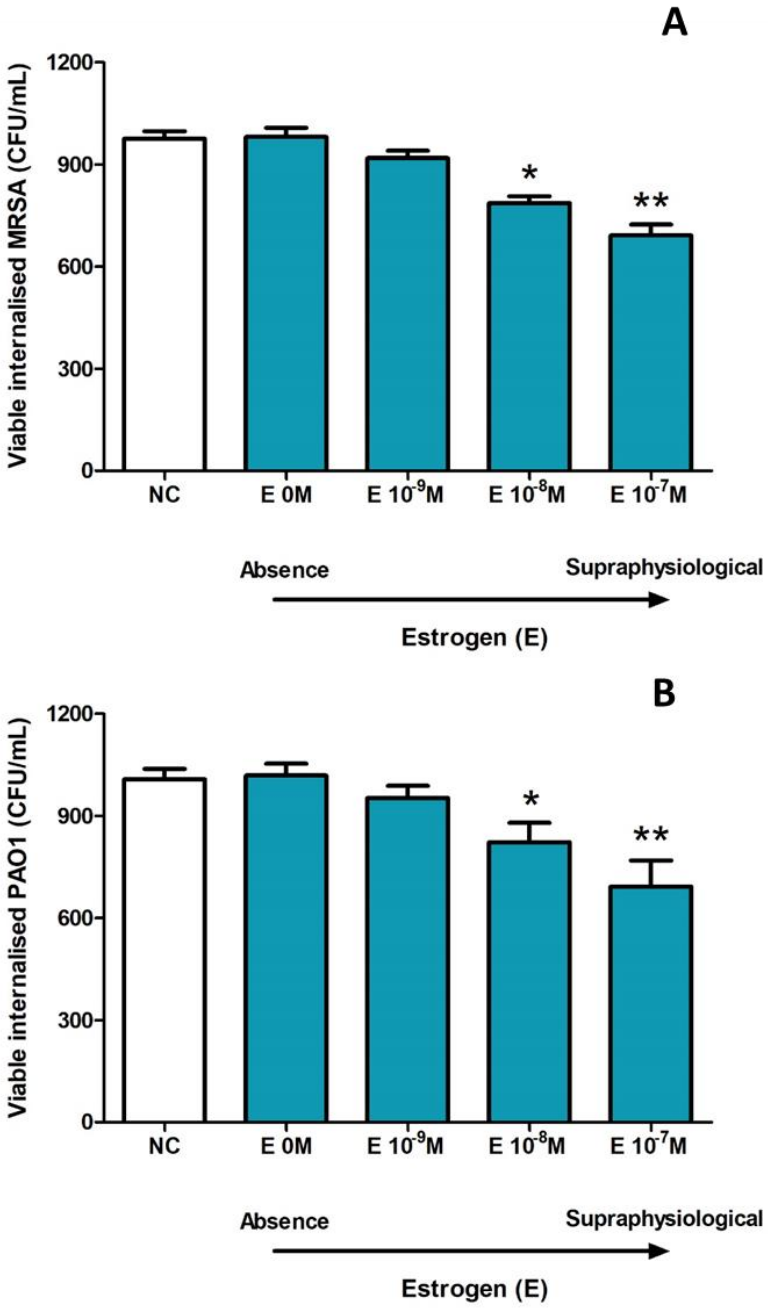

Figure 3.13. Estrogen promotes the killing of MRSA (A) and PAO1 (B) by U937-Derived Macrophages. Physiological (1 x 10^{-8} M) and supraphysiological (1 x 10^{-7} M) levels of estrogen significantly (*: $P<0.05$, **: $P<0.01$) promoted the killing of internalised MRSA (A) and PAO1 (B) by M0 macrophages compared to the negative control (NC). Data indicate mean (n = 6) viable internalised bacteria (CFU/mL) ± standard error of the mean (StEM).

3.4.8 Visualisation of Bacterial Internalisation

3.4.8.1 Epifluorescence Microscopy

In order to visualise and compare the phagocytosis of pathogens, with and without estrogen treatment, U937-derived macrophages were incubated with GFP *S. aureus*

or mCherry *P. aeruginosa* prior to staining for phalloidin–tetramethylrhodamine B isothiocyanate conjugate from *Amanita phalloides* (Figure 3.14.A), or labelling with CD18 mouse monoclonal antibody and stained for goat anti-mouse IgG (H+L) superclonal secondary antibody, alexa fluor® 488 conjugate (Figure 3.14.B). Images were captured using a 100X objective on a Nikon E600 epifluorescence microscope (Section 2.2.11.1).

The absence of estrogen (A2 and B2) had no effect on the bacterial internalisation compared to the BSA negative controls (A1 and B1). However, it is clear that estrogen treated macrophages internalised more of the *S. aureus* and *P. aeruginosa* colonies (A3 and B3) than untreated cells.

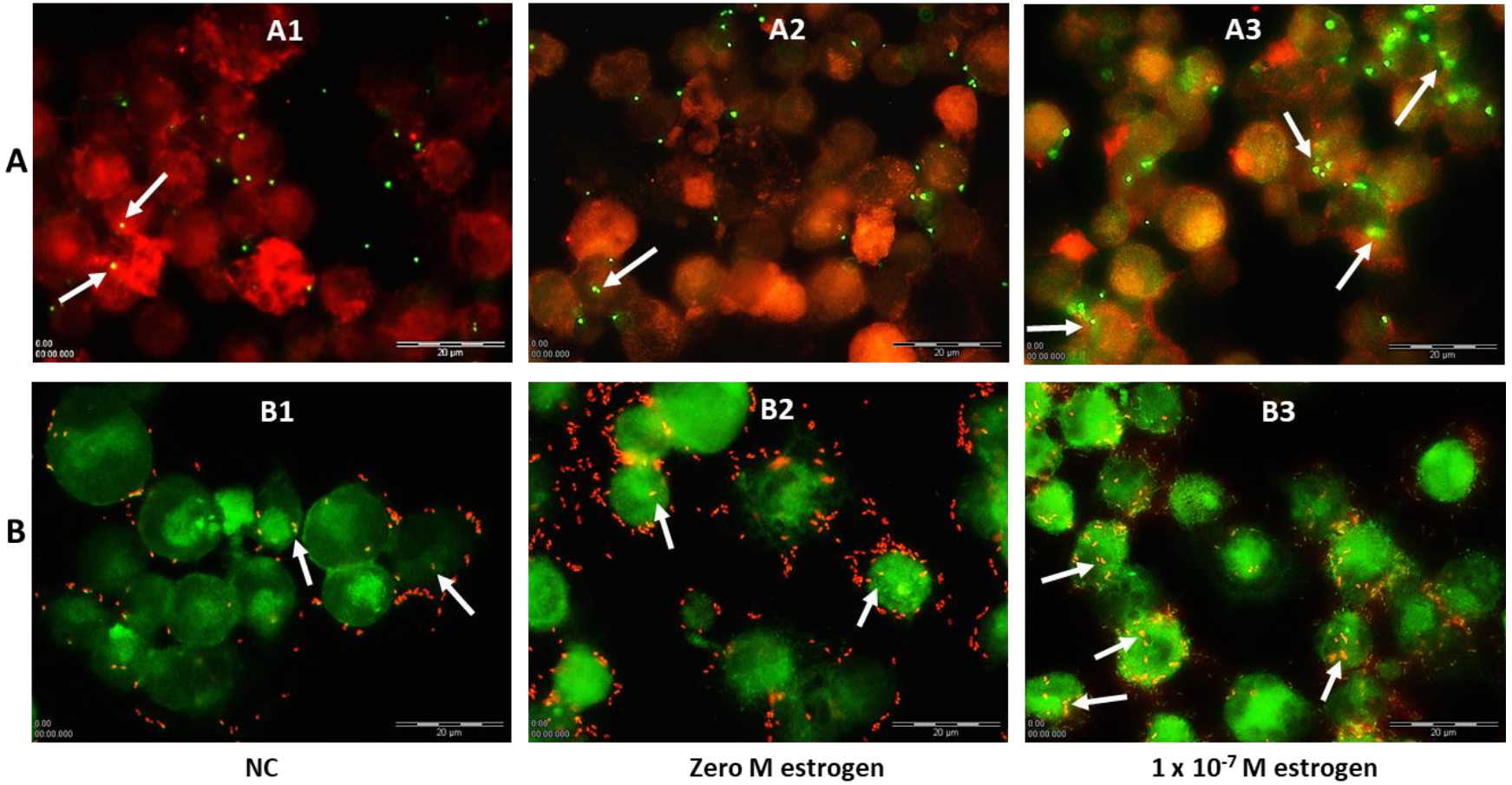

Figure 3.14. Effect of estrogen on the internalisation of GFP labelled-*S, aureus* (A) and mCherry-*P. aeruginosa* by U937 macrophages. U937-derived macrophages were treated with/without supraphysiological (1 x 10-7 M) estrogen supplementation for 24 hours prior to incubation with GFP labelled-*S, aureus* SH1000 (A) or mCherry labeled-PAO1 (B) for 3 hours. U937-derived macrophages were stained for phalloidin–tetramethylrhodamine B isothiocyanate conjugate from Amanita phalloides (A) or mouse anti-human CD18 monoclonal antibody followed by a goat anti-mouse IgG (H+L) superclonal secondary antibody, alexa fluor® 488 conjugate (B). The internalisation of *S. aureus* and *P. aeruginosa* by U937-derived macrophages (white arrows) was promoted by treatment with estrogen (A3 and B3) compared to negative control (NC) (A1 and B1) or macrophages lacking estrogen exposure (A2 and B2).

3.4.8.2 Z-Stack Analysis by Confocal Microscopy

To confirm the internalisation of bacteria by estrogen-treated U937 macrophages, Z-stacks were performed on phalloidin-stained phagocytes to visualise a series of layers through the interior of phagocytes and identify intracellular GFP-*S. aureus* colonies using a 63X objective on a Leica TSC SPE1000 confocal microscope. Z-stacks (example layer shown in (Figure 3.15) spanning the mid-intracellular region of estrogen-treated U937 macrophages (stained red) contained GFP-*S. aureus* colonies that fluoresced green, confirming the intracellular localisation of GFP-*S. aureus* inside (red) macrophages (Panel C). The size of Z-steps was set at 0.04 µm in this experiment.

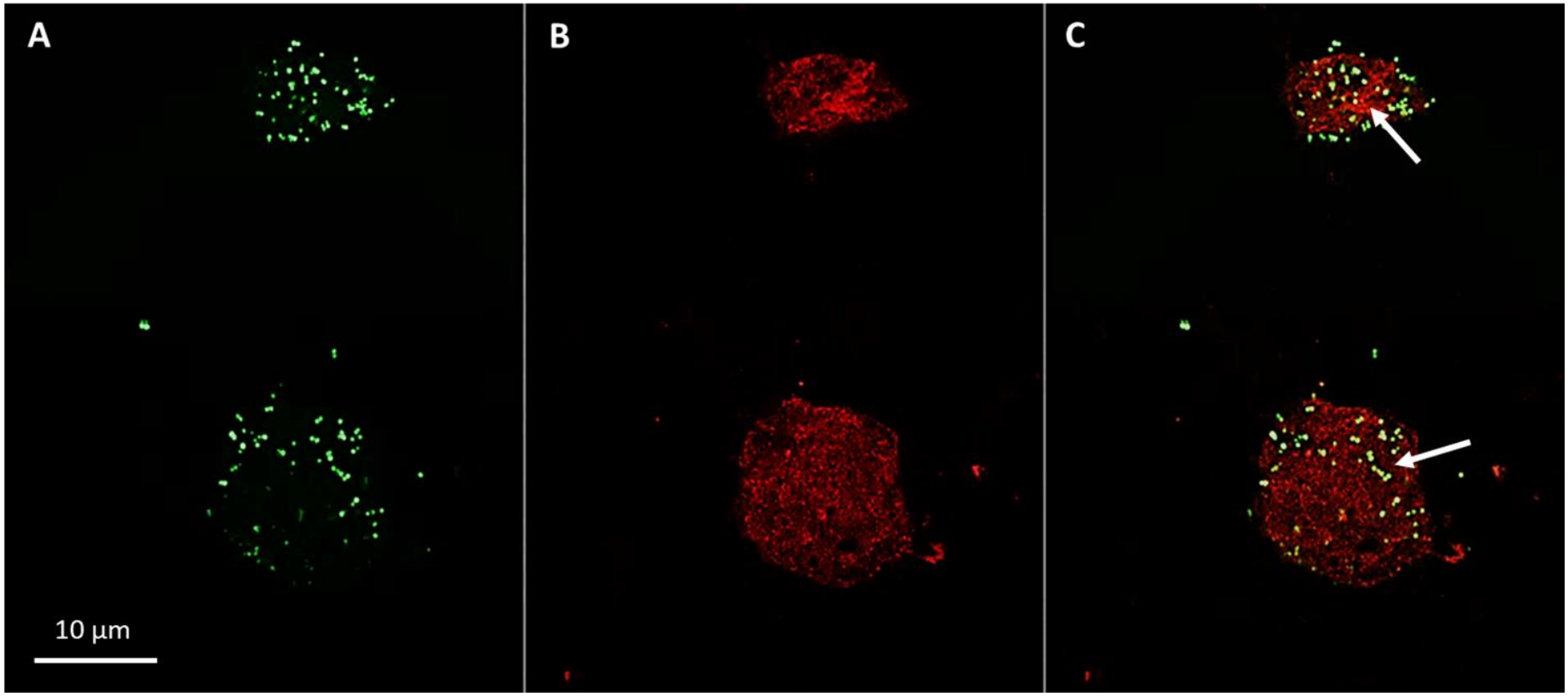

Figure 3.15. Z-Stack Demonstrating GFP-*S. aureus* Localisation inU937-Derived M0 macrophages Following Estrogen Supplementation. Bright green fluorescence was observed from GFP labeled *S. aureus* SH1000 (A). U937-derived M0 macrophages stained with phalloidin–tetramethylrhodamine B isothiocyanate conjugate from Amanita phalloides fluoresced red (B). The merged image confirmed several GFP-*S. aureus* colonies were located in the mid-intracellualr region of estrogen-treated U937 M0 macrophages after 3 hours of host-pathogen interaction (C).

3.4.8.3 Scanning Electron Microscopy

In order to visualise and compare detailed features of the interactions between MRSA or PAO1 and macrophages, samples were visualised using a Supra 40VP scanning electron microscope as described in 2.2.11.2 (Figure 3.16). The moment of the MRSA and PAO1 uptake shows in the images (Panels B and C) as the estrogen treated macrophage sends out pseudopodia to engulf the invading bacteria. Surprisingly, the morphological response of macrophages toward PAO1 (Panewl B) was distinct to and somewhat more pronounced than the response to MRSA (Panel C) in terms of the formation of membrane extensions, warranting further investigation. In concordance with these findings, PAO1 recovery was consistently higher than MRSA recovery in all host-pathogen experiments, supporting the notion of bacteria-specific interactions.

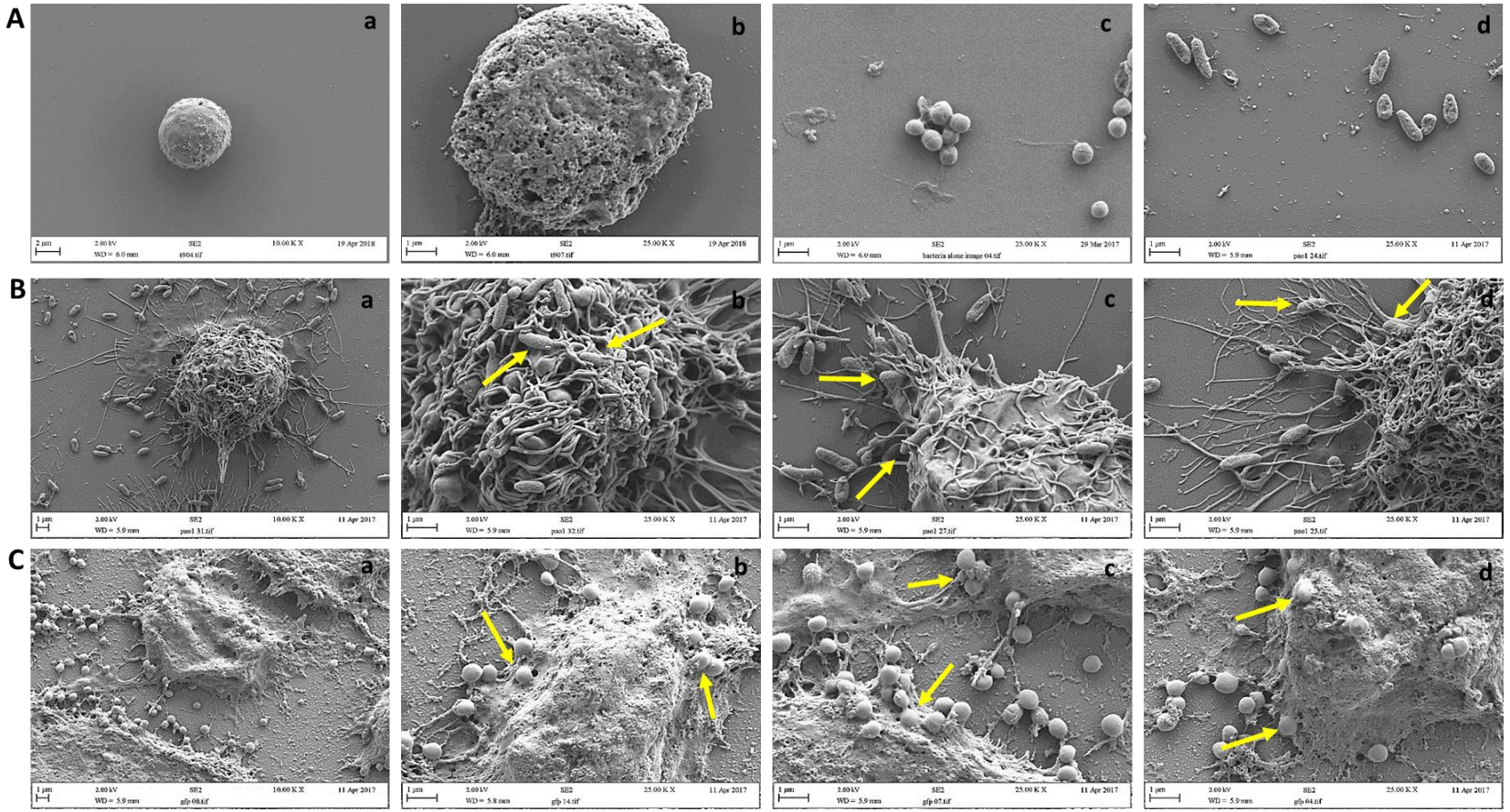

Figure 3.16. The Interaction of U937-derived M0 Macrophages with Gram Positive (MRSA11) and Gram Negative (PAO1) Bacteria Following Estrogen Supplementation. SEM images illustrating morphological changes in membrane extensions in estrogen-treated, U937-derived M0 macrophages after a 1h period of host-pathogen interaction with MRSA11 and PAO1. Control U937-derived M0 macrophages (Aa, Ab), MRSA11 (Ac) and PAO1 (Ad) were visualised before conducting the host-pathogen assay. Following the 1-hour period of host-pathogen interaction, estrogen-treated U937 M0 macrophages sent out morphologically distinct pseudopodial extensions to engage PAO1 (B) and MRSA (C) bacteria (yellow arrows).

3.5 Discussion

Wound infection is commonly quoted as a significant cause of delayed wound healing in the elderly and chronic wounds (Yotis, 1967). Chronic wounds are frequently colonised with a diverse range of Gram-positive and Gram-negative bacteria particularly *S. aureus* and *P. aeruginosa* (Dowd *et al*., 2008). Studies of age-related impaired wound healing suggest that sex steroid hormones, particularly estrogen, may have a significant impact on the inflammatory response *in vivo*. Treatments with topical and systemic estrogen have been indicated to enhance the rate of acute wound healing in both elderly men, and particularly post-menopausal women, by reducing the inflammatory response (Gilliver *et al*., 2007). Although estrogen has been shown to promote human acute wound healing in the elderly (Ashcroft *et al*., 1999a), the effect of this hormone on bacterial clearance and wound infections has received little attention to date.

First, it was very important to develop and generate a successful *in vitro* model of monocyte-derived macrophages. PMA induced the differentiation of both U937 and human peripheral blood monocytes (HPBM) into M0 macrophages (Figure 3.2 and figure 3.3). This was confirmed via a high expression of the CD11c surface marker by macrophages, with monocytes lacking the marker. U937 and HPBM are known precursors of tissue macrophages and dentritic cells *in vitro* (Sintiprungrat *et al*., 2010)**.** PMA was described to induce the conversion of monocytes into macrophage-like cells *in vitro* (Martinez *et al*., 2008; Rios de la Rosa *et al*., 2017). Our results are supported by several reports as recent research reported that PMA-treated monocytes express high levels of CD11b, CD11c and protein kinase-C (PKC) (Sintiprungrat *et al*., 2010). When treated with PMA, monocytes were shown to upregulate cell adhesion molecules including the beta-2integrins (CD11a, CD11c, CD18 and CD11b) allowing the cells to attach to cell culture flask surfaces *in vitro*. PMA triggers calcium and phospholipid-dependent isoforms of PKC and encourages cyclic AMP metabolism, initiating maturation into a macrophage-like morphology (Luscinskas *et al*., 1994).

A 3-hour incubation of both MRSA and *P. aeruginosa* with estrogen had no significant effect on their rate of growth. Therefore, any direct effect of estrogen on MRSA and PAO1 could be excluded during the host-pathogen assays in this study, with any changes in the number of bacteria instead arising solely due to the effect of estrogen on the interaction of macrophages with bacteria. These results are supported with many previous studies showing steroids, particularly estrogen and progesterone, have no effect on bacterial growth (Lev, 1959). However, some non-endogenous steroids have been reported to decrease the growth of many Gram-positive bacteria (Casas-Campillo *et al*., 1961; Yotis, 1967), but the exposure to these steroids periods was for substantially longer periods than 3 hours.

The effect of phenol red on the host-pathogen interaction assays was investigated. Phenol red in the RPMI cell culture medium had no significant effect on MRSA and PAO1 recovery compared to RPMI medium lacking phenol red, regardless of the presence or absence of estrogen. Phenol red was shown to have very weak estrogenic effects on MCF-7 human breast cancer cells, with an affinity that was 0.001% that of 17β-estradiol (Berthois *et al*., 1986). Other studies reported that phenol red had no effect on estrogen-sensitive T47D breast cancer cells, primary immature rat pituitary cells or immature rat uterine cells (Welshons *et al*., 1988). Thus, published findings are in concordance with the data in this study, demonstrating phenol red in the cell culture medium has negligible influence on host-pathogen interactions.

Multiplicity of infection (MOI) assays were then performed to investigate the effect of physiological estrogen (1×10^{-8} M) on phagocytosis over a range of starting bacterial inoculations (1×10^{3} CFU, 1×10^{4} CFU, 1×10^{5} CFU and 1×10^{6} CFU) for both MRSA or PAO1. The SERM tamoxifen, an estrogen-like molecule was found to enhance the bactericidal activity of neutrophils against *P. aeruginosa in vitro* and MRSA *in vivo* where (Corriden *et al*., 2015) conducted *in vitro* host-pathogen assays at MOIs of 0.1. Findings in this study agree with published reports. The bacterial recovery of MRSA and PA01 following estrogen (1×10^{-8} M) supplementation significantly increased as the MOI [bacteria: macrophage ratio] increased from 0.001 to 1. However, the bacterial recovery followed a similar pattern throughout,

regardless of the MOI ratio, with estrogen consistently increasing (P<0.05) bacterial clearance at all MOIs.

The effect of estrogen on the phagocytosis of the two major wound pathogens MRSA and *P. aeruginosa* by macrophages was investigated in this study. The results of the initial *in vitro* host-pathogen interaction assays (3.4.5.1) demonstrated novel data showing estrogen significantly (P<0.05) enhances the internalisation of both MRSA and *P. aeruginosa* in a dose-dependent manner following exposure to activated U937-derived M0 macrophages *in vitro*.

Li *et al*. (2000) extracted microglial cells from brain tissues of patients with Alzheimer disease. Similar to experimental procedures in this chapter, the macrophages were treated with 1 x 10^{-7} M estrogen for 24 hours and 48 hours prior to incubation with amyloid β-peptide (Aβ) for 24 hours. Estrogen was found to enhance the uptake of Aβ by brain macrophages (microglia) in a time- and dose-dependent manner. In order to confirm that estrogen enhances the phagocytic activity of microglia via a general engulfment mechanism, not specific to Aβ only, Li *et al*. (2000) re-conducted the same experiments replacing Aβ with *Escherichia coli*. Intriguingly, estrogen significantly increased the internalisation of *E.coli* in a concentration-dependent manner in comparison with the vehicle controls. These findings highlight the beneficial effects of estrogen on the phagocytic ability of microglia in the brain. However, other types of macrophages were not assessed and only *E.coli* was investigated so it remains unclear whether the effects of estrogen could be observed with varied bacterial species and strains, especially antibiotic-resistant strains.

Crompton *et al*. (2016) investigated the effect of estrogen on cutaneous murine wound healing following exposure to LPS derived from *Klebsiella pneumoniae*. Exposure of immune cells to LPS caused a substantial delay in wound healing. They then co-treated LPS-treated wounds with 17β-estradiol, and interestingly, the delay in wound repair was reversed, with accelerated healing noted in mice following estrogen treatment (Crompton *et al*., 2016). The results of this report were interesting but Crompton *et al*. (2016) only used locally applied *K. pneumonia*-derived LPS in their model of infection. In order to generate realistic models of

infected wounds it is important to develop assays that infect wounds with live bacterial strains, not just bacterial-derived factors such as LPS. It is also important to consider both Gram-positive and Gram-negative models of infection. Moreover, *K. pneumoniae* is not a major wound pathogen compared to *S. aureus* and *P. aeruginosa*, which are the most frequently isolated bacterial species from infected wounds (Giacometti *et al*., 2000; Gjødsbøl *et al*., 2006; Kirketerp-Møller *et al*., 2008; Rybtke *et al*., 2015).

In vitro studies using cell lines provide only limited evidence to predict effective effects in humans. Thus, this study also confirmed findings in *ex vivo* primary human peripheral blood monocytes (HPBM) to establish a body of evidence using both a Gram-positive and a Gram-negative bacterium. Similar to results obtained with U937-derived macrophages *in vitro*, physiological and supraphysiological estrogen significantly increased ($P<0.05$) the internalisation of both MRSA and PAO1 in comparison to the negative control.

Li *et al*. (2000) investigated the effect of estrogen on the uptake of fluorescent-*E.coli* by microglial cells using a fluorescence microplate reader, and showed estrogen enhanced the internalisation of *E.coli* in a concentration- and time-dependant manner. In line with this, and using similar experimental settings, data from the M0 macrophages-GFP *S. aureus* interaction assay strengthened the host-pathogen *in vitro* and *ex vivo* findings, indicating greater bacterial uptake following treatment with physiological and suppraphysiological estrogen. Ashcroft and co-researchers reported that ovariectomized mice presented with high numbers of macrophages in injured tissues compared to wounds of normal healthy mice. Interestingly, in this thesis estrogen increased the number of macrophages that become active phagocytes following exposure to pathogens, thereby providing a justifiable reason for enhanced bacterial clearance described by Gilliver *et al*. (2007).

The effect of estrogen on the absolute killing of bacteria was investigated in the gentamycin protection assay. Gentamicin eradicates extracellular bacteria but it has no effect on intracellular bacteria due to poor ability to permeate the macrophage membrane (Waldbeser *et al*., 1994; Hess *et al*., 2004; Oelschlaeger, 2010; Hamad *et*

al., 2010). Interestingly, there was a significant decrease in the number of viable intracellular viable bacteria recovered following treatment of macrophages with physiological or supraphysiological estrogen, demonstrating estrogen promotes the killing of internalised pathogens, not just the uptake of bacteria by phagocytes.

Published research demonstrated that extended exposure of *ex vivo* macrophages to estrogen augments the expression of iNOS and the production of numerous cytokines, particularly IL-1β, IL-12 and IL-6 following TLR4 activation by LPS *in vitro* (Deshpande *et al*., 1997; Ghisletti *et al*., 2005). The findings of this report combined with findings in Crompton *et al*. (2016) and Li *et al*. (2000) support the data in this Chapter, showing estrogen promotes the microbicidal activities of macrophages against bacteria.

In order to visualise the phagocytosis of *S. aureus* and *P. aeruginosa* with/without estrogen treatment, host-pathogen assays were conducted using fluorescent GFP *S. aureus* or mCherry *P. aeruginosa*. Visualisation of host-pathogen interactions was conducted via epifluorescent microscopy (Figure 3.14) and confocal microscopy (Figure 3.15). The findings demonstrated estrogen increased the internalisation of *S. aureus* and *P. aeruginos*a by macrophages, in concordance with the bacterial recovery data obtained from *in vitro* host-pathogen assays.

Images captured by SEM also confirmed increased host-pathogen interaction following treatment of macrophages with estrogen. It was noted that estrogen-treated macrophages had distinct morphological responses to MRSA and PAO1, in terms of pseudopodial formation. This finding was in concordance with data in the host-pathogen assays showing PAO1 recovery was consistently higher than MRSA recovery. The SEM images represent novel results that merit further investigation to determine the underlying mechansisms that might lead to pathogen-specific differences in pseudopdial formation. In order to better understand the biochemical processes occurring at the host-pathogen interface, future work could determine the effect of estrogen on physical and chemical changes in phagocytes. Fourier Transform Infra-Red (FTIR), spectroscopy and Raman spectroscopy can be used to determine changes in the functional groups on the cell surface of active phagocytes treated with estrogen.

The underlying mechanism by which estrogen affects bacterial phagocytosis has not been investigated yet. It is possible that estrogen promotes bacterial clearance by inducing genes or pathways involved in the processes of phagocytosis via activation of ER proteins. Future investigations will determine the precise ER pathways involved in estrogen-enhanced phagocytosis. Determining the mechanisms by which estrogen induces phagocytosis my lead to novel therapeutic strategies that combat wound infections in the elderly by modulating the host immune response. One potential avenue will be to use selective estrogen receptor modulators (SERMs) such as tamoxifen that act as agonists of ER proteins in a tissue-specific manner.

Tissue macrophages exist in different activation states: either pro-inflammatory (classically activated) by LPS or IFN-γ, referred to as M1 macrophages, or anti-inflammatory (alternatively activated) by IL-13 or IL-4, referred to as M2 macrophages (Aron-Wisnewsky *et al*., 2009). Preliminary assays in this study were performed on PMA-differentiated M0 macrophages. Further work will investigate M1 and M2 macrophages to determine whether estrogen influences phagocytosis in an activation-dependent manner.

Images from the SEM (Figure 3.16) illustrated major morphological changes in the membrane of phagocytes (macrophages) following estrogen treatment, with the establishment of multiple pseudopodial structures suggesting estrogen induces significant alterations in the actin cytoskeleton of phagocytes. These findings suggested the downstream genes mediated by estrogen may include mediators of the actin cytoskeleton. Thus, further investigations could measure differences in activated/total levels of proteins involved in the regulation of the actin cytoskeleton, such as members of the Rho GTPase family.

Findings of this study are novel and support the preliminary work performed by Crompton *et al*. (2016). In summary, bacterial phagocytosis by macrophages was elevated following stimulation with physiological (typical of youth) and supraphysiological estrogen levels compared to estrogen deprivation and an absolute absence of estrogen. This suggests that the ageing process (age-related decline in estrogen levels) may increase the propensity and progression of wound

infections in the elderly, in line with the findings of Ashcroft *et al*. (1999a). The findings of this study suggest local and/or systemic hormone replacement therapy (HRT) might resolve or reduce the likelihood of Gram-positive and Gram-negative wound infections in the elderly.

Chapter 4: Effect of Estrogen on the Phagocytosis of MRSA and *P. aeruginosa* by M1-like and M2-like Macrophages

4.1 Introduction

4.1.1 Macrophages Polarisation and Plasticity

The plasticity of macrophages is established by acquisition of distinct functional and morphological characteristics directed by particular tissues, and/or immunological and environmental stimuli (Mantovani *et al.*, 2005; Gordon and Martinez, 2010; Wynn *et al*., 2013). Macrophages in tissues have several similar characteristics but they are an extremely heterogeneous population of cells in relation to their function and expression of surface markers (Figure 4.1) (Mackaness, 1964; Nathan *et al*., 1983; Stein *et al*., 1992; Mantovani *et al*., 2005; Murray and Wynn, 2011; Murray *et al*., 2014). Macrophages are key effectors of the innate immune system, providing an initial defence against foreign invaders such as bacteria (Duque and Descoteaux, 2015). They also play key roles in initiating and controlling the adaptive immune responses (Duque and Descoteaux, 2015). Macrophages are crucial in tissue repair, haemostasis, inflammation and remodelling (Mosser and Edwards, 2010). As a condition of a favourable resolution of inflammation, macrophages remove apoptotic leukocytes, and dysregulation in this particular macrophage role leads to chronic inflammatory and autoimmune diseases (Balhara and Gounni, 2012). Macrophages have multiple functions and characteristics (pro-inflammatory or anti-inflammatory) in immunity, depending on the type of stimulation and resulting functional phenotype (Martinez *et al*., 2008). Macrophages were initially classified into two major phenotypes based on type 1 helper T-cell (Th1) and type 2 helper T-cell (Th2) polarisation (Hesse *et al*., 2001; Puig-Kröger *et al*., 2004; Yona *et al*., 2013; Martinez and Gordon, 2014). Cytokines mainly released by Th1 cells, such as IFN-γ differentiate macrophages to pro-inflammatory M1 macrophages. However, cytokines released by Th2 cells such as IL-4 have been reported to dampen macrophage activation, resulting in M2 anti-inflammatory macrophages (Raes *et al*., 2002; Kzhyshkowska *et al*., 2006).

4.1.1.1 Classically Activated Macrophages

In response to cytokines such as IFN-γ, and/or bacterial components such as LPS, macrophages are polarized to a pro-inflammatory phenotype and are referred to as classically activated or M1 macrophages. Classical activation or M1 polarisation are terms that were first used during the 1960s when studies on mice reported macrophages with enhanced anti-bacterial activities towards infections with *Mycobacterium bovis bacillus Calmette-Guerin* (BCG) or *Listeria monocytogenes* (Mackaness, 1964). The cytokine IFN-γ produced by TH1 cells was described as the main activator of antimicrobial and cytotoxic activities of macrophages (Mosmann *et al*., 1986). Genetic deficiencies of IFN-γ and/or its receptors confirmed that macrophages polarisation to the M1 phenotype is essential for host defence against pathogenic infections in knockout mice models and in humans. Components of intracellular pathogens (e.g. LPS), TNF, GM-CSF and TLR4 ligands were also described to encourage M1 polarisation (Mantovani *et al*., 2005).

M1 macrophages stimulate TH1 immunity by releasing high levels of IL-12 (Mantovani *et al*., 2004), producing microbicide enzymes such as inducible NO synthase (iNOS) to destroy pathogenic invaders, secreting pro-inflammatory cytokines, such as, IL-1β, IL-6, IL-12, IL-23, TNF-α, and releasing chemokines to attract immune cells to the infection site and MMPs (Mantovani *et al*., 2004).

4.1.1.2 Alternatively Activated Macrophages

In 1990, a study reported for the first time the inhibition of M1 polarisation. This study revealed that the production of IFN-γ induced superoxide (O_2^-) by IL-4 was inhibited (Abramson and Gallin, 1990). Later on the 1990s, Gordon and colleagues described the term “alternatively activated macrophages (Stein *et al*., 1992). It is commonly believed that M2 macrophages are involved in tissue repair, fibrosis and tumour progression (Wynn, 2004). They are identified by expression of surface markers, mainly the macrophage mannose receptor CD206 (Stein *et al*., 1992) and CD163 (Zeyda *et al*., 2007). Arginase (Arg-1) is an enzyme that is involved in the production of proline via conversion of arginine to ornithine (Martinez *et al*., 2008). Proline has been linked with ECM formation and collagen deposition (Hesse *et al*.,

2001). M2 macrophages release anti-inflammatory cytokines, such as, IL-10, and are crucial in resolving chronic inflammation, stimulating angiogenesis and fibrogenesis and promoting tissue remodelling (Mosser and Edwards, 2008).

Depending on the stimuli that induces polarisation of macrophages to the M2 phenotype, M2 macrophages were divided into three subpopulations: M2a encouraged by stimulation with IL-4 and/or IL-13, M2b encouraged by TLR lignans, immune complexes (ICs), or IL-1R and M2c induced by IL-10/TGF-β (Mantovani *et al*., 2004).

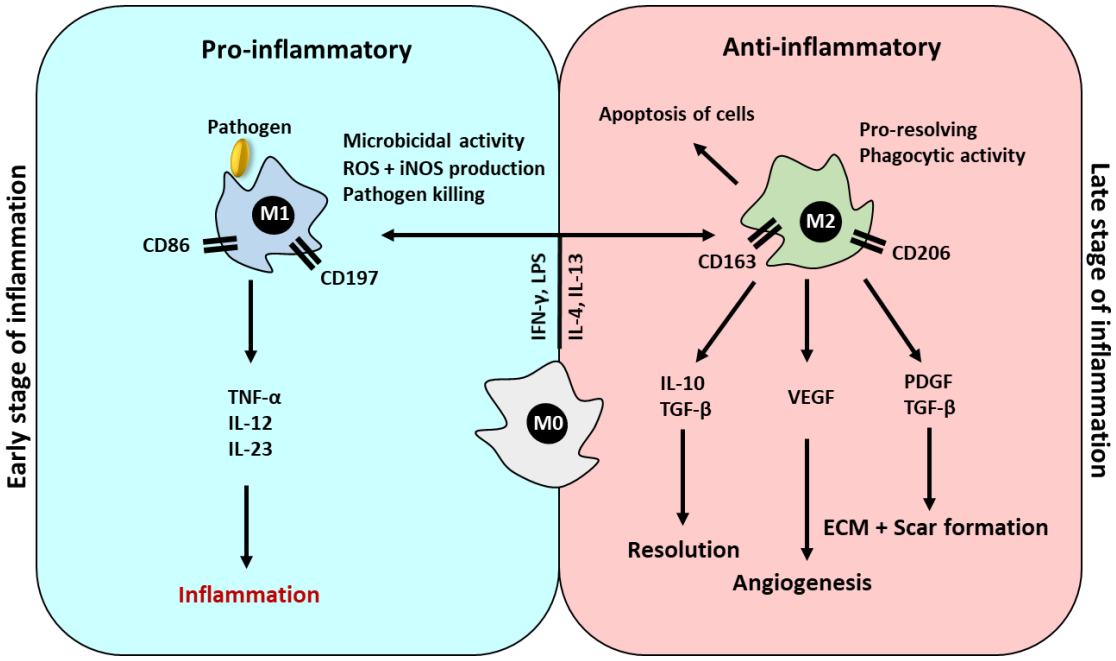

Figure 4.1. Plasticity of macrophages in wound healing. Following an injury, monocytes infiltrate to the wound site and become macrophages (M0). During the early stage of inflammation, macrophages switch to the M1 phenotype. M1 macrophages have a microbiocidal activity and induce TH1 responses via release of IL-12. Close to the end of the inflammatory stage, the microenvironment of the wound changes and the process of clearing apoptotic cells initiates the polarisation of macrophages toward M2 macrophages. M2 cells play a key role in the resolution of inflammation and progression to subsequent stages of wound healing. Both M1 and M2 macrophages are key players in switching from inflammation to proliferation (angiogenesis, re-epithelialisation and matrix production). Figure drawn based on information in (Benoit *et al*., 2008; Krzyszczyk *et al*., 2018).

4.1.2 Macrophage Phenotypes and Plasticity in Wound Healing

The proliferation of primary monocytes is limited *in vitro* but evidence suggests skin macrophages can auto-renew *in vivo* (Davies *et al*., 2013). In contrast, it remains unclear if macrophages are only recruited to the wound site when required and

removed after wound repair, or if macrophages deriving from monocytes can proliferate *in vivo* (Italiani and Boraschi, 2014).

Macrophages were initially classified into two major phenotypes referred to as M1 and M2 macrophages. M1 macrophages are commonly linked with pro-inflammatory events, while M2 macrophages are known to be anti-inflammatory and pro-regenerative. It is essential to understand that M1 and M2 macrophages are not separate types of cells, but they form a scale in which they take varying degrees of M1 or M2 properties. In addition, *in vivo* studies propose that macrophages are a heterogeneous group of cells all displaying a diversity of M1 and M2 characteristics (Martinez and Gordon, 2014; Murray *et al*., 2014; Ogle *et al*., 2016).

Distinct M1 and M2 macrophages classification is not applied when comparing wound healing macrophages *in vivo*. However, this classification is widely used *in vitro* when molecules inducing the polarisation are known and experimentally introduced to the system (Novak and Koh, 2013). The types and suggested roles of macrophages related with wound healing *in vitro* and *in vivo* are illustrated (figure 4.2).

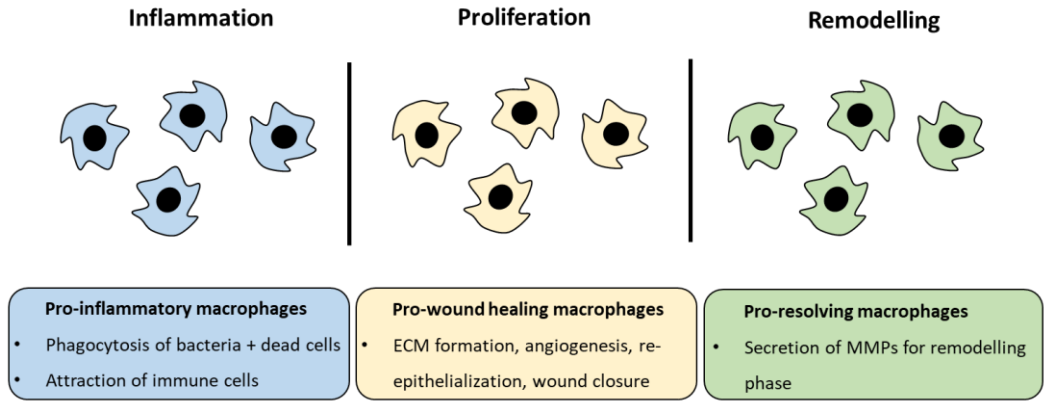

Figure 4.2. The role of macrophage phenotypes in wound repair. During haemostasis and inflammation, pro-inflammatory macrophages invade the wound area and start removing dead cells and bacteria. During the proliferative stage, pro-wound healing macrophages are present and release factors such VEGF, PDGF, TGF-β to aid angiogenesis, ECM and scar formation and re-epithelialization. During remodelling, pro-resolving macrophages are involved in strengthening the new skin barrier.

In line with the wound healing process, pro-inflammatory macrophages are dominant at the wound site after an injury, followed by pro-wound healing

macrophages that stimulate the growth of cells during the proliferative phase, and finally pro-resolving macrophages that dampen immune responses and stimulate the remodelling and maturation of collagen (Vannella and Wynn, 2017).

During wound healing, pro-inflammatory macrophages are a source of NO, ROS, IL-1, IL-6, and TNF-α. They release MMPs in order to disrupt the extracellular matrix and create a space for inflammatory cells to infiltrate the wound (Murray and Wynn, 2011). They also release important levels of growth factors such as PDGF, VEGF, insulin-like growth factor 1 (IGF-1) and TGF-β1 which facilitate the proliferation of cells involved in angiogenesis and ECM deposition (Murray and Wynn, 2011). Pro-resolving macrophages dampen inflammation by upregulating IL-10 and produce MMPs to remodel and reinforce the ECM (Vannella and Wynn, 2017). The main roles of pro-resolving macrophages are to reinstate homeostasis and minimize fibrosis via apoptosis of myofibroblasts, and suppression T cell proliferation (Murray and Wynn, 2011).

Knowing that wound healing is a sequence of overlapping phases, all types of macrophages share many characteristics simultaneously. This is particularly true for pro-wound healing macrophages as they fluctuate between the early and late stages of wound repair, and therefore display similar features to pro-inflammatory and pro-resolving macrophages.

M1 macrophages are the dominant pro-inflammatory macrophages during the early pro-inflammatory stage of wound repair. They are phagocytic, and serve as cleaners of the wound site by removing foreign invaders and dead tissue. *In vitro*, M1 macrophages are induced by intracellular cytokines such as IFN-γ, and/or bacterial elements, such as LPS and peptidoglycans. M1 macrophages are distinguished *in vitro* by the expression of the chemokine receptor CCR7 (CD197) and high levels of the co-stimulatory molecules CD80 and CD86, resulting in efficient antigen presentation capacity (Mantovani *et al*., 2004).

M2 macrophages play a regenerative role in wound healing. They are induced by IL-4 and IL-13 and characterised by a high expression of the mannose receptor (CD206) (Stein *et al*., 1992), the haemoglobin scavenger receptor (CD163) (Zeyda *et*

al., 2007), IL-10, and TGF-β. M2 macrophages release only very low levels of pro-inflammatory mediators such as TNF-α, IL-12, and IL-8. In wounds M2 macrophages have been divided into three subtypes (M2a, M2b and M2c) relating to expression of different surface markers. The three subtypes are commonly identified and used *in vitro* to investigate M2 phenotype characteristics. However, distinct M2 macrophages classification is not widely applied when referring to wound healing *in vivo*. This is due to the heterogeneous populations present, which are generated from a variety of stimuli within wounds (Novak and Koh, 2013). Studies have reported that the wound healing M2 macrophages align with the well-defined subset of M2a (Ogle *et al*., 2016). M2a macrophages are induced by IL-4/IL-13 and distinguished by the expression of CD206. They produce arginase-1, IGF-1, PDGF-BB and a variety of chemokines (CCL17, CCL18, CCL22) (Ogle *et al*., 2016). They are crucial in the proliferative stage of wound healing by aiding ECM formation via production of collagen precursors and stimulation of fibroblasts. M2 macrophages also release high levels of PDGF, which is involved in angiogenesis (Vannella and Wynn, 2017).

Overall, macrophage classification is complex and not yet fully understood with uncertainty whether macrophage phenotypes are distinct, or even appropriate to *in vivo* models of wound healing (Martinez and Gordon, 2014). Although the mechanism is not understood, a balance between the M1 and M2 phenotypes is essential to preserve a homeostatic environment, and imbalance leads to disturbed inflammation.

Exogenous estrogen supplementation enhances healing in elderly people, indicating that the systemic and peripheral age-related decrease in endogenous estrogen levels impairs healing in both sexes (Ashcroft *et al*., 1997b; Hardman and Ashcroft, 2005). Known for its anti-inflammatory proprieties, estrogen is reported to resolve excessive inflammation by directly effecting inflammatory responses of monocytes and macrophages (Ashcroft *et al*., 2003; Guo and DiPietro, 2010). Estrogen has been reported to accelerate re-epithelialisation, promote angiogenesis, increase matrix deposition and wound contraction, dampen the inflammatory response and inhibit the release of pro-inflammatory cytokines (Ashcroft and Ashworth, 2003).

These findings suggest estrogen modulated both pro-inflammatory (M1) and anti-inflammatory / pro-regenerative (M2) macrophages during wound repair. Estrogen is also believed to be a key player in regulating immune responses against bacterial infections in wounds (Cutolo *et al*., 2004). Data in the previous chapter showed that estrogen significantly enhanced the phagocytosis of MRSA and *P. aeruginosa* by classical M0 macrophages, in a concentration-dependant manner when above levels described as estrogen deprivation. Using models of macrophage polarisation into M1 and M2 phenotypes, the effect of estrogen on the phagocytosis of MRSA and *P. aeruginosa* by M1-like and M2-like macrophages was investigated.

4.2 Aim and Objectives

4.2.1 Aim

To investigate the effect of estrogen (age-related changes in estrogen concentrations) on the phagocytic function of both pro-inflammatory (M1) and anti-inflammatory (M2) macrophages using *in vitro* and *ex vivo* models of host-pathogen interactions.

4.2.2 Objectives

- Generate an *in vitro* model of HPBM and U937 monocyte differentiation into M1-like and M2-like macrophages.

- Compare the phagocytic function of M1 and M2 macrophages under conditions of physiological and supraphysiological levels of estrogen with conditions of estrogen deprivation or an absolute absence of estrogen.

- Compare the effect of age-related changes in estrogen levels on the phagocytosis of Gram-positive and Gram-negative bacteria by M1-like and M2-like macrophages.

4.3 Methods

4.3.1 Flow Cytometry

U937 and HPBM were differentiated into M1-like and M2-like macrophages *in vitro* (Figure 4.3) as described in section 2.2.5. The expression of surface markers specific for M1 (CD197) and M2 (CD206) macrophages (relative MFI and % fluorescence) on M1- and M2-like cells were compared with untreated M0 control macrophages.

4.3.2 *In vitro* and *Ex Vivo* Models of Host-Pathogen Interactions

U937 and HPBM M1-like/M2-like macrophages were adhered to wells and treated with different concentrations (1 x 10^{-7} M, 1 x 10^{-8} M, 1 x 10^{-9} M and zero M) of estrogen in antibiotic-free medium for 24 hours to represent the models of age-related changes in estrogen levels described in Sections 2.2.2 and 2.2.8.2. Cells were incubated with MRSA or *P. aeruginosa* (1 x 10^4 CFU) for 3 hours at 37 °C and 5% CO_2 prior to counting bacterial colonies (CFU/mL) following the methods described in Section 2.2.8.2.

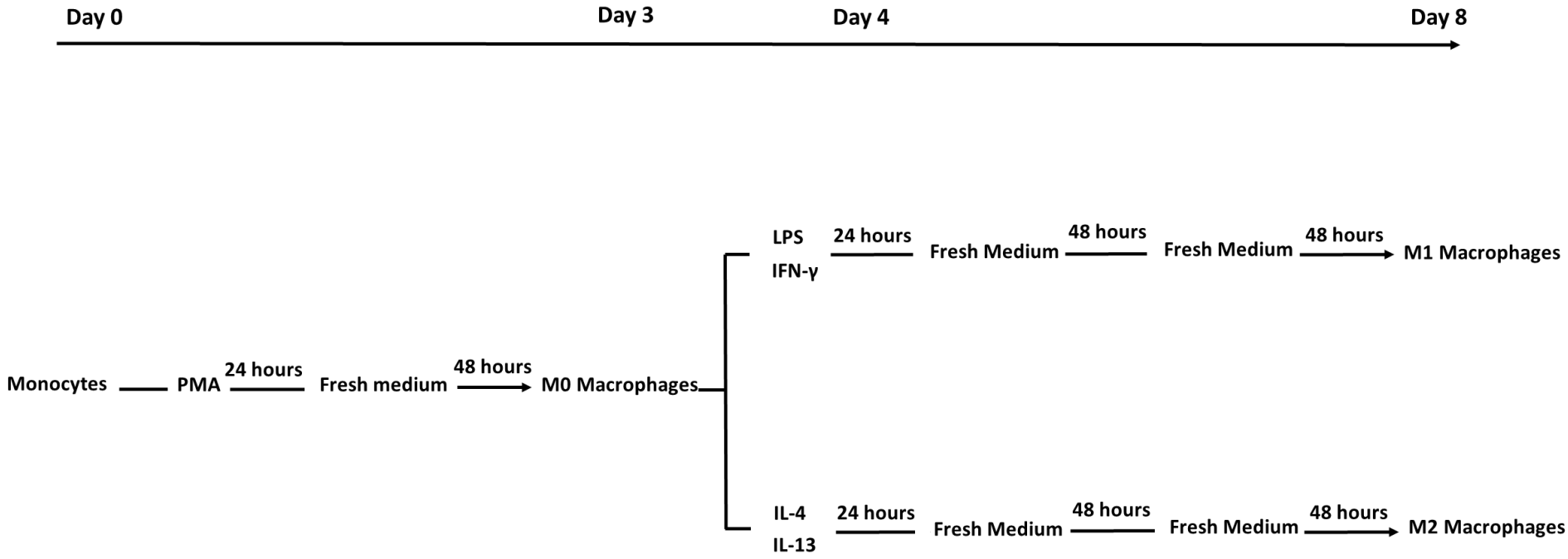

Figure 4.3. Experimental Generation of M1 and M2 Macrophages. U937 and $CD14^+$ HPBM were differentiated into adherent M0 macrophages using PMA for 24 hours (with medium being changed after 24 hours). Further polarisation into classically activated (M1) or alternatively activated (M2) macrophages was performed using LPS + IFN-γ or IL-4 + IL-13 for 24 hours, respectively. The medium was changed every other day to generate resting M1-like and M2-like macrophages.

4.4 Results

4.4.1 Differentiation of Monocytes into M1 and M2 Macrophages

The expression of CD197 and CD206, which are surface markers for M1 (Mantovani *et al*., 2004) and M2 (Stein *et al*., 1992) macrophages respectively, were examined by flow cytometry (Figure 4.4). The findings confirmed the successful conversion of U937 M0 macrophages into $CD197^+$ M1 and $CD206^+$ M2 macrophages. The U937 macrophages treated with LPS/IFN-γ were 94.7% $CD197^+$ and 0.4% $CD206^+$, whereas the U937 macrophages treated with IL-4/IL-13 were 6.6% $CD197^+$ and 79.8% $CD206^+$. The MFI for M1 surface marker CD197 was significantly (P<0.01) higher in U937 macrophages treated with LPS/IFN-γ compared to untreated U937 macrophages or U937 macrophages treated with IL-4/IL-13. In contrast, the MFI for the M2 surface marker CD206 was significantly (P<0.01) higher in U937 macrophages treated with IL-4/IL-13 compared to untreated U937 macrophages or macrophages treated with LPS/IFN-γ.

Similarly, the findings confirmed the successful conversion of HPBM-derived macrophages into $CD197^+$ M1 and $CD206^+$ M2 macrophages (Figure 4.5). There was substantial expression of the M1 surface marker CD197 (67.2%) by HPBM-derived macrophages treated with LPS/IFN-γ, whereas CD197 was almost absent in HPBM-derived M0 macrophages (1%) and at very low levels (5.7%) in HPBM-derived macrophages treated with IL-4/IL-13. The M2 surface marker CD206 was substantially expressed (73.4%) by HPBM-derived macrophages treated with IL-4/IL-13 whereas CD206 was almost absent from HPBM-derived M0 macrophages (0.1%) and HPBM-derived macrophages treated with LPS/IFN-γ (0.2%). The MFI for CD197 from HPBM-derived macrophages treated with LPS/IFN-γ was significantly (P<0.01) greater than from HPBM-derived M0 macrophages or HPBM-derived macrophages treated with IL-4/IL-13.In contrast, the MFI for CD206 from HPBM-derived macrophages treated with IL-4/IL-13 was significantly (P<0.01) higher than from HPBM-derived M0 macrophages or HPBM-derived macrophages treated with LPS/IFN-γ.

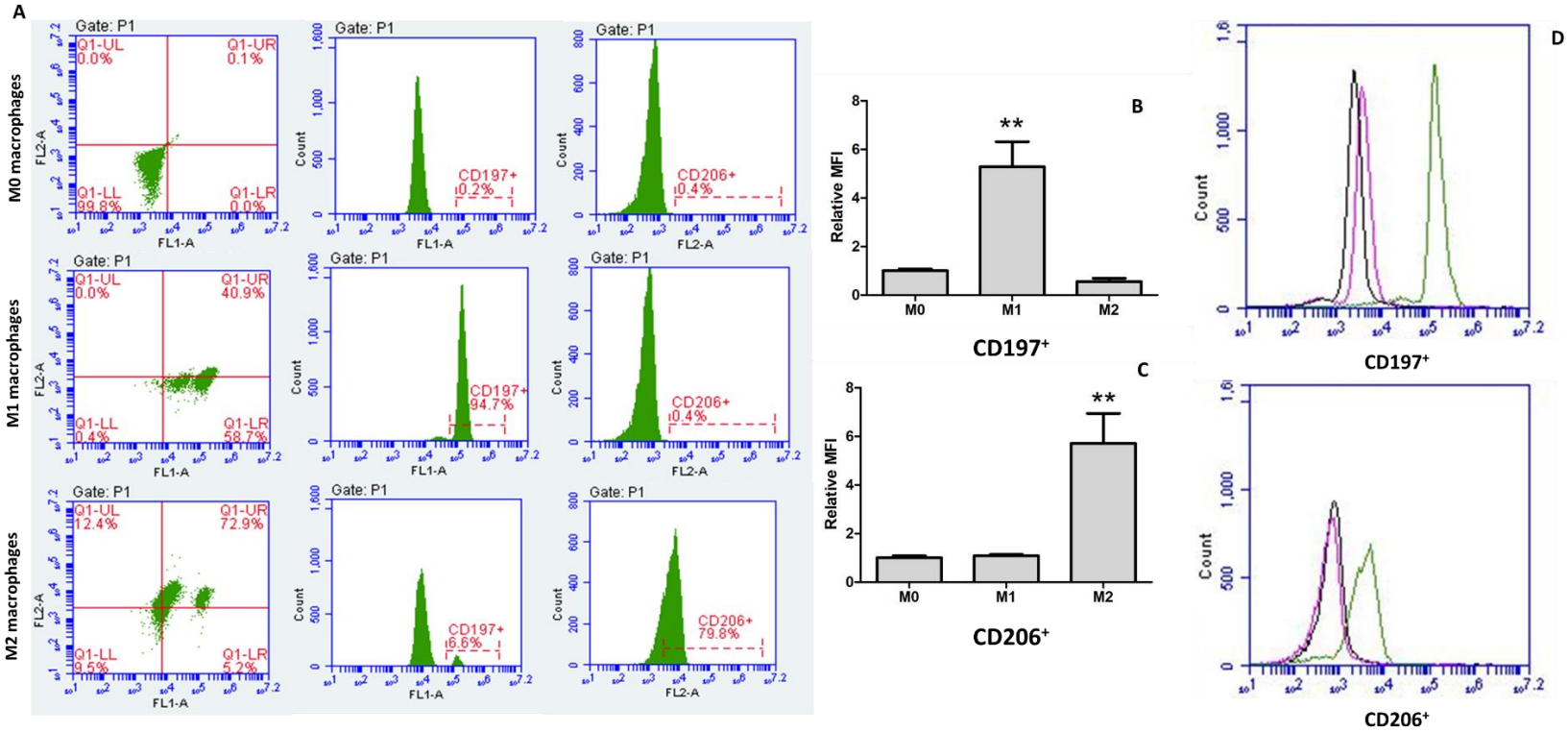

Figure 4.4. Differentiation of U937 M0 macrophages onto M1/M2 macrophages. The polarisation of U937 M0 macrophages into M1-like and M2-like macrophages was confirmed by through detection of CD197 and CD206 surface markers by flow cytometry respectively. M0 macrophages did not express CD197 or CD206 markers (0.2% and 0.4% respectively). U937 macrophages treated with LPS/IFN-γ expressed high levels of CD197 (94.7%) but almost lacked CD206 (0.4%). U937 macrophages treated with IL-4/IL-13 expressed CD206 (79.8%) but very low levels of CD197 (6.6%) (A). Three distinct populations of U937 macrophages were identified (D) with significantly higher MFI for CD197 from M1 macrophages compared to M0 and M2 macrophages (B), and a significantly higher MFI for CD206 from M2 macrophages compared to M0 and M1 macrophages. Data represent averages of n = 3 experiments. ** Indicates significant difference (One-way ANOVA) in MFI (P<0.01) compared to M0 macrophages. Error bars represent the standard error of the mean (StEM).

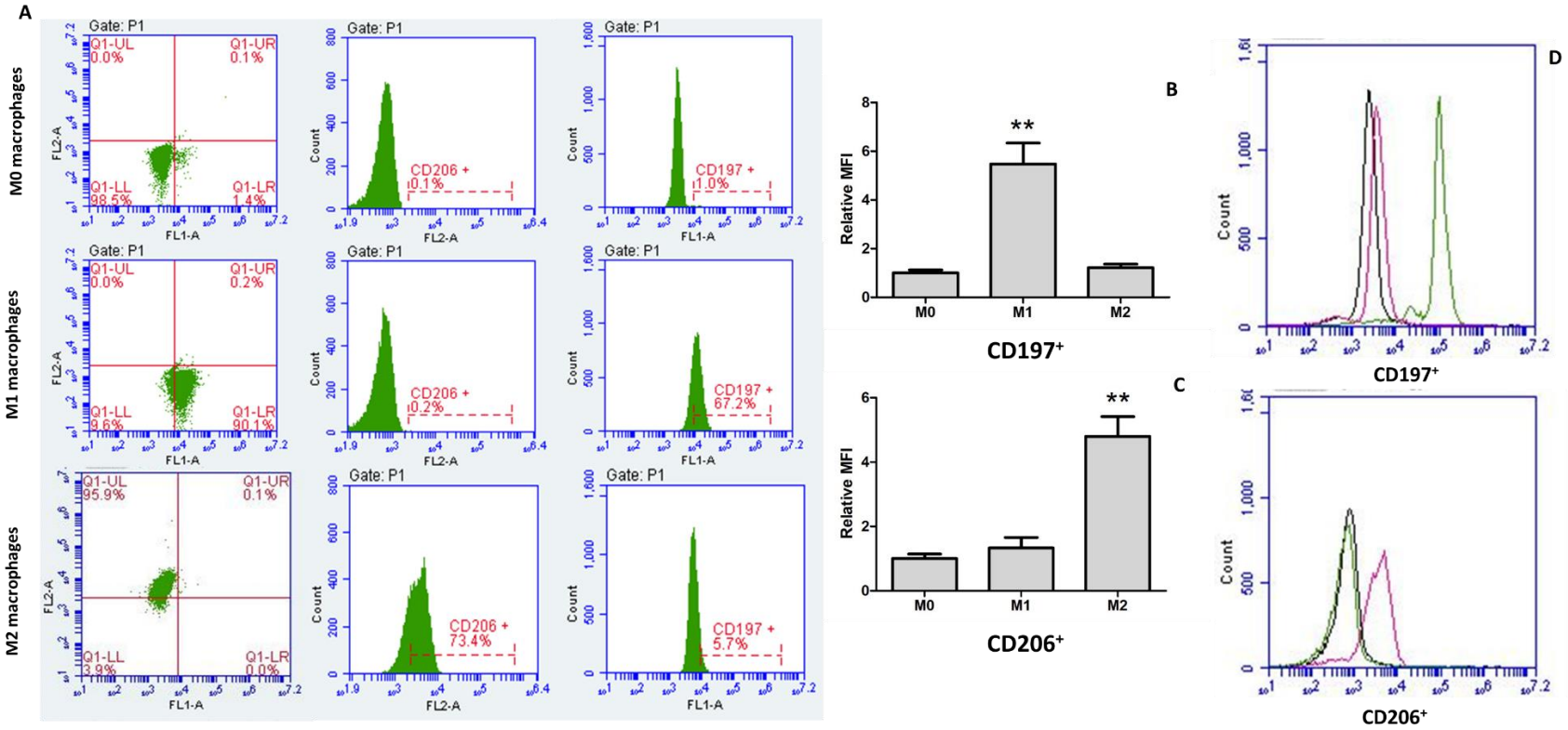

Figure 4.5. Polarisation of HPBM M0 macrophages into M1/M2 macrophages. The polarisation of HPBM M0 macrophages into M1 and M2 macrophages was confirmed via detection of CD197 and CD206 surface markers by flow cytometry respectively. PMA-differentiated M0 macrophages almost lacked both CD197 (1%) and CD206 (0.1%). In contrast, macrophages treated with LPS/IFN-γ expressed CD197 (67.2%) but almost lacked CD206 (0.2%). Macrophages treated with IL-4/IL-13 expressed CD206 (73.4%) but very low levels of CD197 (5.7%) (A). Three distinct populations of HBPM macrophages were detected (D) with significantly higher MFI for CD197 from M1 macrophages compared to M0 and M2 macrophages (B), and significantly higher MFI for CD206 from M2 macrophages compared to M0 and M1 macrophages. Data represent an average of n = 3 experiments. ** Indicates significant difference (One-way ANOVA) in MFI (P<0.01) compared to M0 macrophages. Error bars represent the standard error of the mean (StEM).

4.4.2 Effect of Age-Related Changes in Estrogen Levels on *In vitro* Phagocytosis of MRSA and *P. aeruginosa* by U937-derived M1 and M2 Macrophages

The effect of age-related changes in estrogen levels on the clearance of MRSA and *P. aeruginosa* by U937-derived M1 and M2 macrophages was investigated (Figure 4.6). Compared to the negative control, conditions representing an absolute absence of estrogen (zero M) and estrogen deprivation (1×10^{-9} M) had no significant effect ($P>0.05$) on the clearance of MRSA or PAO1 by both U937-derived M1 and M2 macrophages.

In contrast, physiological and supraphysiological levels of estrogen significantly ($P<0.01$) reduced the recovery of MRSA and *P. aeruginosa* by both U937-derived M1 and M2 macrophages in a dose-dependent manner compared to the negative control. After a 3-hour incubation with U937-derived M1 macrophages, the MRSA recovery was significantly ($P<0.05$) reduced to 1586 CFU/mL and 1476 CFU/mL following treatment with physiological and supraphysiological levels of estrogen respectively, compared to the negative control (2117 CFU/mL). Although somewhat less effective, after a 3-hour incubation with U937-derived M2 macrophages with physiological or supraphysiological levels of estrogen still significantly ($P<0.05$) reduced the recovery of MRSA to 2927 CFU/mL and 2533 CFU/mL respectively compared to the negative control (3538 CFU/ml).

Similarly, treating U937-derived M1 macrophages with physiological or supraphysiological levels of estrogen significantly ($P<0.05$) reduced PAO1 recovery to 2405 CFU/mL and 1920 CFU/mL respectively compared to the negative control (2660 CFU/mL). Although somewhat less effective, treating U937-derived M2 macrophages with physiological or supraphysiological levels of estrogen still significantly ($P<0.05$) reduced the recovery of PA01 to 3052 CFU/mL and 2594 CFU/mL respectively compared to the negative control (3790 CFU/mL).

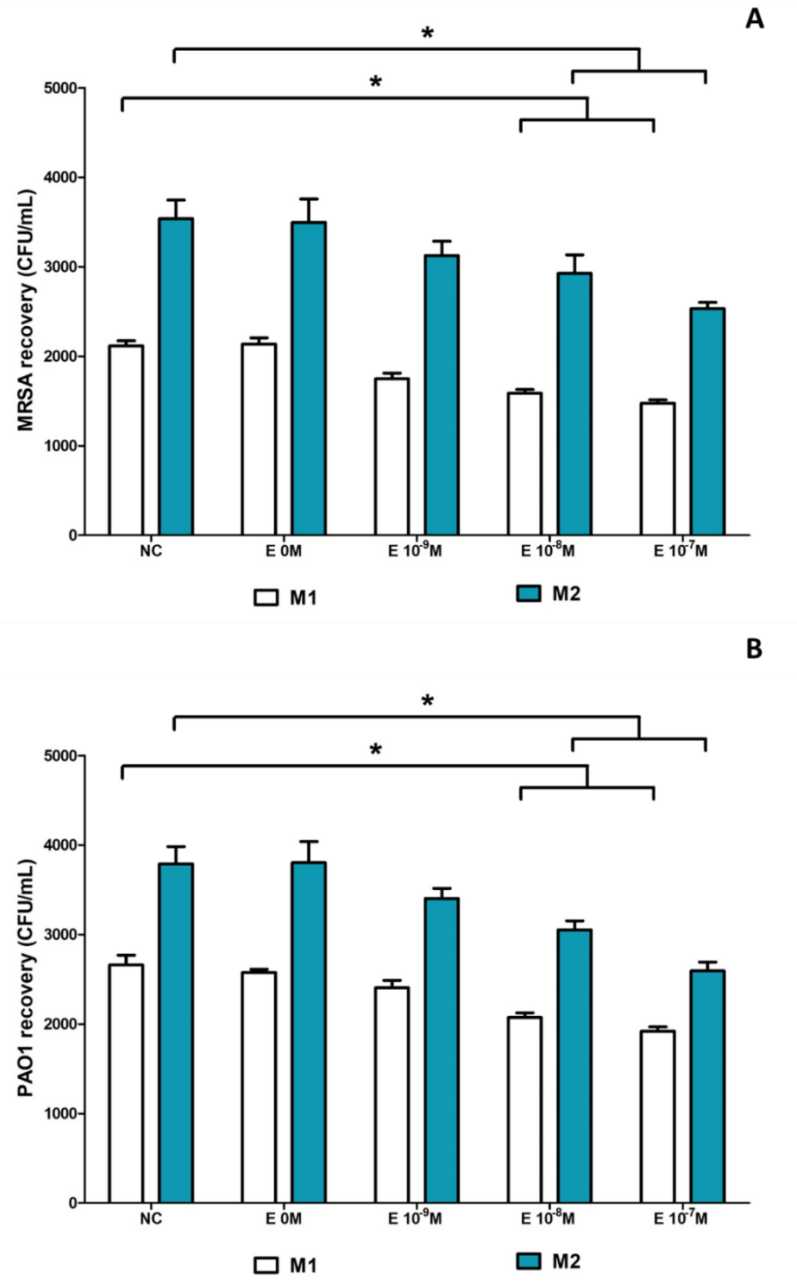

Figure 4.6. Estrogen enhances the phagocytosis of MRSA (A) and PAO1 (B) by U937-derived M1/M2 macrophages *in vitro*. The recovery of MRSA (A) and PAO1 (B) after incubation with U937-derived M1/M2 macrophages were significantly (*: $P<0.05$, **: $P<0.01$) decreased following treatment with physiological (1×10^{-8} M) or supraphysiological (1×10^{-7} M) levels of estrogen compared to the negative control. U937-derived M1 macrophages internalised significantly ($P<0.05$) more MRSA/PAO1 respectively than U937-derived M2 macrophages. Data is mean ± StEM, n = 10.

4.4.3 Effect of Age-Related Changes in Estrogen Levels on the Phagocytosis of MRSA and *P. aeruginosa* by *Ex Vivo* M1 and M2 Human Macrophages

The effect of estrogen on the phagocytosis of MRSA and *P. aeruginosa* by *ex vivo* HPBM-derived M1/M2 macrophages was investigated (Figure 4.7). After a 3-hour

incubation of MRSA or *P. aeruginosa* with HPBM-derived M1/M2 macrophages, the number of recovered MRSA and *P. aeruginosa* under conditions representing an absolute absence of estrogen (zero M) and estrogen deprivation (1×10^{-9} M) were not significantly different ($P>0.05$) to the negative control.

Physiological and supraphysiological levels of estrogen significantly ($P<0.05$) reduced MRSA and *P. aeruginosa* recovery by HPBM-derived M1 and M2 macrophages compared to the negative control. After a 3-hour incubation with HPBM-derived M1 macrophages, the MRSA recovery significantly ($P<0.05$) decreased from 2825 CFU/mL in the negative control to 2023 CFU/mL and 1343 CFU/mL following treatment with physiological and supraphysiological levels of estrogen respectively. Although significantly less effective than M1 macrophages, treatment of HPBM-derived M2 macrophages with physiological or supraphysiological levels of estrogen still significantly ($P<0.05$) reduced the recovery of MRSA to 2745 CFU/mL and 2437 CFU/mL respectively compared to the negative control (3635 CFU/mL).

Similarly, treating HPBM-derived M1 macrophages with physiological or supraphysiological levels of estrogen significantly ($P<0.05$) reduced *P. aeruginosa* recovery to 2430 CFU/mL and 2083 CFU/mL respectively compared to the negative control (3352 CFU/mL). Although somewhat less effective, treating HPBM-derived M2 macrophages with physiological or supraphysiological levels of estrogen still significantly ($P<0.05$) reduced the recovery of *P. aeruginosa* to 3062 CFU/mL and 2339 CFU/mL respectively compared to the negative control (4052 CFU/mL).

Intriguingly, the MRSA and *P. aeruginosa* uptake was significantly ($P<0.05$) greater with both U937-derived and HPBM-derived M1 macrophages compared to M2 macrophages.

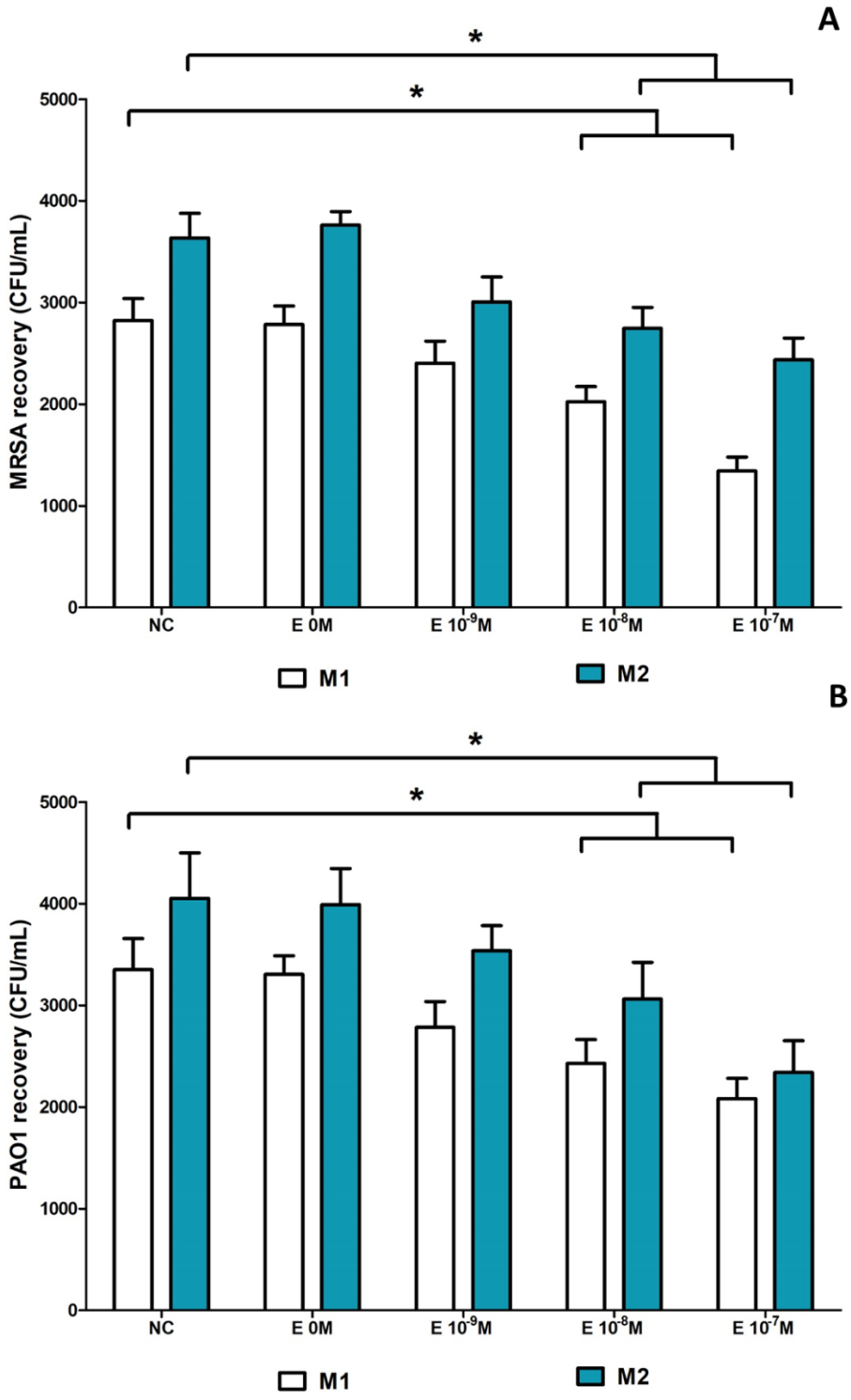

Figure 4.7. Estrogen improves the phagocytosis of MRSA (A) and PAO1 (B) by *ex vivo* HPBM-derived M1/M2 macrophages. The recovery of MRSA (A) and *P. aeruginosa* (B) after incubation with HPBM-derived M1/M2 macrophages were significantly (*: $P<0.05$, **: $P<0.01$) decreased following treatment with physiological (1×10^{-8} M) or supraphysiological (1×10^{-7} M) levels of estrogen compared to the negative control. HPBM-derived M1 macrophages internalised significantly ($P<0.05$) more MRSA/PAO1 respectively than HPBM-derived M2 macrophages. Data is mean ± StEM, n = 10.

4.5 Discussion

Plasticity of macrophages is a crucial process for an effective inflammatory phase during wound healing. Macrophages ability to continuously switch from a phenotype to another was demonstrated in mice (Thomas *et al*., 1992; Erwig *et al*., 1998). Experiments in this study were conducted using pro-inflammatory (M1-like) and anti-inflammatory (M2-like) macrophages. *In vitro* M1 macrophages can be induced by differentiation with IFN-γ and/or LPS, and distinguished through the expression of the surface markers CD197 and/or CD86 (Mantovani *et al*., 2004). In contrast, M2 macrophages can be induced by differentiation with IL-4 and/or IL-13, and distinguished through the expression of the macrophage mannose receptors, CD206 (Stein *et al.,* 1992; Daigneault *et al.*, 2010) and/or CD163 (Zeyda *et al*., 2007). Successful polarisation of U937-derived, and HPBM-derived M0 macrophages into M1-like and M2-like macrophages was achieved in this study, with high expression of CD197 confirming polarisation into M1-like macrophages by IFN-γ/LPS, and high CD206 expression confirming polarisation into M2-like macrophages by IL-4/IL-13.

The effect of estrogen on the phagocytosis of MRSA and *P. aeruginosa* by U937-derived M1-like and M2-like macrophages was investigated *in vitro*. In line with findings of chapter 3, the results from this chapter demonstrated that the physiological and supraphysiological levels of estrogen enhanced the clearance of both MRSA and PAO1 in a dose-dependent manner in both U937-derived M1 and M2 macrophages. Interestingly, the bacterial recovery followed a similar pattern throughout, regardless of the macrophage phenotype, with estrogen consistently increasing (P<0.05) the phagocytosis of bacteria. These results are supported by the findings of Crompton *et al*. (2016), when estrogen was found to significantly enhance the rate of wound repair in mice in a *K. pneumonia* LPS model of infection, and by Li *et al*. (2000) when a 48-hour stimulation with estrogen was found to enhance the uptake of amyloid β-peptide and *E. coli* by macrophages extracted from human brain tissues (microglial cells). Cutolo *et al*. (2004) also described estrogen as a key regulator of immune responses against bacterial infections in macrophages as estrogen was found to enhance the expression of growth and proliferation markers in macrophages in patients with autoimmune diseases.

It is believed that during early stages of wound healing, M1 macrophages are highly phagocytic, and serve as cleaners of the wound site by removing foreign invaders and dead tissue (Stein *et al*., 1992) whereas M2 macrophages have anti-inflammatory properties and play a more regenerative role as the inflammatory response phase subsides (Mantovani *et al*., 2004)**.** In line with this, M1 macrophages were described to be highly phagocytic *in vitro*, and produce microbicide enzymes such as iNOS, essential for pathogen destruction. They also release many pro-inflammatory cytokines, such as, IL-1β, IL-6, IL-12, IL-23 and TNF-α (Mantovani *et al*., 2004). Werner and Grose (2003) reported an up-regulation of these cytokines during the inflammatory phase of wound repair. In addition, research indicated that genetic deficiencies of IFN-γ and/or its receptors confirmed that macrophages polarisation to the M1 phenotype is essential for host defence against pathogenic infections in knockout mice models and in humans. Components of intracellular pathogens (e.g. LPS), TNF, GM-CSF and TLR4 ligands were also described to encourage M1 polarisation (Mantovani *et al*., 2005). Collectively, data from the host-pathogen assays support these reports, with significantly enhanced clearance of MRSA and *P. aeruginosa* by M1 macrophages compared to M2 macrophages or M0 macrophages (results in chapter 3).

The assays were conducted first using the immortal cell line "U937 monocytes". *In vitro* results would provide an insufficient evidence to predict effects *in vivo*. To avoid this limitation, primary monocytes derived from human peripheral blood were used to conduct an *ex vivo* model of host-pathogen interactions. Unsurprisingly, the findings were consistent with the *in vitro* assays. The bacterial recovery was shown to decrease with increasing estrogen concentrations with both HPBM-derived M1 and M2 macrophages, with a significantly greater uptake of bacteria with M1 macrophages compared to M2 macrophages. This suggests enhanced phagocytosis is a feature of M1 macrophages, regardless of their source. In support of these results, Deshpande *et al*. (1997) reported that estrogen causes an increase in the production of iNOS by murine macrophages as well as many other cytokines, particularly, IL-1β, IL-12 and IL-6. This is due to TLR4 activation with LPS in murine macrophages. In contrast, other studies reported many anti-

inflammatory effects of estrogens on macrophages *in vitro*. Indeed, a 2-hour estrogen treatment of macrophages (RAW 264.7 cell line) *in vitro* caused a substantial diminution in the expression of inflammatory mediators, mainly NF-κB, subsequent to TLR4 activation (Ghisletti *et al*., 2005). Long-time (chronic) exposure to estrogen was found to promote the alternative activation of macrophages and to dampen the IFN-γ/LPS actions on human macrophages (Campbell *et al*., 2014; Toniolo *et al*., 2015). In this study, a 24-hour incubation with estrogen was shown to increase the rate of bacterial phagocytosis in all macrophages regardless the macrophage subtype. Further investigations are warranted to determine whether estrogen have an effect on the polarisation of macrophages *in vitro*. One application will be to measure the expression of the M1-like and M2-like specific markers (e.g. CD197 and CD206 respectively) before and after treatment of macrophages with estrogen.

Human trials have confirmed that estrogen supplementation accelerates healing by reducing inflammatory response in the elderly (Deshpande *et al*., 1997; Srivastava *et al*., 1999) but further *in vivo* investigations are needed to demonstrate the effect of estrogen on bacterial clearance in elderly subjects. Investigations could also be conducted using existing animal models of age-related impaired healing (Deshpande *et al*., 1997; Ghisletti *et al*., 2005) to confirm *in vivo* effects of estrogen on the phagocytic function of M1 and M2 macrophages.

Billiau and Vandekerckhove (1991) reported that bacterial clearance in wounds is reliant on good regulation of TNF-α. Interestingly, estradiol was shown to suppress the production of TNF-α in murine macrophages (Deshpande *et al*., 1997; Srivastava *et al*., 1999). The work presented in this chapter suggests that TNF-α could be associated with the mechanism of estrogen-enhanced phagocytosis in M1 macrophages. Further research is warranted to investigate the change in TNF-α levels and other pro-inflammatory cytokines (e.g. MIF) involved in the estrogen-increased phagocytosis in macrophages.

Compared to results reported in chapter 3, M1-like macrophages were shown to be more phagocytic than M0-like macrophages regardless of the source of macrophage. Further work investigating mechanisms of estrogen-enhanced

phagocytosis in this thesis will therefore be conducted using pro-inflammatory M1-like macrophages instead of M0-like or M2-like macrophages. Investigations were conducted (chapter 5) to determine the ER pathways linked with the estrogen-mediated enhancement of phagocytosis through the use of: selective estrogen receptor modulators (SERMs) (e.g. Tamoxifen), selective estrogen receptor degraders (SERDs) (e.g. Fulvestrant), selective ER-α and/or ER-β agonists (e.g. PPT, DPN) to determine the exact ER activation profile involved in the estrogen-enhanced phagocytosis. In order to investigate the mechanism by which estrogen enhanced bacterial clearance in M1 macrophages, the changes in the levels of proteins that regulate the actin cytoskeleton dynamics including Rho GTPase proteins, and focal adhesion proteins on the cell membrane of estrogen-treated M1 macrophages are presented (detailed in chapter 6).

In summary, these findings confirm for the first time that estrogen deprivation inhibits phagocytosis by both M1-like and M2-like human macrophages whereas physiological/supraphysiological levels of estrogen stimulate the phagocytic function of M2 but particularly M1 human macrophages against Gram-positive and Gram-negative wound pathogens. Together, the results suggest topical or systemic estrogen supplementation may potentially resolve or reduce the likelihood of wound infections in the elderly.

Chapter 5: Estrogen Enhances the Phagocytosis of MRSA and *P. aeruginosa* by Human M1 Macrophages via Binding to ER-α

5.1 Introduction

5.1.1 Estrogen Receptors

Estrogen signals principally through the estrogen receptor (ER) proteins that are part of the nuclear receptor (NR) family. ERs display a structure that is typical of the NR family, containing six domains (A-F) (Kuiper *et al*., 1998; Klinge, 2000; Begam *et al*., 2017) (Figure 5.1).

ER-alpha (ER-α) was first discovered in 1958 and cloned in 1985 (Jensen, 1962; Kuiper *et al*., 1998). However, ER-beta (ER-β) was first characterised in 1996 in rat prostate and ovary. Later in 1998, a human ER-β was identified (Mosselman *et al.*, 1996; Kuiper *et al*., 1998). Both ERs are located in various tissues (Campbell *et al*., 2010). ER-α is highly expressed in ovaries, cancer breast tissues and reproductive tissues *(*Kuiper *et al*., 1997; Ali and Coombes, 2000; Campbell *et al.*, 2010). ER-β is mainly found in peripheral tissues such as bone, heart and hypothalamus**,** but it can also be found in healthy breast tissue, the prostate and testis *(Kuiper et al*., 1997; Ali and Coombes, 2000; Campbell *et al.*, 2010). ERs are expressed in the skin, suggesting that estrogen regulates skin functions and development (Ashworth, 2005). Both ER-α and ER-β have been identified in the epidermis of young and old women, with isoforms observed in epithelial cells of the hair follicles (Ashworth, 2005).

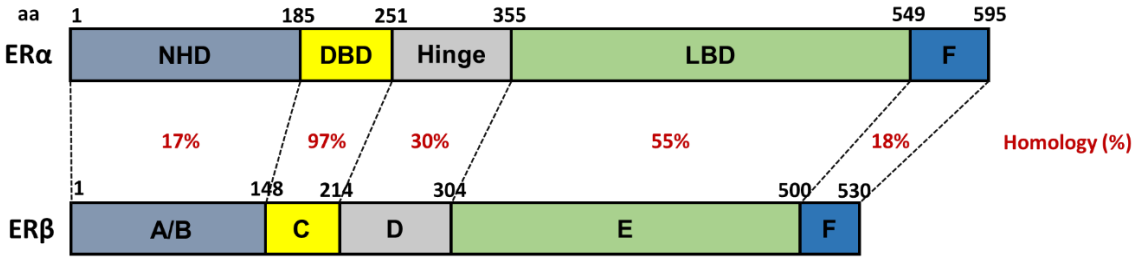

Figure 5.1. Schematic representation comparing the structure of human ER-α with ER-β. Homology between domains (A-F) is represented as percentage (%) similarity. NHD = N-terminal homology domain, DBD = DNA-binding domain, LBD = ligand-binding domain. Figure adapted from (Webb *et al.*, 1999; Klinge, 2000; Begam *et al*., 2017).

Estrogen signals predominantly by binding to inactive ERs in the nucleus of the cell (Klinge, 2000). When estrogen binds to ERs, they become activated and dimerize (Klinge, 2000). Each ER has a DNA-binding domain (DBD) which binds to the target

gene at a site called the estrogen response element (ERE) (Kuiper *et al*., 1998; Klinge, 2000). In cells expressing a single ER subtype, homodimers of ER-α or ER-β are formed (Kuiper *et al.*, 1998). In cells that express both ER subtypes, a heterodimer containing one ER-α and one ER-β may form (Kuiper *et al.*, 1998). ERs heterodimers and ER-α homodimers bind to DNA with a similar affinity. However, ER-β homodimers bind to DNA with a lower affinity (Kuiper *et al.*, 1998). DNA-bound homodimers and heterodimers bind to steroid receptor coactivator-1 and trigger gene transcription (Marino *et al*., 2006).

Both ER-α and ER-β enhance acute wound repair, however, their functions are different; ER-α is described to control and regulate inflammation, however, ER-β is linked with the modulation of wound healing (Campbell *et al.*, 2010). While ER-α and ER-β express a 97% homology in the DBD region, they only have 55% homology in the LBD region (Barkhem *et al*., 1998; Webb *et al.*, 1999; Klinge, 2000). This made possible the design of artificial estrogens such as SERMs with specific binding affinity to one isoform over another.

5.1.2 Estrogen Receptor Agonists

Estrogen receptor agonists are molecules that induce one or both ER subtypes in order to partially or fully induce a pharmacological effect (Sun *et al*., 1999)**.** Selective agonists of ER-α and ER-β are extensively used to investigate the distinct functions of the ERs in several human diseases. Propyl pyrazole triol (PPT) and Diarylpropionitrile (DPN) are the most frequently used selective ERs agonists. PPT is a selective ER-α agonist (Thammacharoen *et al*., 2009) that has 410-fold more selectivity for ER-α than ER-β (Thammacharoen *et al*., 2009). In 1999, PPT was used for the first time to investigate the role of ER-α in several tissues (Sun *et al*., 1999). DPN is a selective ER-β agonist (Minutolo *et al*., 2011) that has 70-fold more selectivity for ER-β than ER-α (Meyers *et al*., 2001). DPN was first used in 1999 in a purpose to develop new ligands selective for ER-β (Sun *et al*., 1999; Meyers *et al*., 2001) (Figure 5.2).

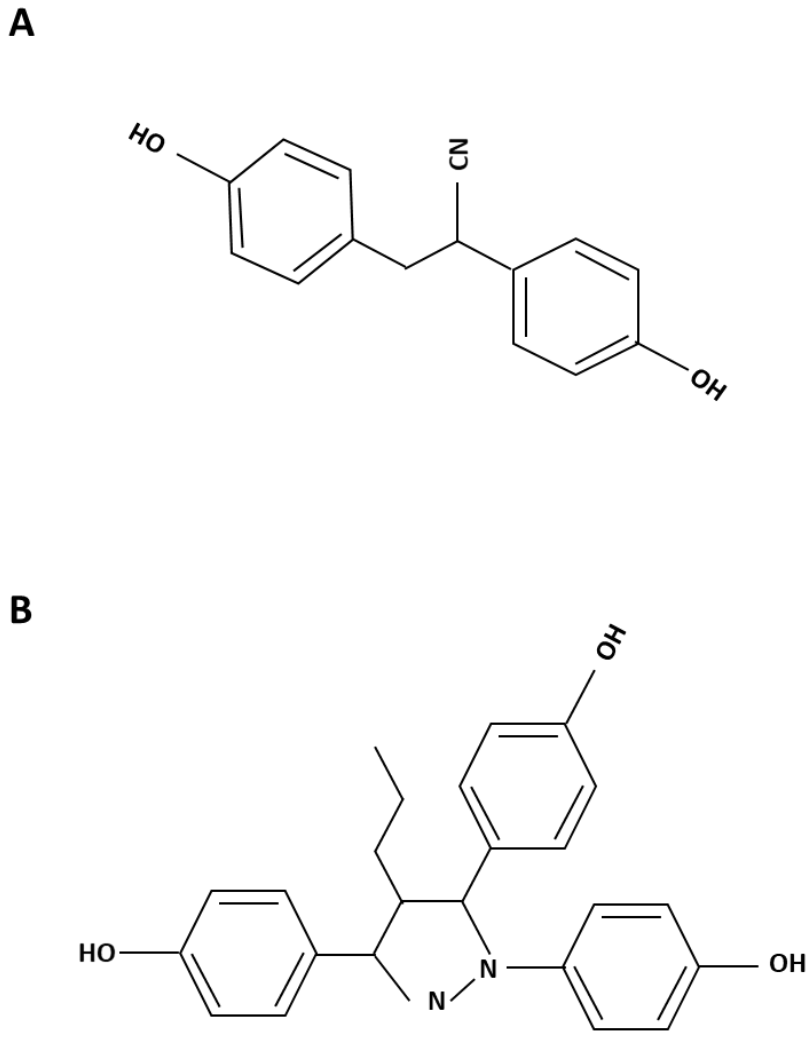

Figure 5.2. Structure of DPN (A) and PPT (B).

5.1.3 Estrogen Receptor Antagonists

Estrogen receptor antagonists are compounds characterised to have high affinity to bind to one or both of the ER subtypes, without the potential of inducing the normal pharmacological effects typically mediated by the receptors (Wakeling *et al.*, 1991; Barkhem *et al*., 1998). ER antagonists compete with estrogen to bind to the specific receptor site (Morris and Wakeling, 2002; Osborne *et al*., 2004). Therefore, the physiological effect produced in response to estrogen will be dampened in the presence of ER antagonists. ER antagonists have been used clinically to control fertility or treat various cancers (Morris and Wakeling, 2002; Osborne *et al*., 2004).

Theophylline, 8-[(benzylthio)methyl]-(7Cl,8Cl) (TPBM) is a potent inhibitor of ER-α via blocking the binding of ER-α to the ERE DNA (Mao *et al*., 2008). TPBM is highly selective for ER-α compared to ER-β, with a greater selectivity for blocking gene transcription mediated by ER-α relative to ER-β (Mao *et al.*, 2008). TPBM has been used to investigate the role of ER-α in many biological processes and disorders including cancer (Mann, 2008; Plant and Zeleznik, 2014).

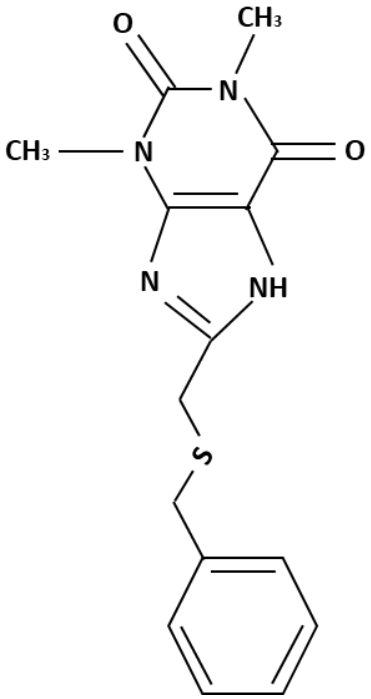

Figure 5.3. Structure of TPBM.

5.1.4 Selective Estrogen Receptor Modulators (SERMs)

Selective estrogen receptor modulators (SERMs) are ER-interacting molecules that have the ability to bind the ERs and act as agonists in specific tissues whilst acting as antagonists in different tissues (Cho and Nuttall, 2001). SERMs have been used clinically to promote the beneficial effects of estrogen in target tissues whilst avoiding/decreasing detrimental effects of estrogen in other non-target tissues such as increasing the risk of breast cancer in breast tissues (Mirkin and Pickar, 2015). SERMs can be differentiated from ER agonists/antagonists due to their opposing effects in tissues (Park and Jordan, 2002). SERMs are structurally different to estrogens but their chemical structures allow them to bind to the LBD of ERs (Brzozowski *et al*., 1997).

Tamoxifen (Figure 5.4) is one of the most documented and frequently used SERMs. It is known to have estrogenic effects in numerous peripheral tissues such as bone, but is anti-estrogenic in the breast tissue and therefore used extensively in breast cancer research (Furr and Jordan, 1984; Morris and Wakeling, 2002; Park and Jordan, 2002; Mirkin and Pickar, 2015). Tamoxifen was discovered and reported by the Food and Drug Administration (FDA) in 1977 (Park and Jordan, 2002; Jordan, 2006; Mirkin and Pickar, 2015; Quirke, 2017). Tamoxifen binds to both ERs, and its effects depend on the cell and tissue types. It is anti-estrogenic in the breast, and is therefore commonly used to prevent and/or treat breast cancer in postmenopausal and premenopausal females (Zidan *et al.,* 2004; Quirke, 2017). Tamoxifen has also been reported to maintain the density of bone in rats and humans (Jordan *et al*., 1987; Zidan *et al*., 2004). However, it has multiple side effects and is frequently linked with endometrial cancer due to its estrogenic effects in the uterus (Kedar *et al*., 1994).

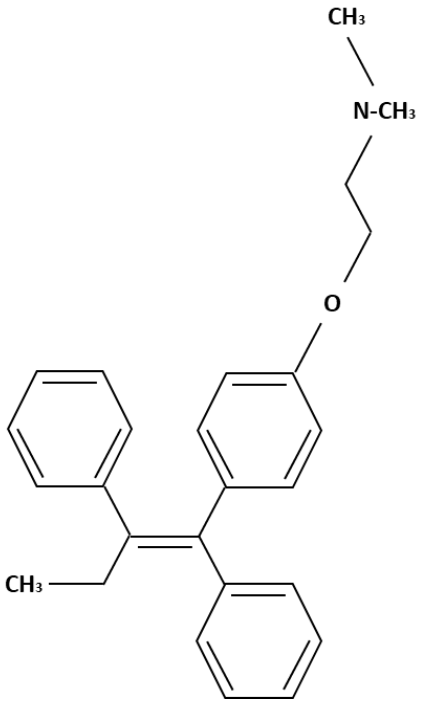

Figure 5.4. Structure of Tamoxifen.

5.1.5 Selective Estrogen Receptor Degraders (SERDs)

Selective estrogen receptor degraders (SERDs) are molecules that have high affinity to bind ERs but lead to their degradation, leading to reduced levels of functional

ERs (Lee *et al*., 2017). Along with SERMs and aromatase inhibitors, SERDs are mainly used to treat breast cancer (Lee *et al*., 2017). Fulvestrant, the most commonly used SERD, was officially discovered in 2002 (Lee *et al*., 2017). In November 2016, other SERDs such as elacestrant and brilanestrant were developed (Lai and Crews, 2017). Fulvestrant is an analogue and competitive inhibitor of 17β-estradiol (Osborne *et al*., 2004), with a high selective binding affinity (89% that of estrogen) to the ER (Morris and Wakeling, 2002). The binding of fulvestrant to the ER weakens the receptor dimerization and blocks the nuclear localisation of ER (Morris and Wakeling, 2002; Osborne *et al*., 2004). In addition, the fulvestrant-ER complex is unstable, which leads to its rapid degradation leading to enhanced cellular ER deprivation compared with estrogen or other SERMs (e.g. tamoxifen) (Morris and Wakeling, 2002). The reduced levels of functional ER protein does not cause any decrease in the levels of ER mRNA. Therefore, after binding to, blocking and accelerating the destruction of the ER protein, fulvestrant inhibits estrogen signalling (Morris and Wakeling, 2002; Osborne *et al*., 2004).

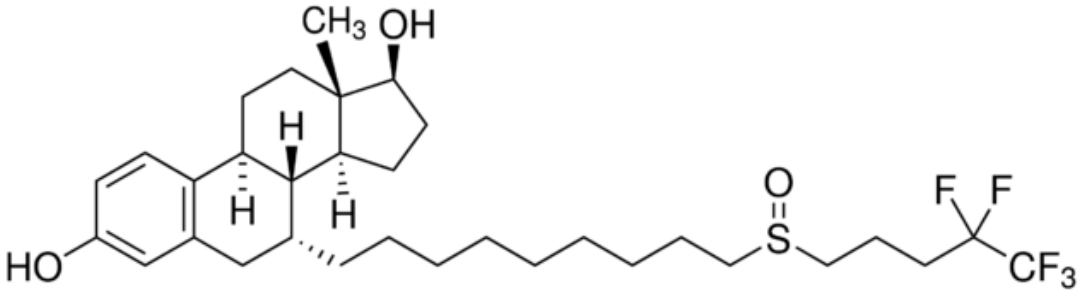

Figure 5.5. Structure of Fulvestrant.

5.1.6 Estrogen Receptors and Wound Infection

ER-α and ER-β are both expressed by inflammatory cells, including neutrophils and macrophages (Ashcroft *et al*., 2003). More widely, ER-α is expressed in human skin, fibroblasts and the adipose tissue (Kuiper *et al*., 1997; Kuiper *et al*., 1998; Campbell *et al*., 2010; Campbell *et al*., 2014). ER-β is expressed in the dermis, epidermis, fibroblasts, endothelial cells and muscle cells (Thornton *et al*., 2003).

Estrogen is key molecule that enhances wound healing in both genders and these actions appear to be mediated via the ERs (Ashcroft *et al*., 1999a). Age-related

estrogen deprivation is frequently linked with delayed wound healing and might contribute to the development of chronic wounds in the elderly (Ashcroft and Ashworth., 2003). In particular, polymorphisms of the ER-β gene are associated with venous ulceration and a pronounced inflammatory response (Ashworth, 2005). ER-α has been implicated in reducing the inflammatory response in wounds by dampening levels of pro-inflammatory cytokines such as macrophage migration inhibitory factor (MIF) (Campbell *et al.*, 2010). In contrast, ER-α activation by PPT has been shown to considerably reduce early wound strength in models of ovariectomized mice compared to untreated ovariectomized mice, whereas activation of ER-β with DPN had no effect on early wound strength (Gál *et al*., 2010). Although the involvement of ER subtypes in the wound healing process is documented, there is a lack in knowledge regarding the precise roles of ER-α and ER-β in the clearance of bacteria from infected wounds.

Ashcroft *et al*. (1999a) showed that estrogen replacement therapy accelerates wound repair in postmenopausal females. Furthermore, this study has showed estrogen enhances the clearance of the typical wound pathogens MRSA and *P. aeruginosa* (chapter 3 and 4). However, Anderson *et al*. (2004) reported that estrogen supplementation in postmenopausal females is associated with a higher risk of breast cancer and heart diseases. Thus, rather than using systemic estrogen supplementation, the use of appropriate ERs agonists/antagonists, SERDs and particularly SERMs could be beneficial in treatment of age related impaired healing and infected wounds.

5.2 Aim and Objectives

5.2.1 Aim

To investigate the involvement of ERs in mediating the beneficial effects of estrogen on promoting phagocytosis by human M1 macrophages using *in vitro* and *ex vivo* models of host-pathogen interactions.

5.2.2 Objectives

- Determine the effect of ERs agonists and antagonists, SERDs and SERMs on the phagocytosis of Gram-positive (MRSA) and Gram-negative (*P. aeruginosa*) bacteria by U937- and HPBM-derived M1 macrophages.

5.3 Methods

5.3.1 Estrogen Receptors Stimulation/Blockade

In order to test the effect of ERs agonists/antagonists, SERMs and SERDs on the MRSA and *P. aeruginosa* clearance, *in vitro* and *ex vivo* host-pathogen assays were conducted as described in section 2.2.12.

5.4 Results

Similar to previous results, supraphysiological estrogen levels significantly decreased (P<0.01) MRSA and *P. aeruginosa* recovery compared to the negative control and the absence of estrogen (Figure 5.6). Treatment with the ER agonist tamoxifen mirrored the effects of estrogen, significantly (P<0.01) decreasing the bacterial recovery of both MRSA and *P. aeruginosa*. In contrast, treatment with the ER degrader fulvestrant blocked the effects of supraphysiological levels of estrogen on the phagocytosis of MRSA and *P. aeruginosa*, with no significant difference in bacterial recovery (P>0.05) detected compared to the negative control and the absence of estrogen. Of interest, ER-α agonism with PPT significantly reduced (P<0.01) the recovery of both MRSA and *P. aeruginosa* in fashion similar to supraphysiological estrogen levels when compared to the negative control and the absence of estrogen. In contrast, ER-β stimulation with DPN did not significantly (P>0.05) affect the uptake of MRSA and *P. aeruginosa* by M1 macrophage compared to the negative control or the absence of estrogen. Intriguingly, the selective ER-α antagonist, TPBM, reversed the effect of estrogen and PPT on the phagocytosis of MRSA and *P. aeruginosa*, with no significant difference (P>0.05) in bacterial recovery detected compared to the negative control or the absence of estrogen.

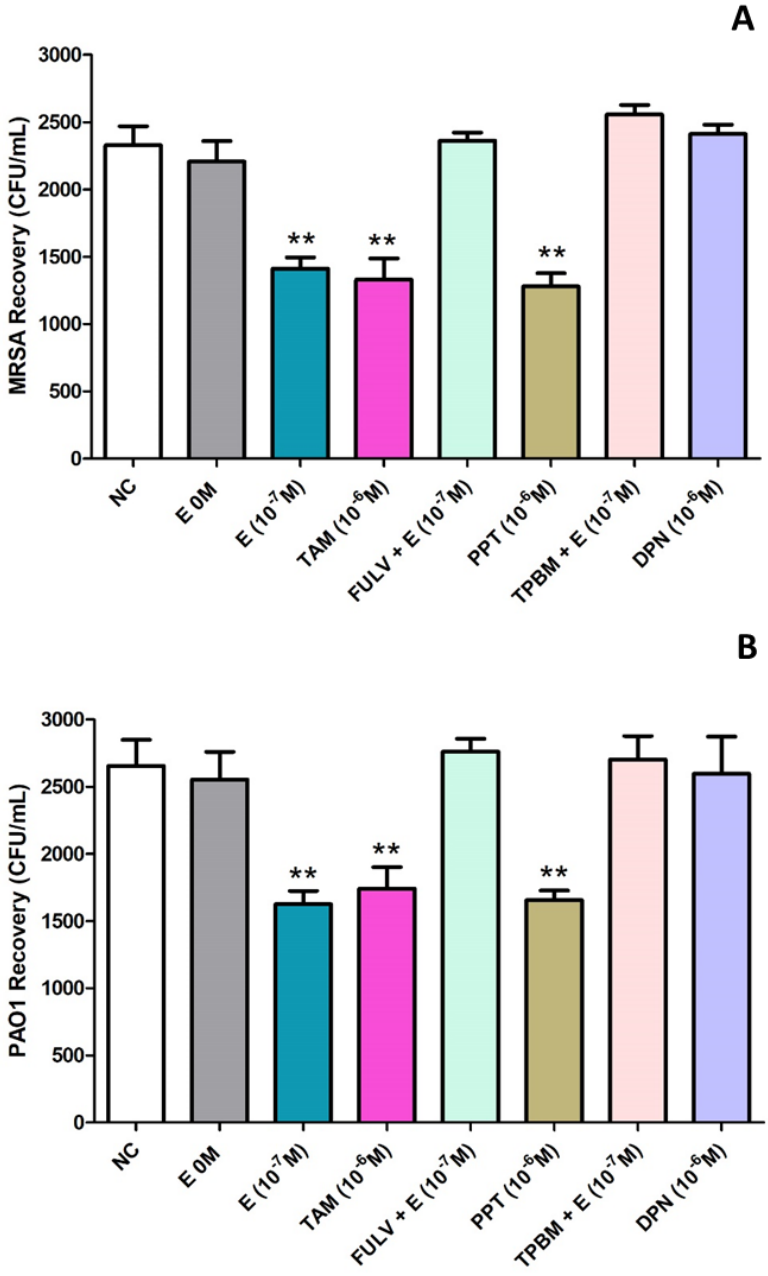

Figure 5.6. Estrogen, tamoxifen and the selective ER-α agonist, PPT, enhances the phagocytosis of MRSA (A) and *P. aeruginosa* (B) by U937 M1 macrophages. Estrogen, tamoxifen and PPT significantly decreased the MRSA (A) and *P. aeruginosa* (B) recovery compared to the negative control or the absence of estrogen (E 0M). Fulvestrant and TPBM significantly blocked the effects of estrogen on the phagocytosis of both MRSA and *P. aeruginosa*, with no significant difference in bacterial recovery detected compared to the negative control or the absence of estrogen. DPN did not significantly affect bacterial uptake compared to the negative control or the absence of estrogen. Data represent an average of n = 6 experiments. ** Indicates significant difference (P<0.01). Error bars represent the standard error of the mean (StEM).

The *in vitro* host-pathogen assays were repeated using *ex vivo* primary HPBM-derived M1 macrophages in order to confirm these initial findings (Figure 5.7). The

results were similar to those obtained with U937-derived M1 macrophages, with estrogen, tamoxifen and PPT significantly (P<0.01) reducing MRSA and *P. aeruginosa* recovery compared to the negative control and the absence of estrogen. Again, fulvestrant and ER-α antagonism with TPBM blocked the effects of estrogen whereas ER-β agonism with DPN had no significant (P>0.05) effect on the phagocytosis of MRSA and *P. aeruginosa* compared to the negative control or the absence of estrogen.

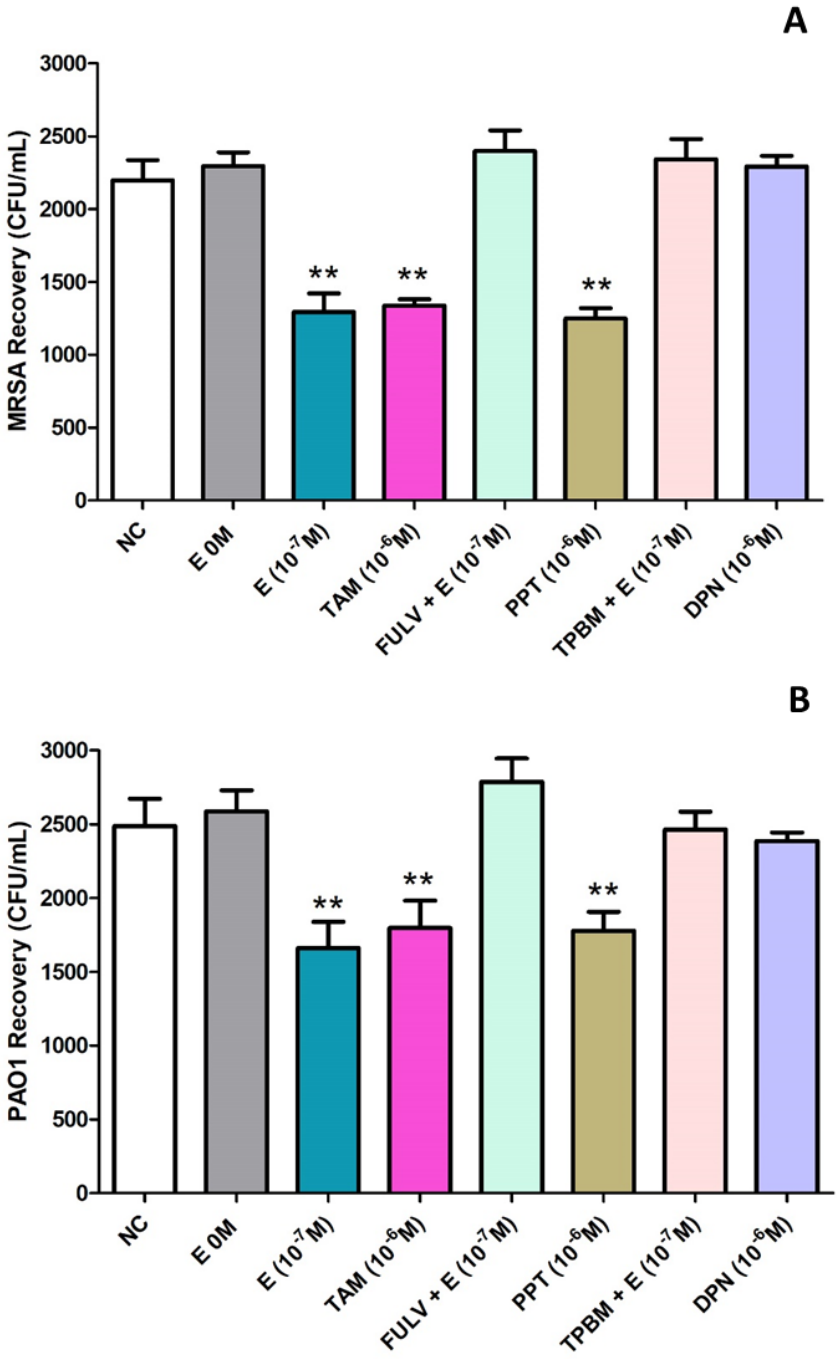

Figure 5.7. Estrogen, tamoxifen and the selective ER-α agonist, PPT, enhance MRSA (A) and *P. aeruginosa* (B) uptake by HPBM M1 macrophages. Estrogen, tamoxifen and PPT significantly decreased the recovery of MRSA (A) and *P. aeruginosa* (B) compared to the negative control and the absence of estrogen (E 0M). Fulvestrant and TPBM significantly blocked the effects of estrogen on the phagocytosis of both MRSA and *P. aeruginosa*, with no significant difference in bacterial recovery detected compared to the negative control or the absence of estrogen. DPN did not significantly affect bacterial uptake compared to the negative control or the absence of estrogen. Data represent an average of n = 6 experiments. ** Indicates significant difference (P<0.01). Error bars represent the standard error of the mean (StEM).

5.5 Discussion

The age-related change in estrogen levels is linked with skin degeneration and impaired wound healing (Ashcroft *et al*., 1999b). Estrogen is frequently linked with pathological wounds (Ashcroft and Ashworth., 2003) and a case-cohort study reported that elderly (over 65 years old) patients treated with estrogen were 30-40% less likely to develop a venous leg ulcer than untreated patients (Margolis *et al*., 2002).. Moreover, estrogen deprivation diminishes the phagocytic capability of neutrophils and macrophages, and increases the risk of infection and a postponement in healing (Ashcroft and Ashworth., 2003).

Due to their possession of nuclear ERs, the function of inflammatory cells such as macrophages are known to be influenced directly by estrogen (Weusten *et al*., 1986; Suenaga *et al.*, 1996; Suenaga *et al.*, 1998) through its binding to ER-α and/or ER-β (Kuiper *et al*., 1998; Klinge, 2000). SERMs and SERDs are ER-interacting molecules that are able to bind to the ERs and achieve normal mechanisms of estrogen signalling to function as agonists or antagonists of estrogen in a tissue-specific manner (Cho and Nuttall, 2001). SERMs and SERDs are compounds typically used to treat several ER-mediated pathologies (e.g. breast cancer) due to their tissue-specific responses (Mirkin and Pickar, 2015; Lee *et al*., 2017). Estrogen stimulated the phagocytic function of human macrophages against MRSA and *P. aeruginosa* (Chapter 3 and 4). This study used SERMs and SERDs to investigate which ERs are involved in the estrogen-mediated stimulation of bacterial clearance by M1-like macrophages.

An accumulating body of evidence demonstrates that tamoxifen acts as ER antagonist in breast tissue, but is agonist in the uterus and peripheral tissues such as bone (Fisher *et al*., 2005; DeMichele *et al.*, 2008). Tamoxifen enhanced the phagocytosis of MRSA and *P. aeruginosa* by human M1 macrophages suggesting tamoxifen was acting in a similar manner to estrogen. In support of this, tamoxifen was shown to enhance neutrophils chemotaxis and phagocytosis functions, boost human neutrophil bactericidal ability against a range of bacteria *in vitro* and

improve clearance of the leading nosocomial antibiotic-resistant bacteria, MRSA, *in vivo* (Corriden *et al*., 2015).

Tamoxifen has been shown to accelerate wound healing in ovariectomized mice, suggesting that it can potentially be used to treat impaired wound repair in the elderly (Hardman *et al*., 2007). This study adds weight to this theory by showing tamoxifen may be beneficial in the treatment of infected wounds in the elderly. Pickar *et al.* (2010) have detailed the beneficial effects of SERMs on females health after menopause, SERMs such as tamoxifen are more beneficial during wound healing when they are applied locally on wounds rather than orally. A new group of SERMs, such as Ophena, are now developed to mimic the positive biological and tissue specific estrogen functions but not the undesirable side effects such as the risk of breast and uterine cancers (Pickar *et al*., 2010).

Fulvestrant is a compound structurally different to SERMs that due to its high binding affinity to the ERs, binds in competition with estrogen to the ERs (Morris and Wakeling, 2002). When bound to ERs, fulvestrant deteriorates the receptor dimerization and blocks nuclear localisation of the ERs (Morris and Wakeling, 2002; Osborne *et al*., 2004). The findings showed that, unlike treatment with estrogen or tamoxifen, pre-treatement of M1 macrophages with fulvestrant blocked stimulation of bacterial clearance by estrogen. This provided additional evidence that estrogen may be acting through the ERs to promote phagocytosis and that disruption of the ERs prevents the beneficial effect of estrogen on bacterial clearance. Collectively, data generated from the use of tamoxifen and fulvestrant provide evidence that estrogen is enhancing the phagocytosis of bacteria, by pro-inflammatory M1 macrophages via binding and activation of the ERs.

It has been reported that the anti-proliferative effects of fulvestrant on cells in breast are due to the degradation of the ER-α protein (Long and Nephew, 2006). Interestingly, it has been reported that ER-α is the key receptor in regulating the thickness of epidermis during wound healing (Stumpf *et al*., 1974; Bidmon *et al*., 1990). Toutain *et al*. (2009) reported that ER-α is also the main isoform involved in promoting the beneficial effects of 17β-estradiol in a model of skin necrosis. On the other hand, ER-β was described to be the key receptor in the protection against

skin damage resulted from exposure to UV (Chang *et al.*, 2010). Both ER subtypes are expressed on fibroblasts and inflammatory cells such as neutrophils and macrophages (Campbell *et al*., 2010). ER-α has been noted to control and regulate inflammation, whereas ER-β is mainly linked with modulating the later phases of wound healing (Campbell *et al.*, 2010). This study used two major agonists of ER-α (PPT) and ER-β (DPN) to investigate the effect of activating ER isoforms on the phagocytosis of MRSA and *P. aeruginosa*. PPT is an ER-α agonist that is 410-fold more selective for ER-α than ER-β whereas DPN is an ER-β agonist with a 70-fold higher selectivity for ER-β than ER-α (Campbell *et al*., 2010). Intriguingly, selective activation of ER-α with PPT mirrored the effects of estrogen by significantly (P<0.01) increasing the phagocytosis of both MRSA and *P. aeruginosa* by U937-derived and HPBM-derived M1 macrophages. In contrast, stimulation with the ER-β agonist DPN failed to improve the phagocytosis of either MRSA or *P. aeruginosa* by macrophages. Of interest, blocking the ER-α with TPBM overturned the effect of PPT on the bacterial clearance. The results of this study are in concordance with published findings implicating ER-α as the key mediator of the inflammatory response, with activation of ER-α in M1 macrophages with PPT increasing bacterial clearance whereas activation of ER-β by DPN had no effect on the phagocytosis of MRSA and PAO1. Moreover, blockade of ER-α with TPBM reversed the effect of PPT. Collectively, these novel findings suggest that estrogen stimulates promotion of phagocytosis by human macrophages through ER-α rather than ER-β.

Tamoxifen is estrogenic in the uterus, therefore, this molecule is frequently linked with a high risk of uterine cancer (Jordan and Morrow, 1999; Bergman *et al.*, 2000). Another commonly used SERM, called raloxifene, is in contrast estrogenic in bone (Heaney and Draper, 1997) and ovaries (Neven *et al*., 2002), but displays anti-estrogenic effects in the uterus (Bryant *et al*., 1996). Hardman and Ashcroft (2008) indicated that estrogen, tamoxifen and raloxifen all accelerated wound healing in ovariectomised mice. Future research could investigate a range of SERMs including raloxifen to determine the most effective mediator(s) of bacterial clearance in macrophages. This might potentially help detecting the most promising topical SERM to treat wound infections in the elderly.

In summary, novel findings reported in this chapter provide clear evidence that ER-α is the ER isoform through which estrogen stimulates phagocytosis by U937- and HPBM-derived M1 macrophages. Selective targeting of ER-α with agents such as PPT may potentially lead to the development of therapeutic approaches to combat wound infections in the elderly.

Chapter 6: Estrogen Enhances the Phagocytic Activity of Human Macrophages via Regulation of Actin Cytoskeleton Proteins

6.1 Introduction

Macrophages are immune cells characterised by the ability to engulf and digest bacteria, damaged cells and any foreign substance that does not express on its surface normal host-cell specific proteins via a process known as phagocytosis (Duque and Descoteaux, 2015). Phagocytosis is a vital process for protecting the host against foreign dangers such as bacterial invasion (Desjardins *et al*., 2005). This process needs to be performed efficiently because unsuccessful or imperfect clearance of bacteria can have detrimental circumstances on the host, and is linked with the progression of several pathological conditions such as chronic inflammation, wound infections and several autoimmune diseases (Engelich *et al*., 2001). Attracted to sites of infection via chemotaxis, phagocytes such as macrophages recognize and attach to microorganisms. This results in actin polymerisation within the phagocyte, re-arrangement of the actin cytoskeleton to form pseudopodia followed by engulfment of the microorganism to form a phagosome and subsequent phagolysosome formation on fusion with a lysosome (Freeman and Grinstein, 2016; Garcia-Gomez *et al*., 2016). Finally, the destruction of bacteria is mediated by lysosomal enzymes and ROS within the phagolysosome (Pauwels *et al*., 2017). Successful engulfment of pathogens requires continuous reorganization of the phagocyte cell membrane that involves dynamic rearrangement of the actin cytoskeleton. The control of these mechanical changes on the cell membrane during cell movement and pathogen engulfment involves Rho GTPase proteins that regulate the polymerisation of actin filaments (Etienne-Manneville and Hall, 2002; Groves *et al*., 2008).

In this study it has been shown that estrogen enhances the phagocytosis of both MRSA and *P. aeruginosa* by human macrophages. The interaction between macrophages and bacteria were visualised by SEM (Figure 3.16), demonstrating estrogen promotes the formation of pseudopodia by macrophages during the uptake of both MRSA and *P. aeruginosa*. These findings suggest estrogen may be regulating the levels or activation of one or more proteins involved in the regulation of the actin cytoskeleton during phagocytosis.

6.1.1 The Actin Cytoskeleton and Phagocytosis:

The process of engulfing a foreign particles, phagocytosis, is a crucial process for the wide diversity of organisms. From simple unicellular organisms that use phagocytosis for nutrition, to complex ones in which phagocytic cells play a fundamental role in the immune response. Phagocytosis is a step-wise process in which particles are first recognized and bind to the cell surface, followed by internalisation and formation of membrane-bound vacuoles known as phagosomes (Jilkine, 2009). After a target particle is recognised the phagocytic process requires a dynamic assemblage of the actin cytoskeleton to promote the formation of pseudopodia (Alberts *et al*., 2008; Freeman and Grinstein, 2016; Garcia-Gomez *et al*., 2016; Pollard *et al*., 2016; Pauwels *et al*., 2017).

The actin cytoskeleton is constructed by a wide range of proteins (Insall and Machesky, 2009). Actin is an abundant protein found in all cells and it exists in two different forms; globular monomers named G-actin, and polymeric chains of the two G-actin subunits, F-actin and filamentous actin (Holmes *et al*., 1990). The F-actin filaments are structurally polarised (Wegner, 1976). The negative (known as "pointed") end holds a subunit with an open ATP binding site, whereas the positive (known as "barbed") end is open to adjacent G-actin monomers (Wegner, 1976). The polymerisation/depolymerisation of actin is a continuous constant process (Wegner, 1976) during which, the energy generated from ATP conversion to ADP stimulates the accumulation of G-actin monomers at the barbed end before polymerisation to F-actin filaments (Wegner, 1976; Alberts *et al*., 2008) (Figure 6.1). After the complete establishment of F-actin filaments, actin-binding proteins add an extra layer of control over the actin cytoskeleton network. For instance, actin severing proteins, such as profilin and cofilin, can bind the actin polymers and alter the filaments length and strength (T. D. Pollard and Borisy, 2003), and increase/decrease the amount of barbed ends by cleaving actin filaments (Devreotes and Horwitz, 2015).

Capping proteins can also bind to the termination of the filaments regulating their attachment and detachment (Isenberg *et al.*, 1980). Additionally, F-actin filaments

bind to integrin and myosin II filaments producing focal adhesive complexes, which allow cells to slide past each other and connect with the extracellular matrix and other adjacent cells via the cytoskeleton network, therefore allowing cell-cell and cell-matrix exchange (Burridge *et al*., 1988; Peskin *et al*., 1993; Pollard and Borisy., 2003; Alberts *et al*., 2008).

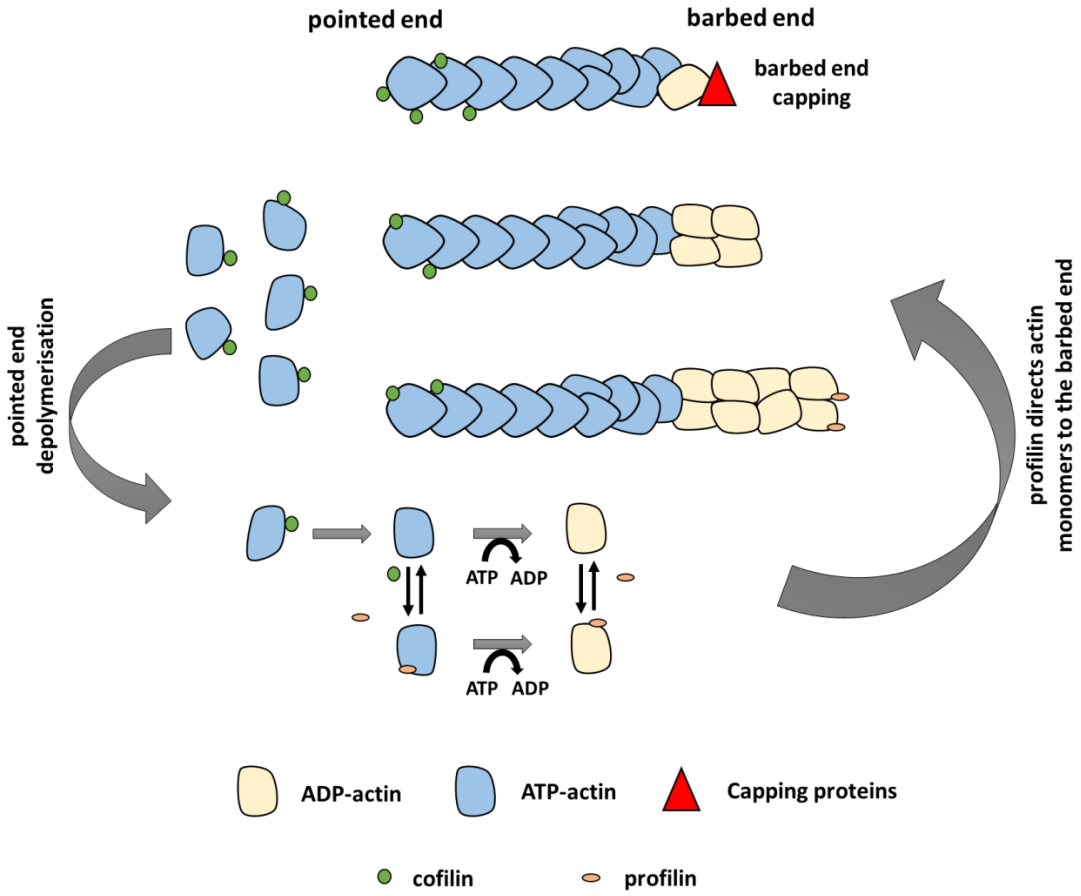

Figure 6.1. Actin cycle. F-actin filaments are separated due to Cofilin binding at the pointed end. Profilin binds to F-actin filaments at the barbed end before capping proteins are attached, allowing the attachment and detachment of filaments. Figure drawn based on details of the actin cycle described by Brown (2016).

6.1.2 Regulation of the Cytoskeleton Organisation by Rho GTPases

Estrogen was shown to promote the phagocytosis of the two major wound pathogens MRSA and *P. aeruginosa* (Chapters 3, 4 and 5). However, the link between estrogen and regulators of the actin cytoskeleton such as Rho GTPases during phagocytosis has not been investigated to date.

The organised movement of membranes and actin cytoskeleton are coordinated in phagocytosis by a family of proteins known as Rho GTPase (Etienne-Manneville and Hall, 2002). The small GTPases of the Rho-family (Rho-GTPases) are members of the Ras superfamily of small GTPases. The Rho-GTPase family includes 20 members distributed into 8 subfamilies (Figure 6.2) (Etienne-Manneville and Hall, 2002). While RhoA, Cdc42 and Rac1 are the most highly conserved and well-studied members in eukaryotic species, the 17 other members of the Rho GTPase family are less studied and their functions and effects have not been fully investigated and understood yet (Etienne-Manneville and Hall, 2002). Rac1, Cdc42 and RhoA are best known for their functions in regulating the actin cytoskeleton (Nobes and Hall, 1995; Machesky and Hall, 1996; A. Hall, 1998; Machesky and Insall, 1999; Etienne-Manneville and Hall, 2002). Working on 2D tissue-culture fibroblast models, (Ridley and Hall, 1992; Nobes and Hall, 1995) it has been reported that RhoA was mainly involved in rearranging actin and myosin filaments to form new stress fibres, however, Cdc42 and Rac1 induced actin polymerisation causing the formation of lamellipodia and filopodia. RhoA, Cdc42 and Rac1 have all emerged as master regulators of cell polarity and contractility, progression of the G1 cell cycle, gene transcription and microtubule dynamics (Etienne-Manneville and Hall, 2002). In addition to regulating the actin cytoskeleton dynamics, Rho GTPases have been linked with many biological functions such as cytoskeletal regulation, phagocytosis, cell polarity establishment, cell proliferation and motility, reactive oxygen species (ROS) production, and tumorigenesis (Etienne-Manneville and Hall, 2002; Jaffe and Hall, 2005).

Proteins of the Rho GTPase family are key regulators of the polymerisation of actin filaments resulting in cytoskeleton reorganisation (Etienne-Manneville and Hall, 2002). These proteins are parts of the GTP-binding proteins (G-proteins) family, which are conserved in eukaryotic organisms from yeast and amoeba to mammals (Etienne-Manneville and Hall, 2002). G-proteins contain a GTP-binding domain and function as molecular switches (Raftopoulou and Hall, 2004). In order to become active, the protein must bind to GTP causing its hydrolysation (Raftopoulou and Hall, 2004). The process of GTP hydrolysation is the origin of the name GTPase. As a

consequence of GTP hydrolysation to GDP, the protein changes structurally and converts into an inactive form (Raftopoulou and Hall, 2004). The Rho GTPase proteins receive signals from cell surface receptors, and interact indirectly with actin, via binding to effector molecules, which causes rearrangement of the cytoskeleton and enable phagocytosis (Raftopoulou and Hall, 2004).

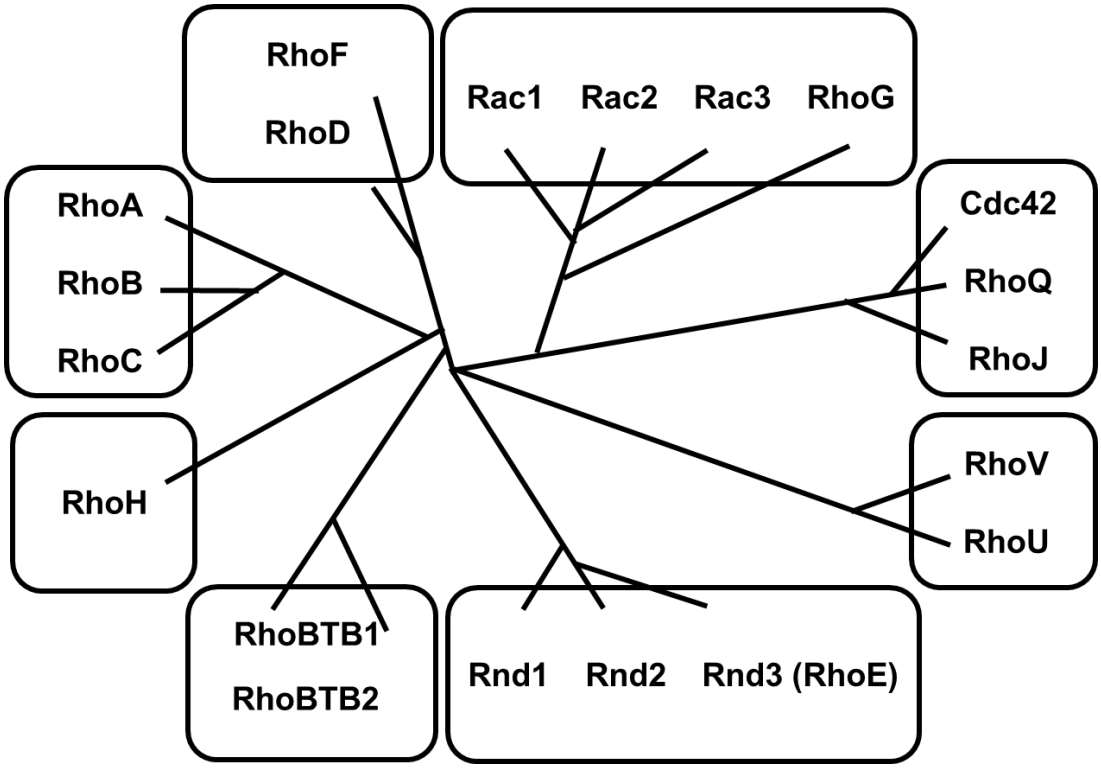

Figure 6.2. Phylogenetic tree representation of the Rho GTPases family. Rho GTPases family comprises 20 proteins divided into eight major groups according to sequence and phylogenetic similarities: Rho, Rac, Cdc42, RhoD/F, Rnd, RhoU/V, RhoH and RhoBTB. Figure based on published details of Rho GTPases (Aspenström *et al.*, 2007; Heasman and Ridley, 2008).

6.1.2.1 Cdc42

Cdc42 is an important Rho GTPase protein that controls signalling pathways regulating many cell functions essentially phagocytosis, cell cycle development and cell migration (Ridley *et al*., 1992; Kozma *et al*., 1995; Qadir *et al*., 2015). Cdc42 is a key regulator of the cytoskeleton dynamics, which is essential for the phagocytosis process (Ridley *et al*., 1992; Kozma *et al*., 1995). In order to elucidate the functions of Cdc42 in humans, researchers studied the dominant negative (DN) and the constitutively active (CA) forms of Cdc42 (Van Aelst and D'Souza-Schorey, 1997), and demonstrated that cdc42 is potentially required in the initiation of cell signals

crucial for the regulation of actin cytoskeleton (Van Aelst and D'Souza-Schorey, 1997; Johnson, 1999). The Cdc42-related proteins stimulate the formation of filopodia, through binding to and activation of an effector protein known as "Wiskott Aldrish Syndrome Protein (WASP)" via Cdc42- and Rac-interacting and binding domain (CRID) (Johnson, 1999; Rohatgi *et al*., 1999; Carlier *et al*., 2003). This leads to the recruitment of G-actin and a specific actin protein called the "Arp2/3 complex", which bind to the existing actin filaments resulting in a new filament (Rohatgi *et al*., 1999; Carlier *et al*., 2003).

Similar to WASP, other proteins such as the tyrosine p21-activated kinases (PAK1 and PAK2) and focal adhesive kinase (FAK), attach to Cdc42 via the CRIB domain and initiate the cytoskeleton reorganisation leading to membrane deformation, essential for phagocytosis (Hofmann *et al*., 2004).

Cdc42 treadmills continuously and reversibly from an active GTP-bound form to an inactive GDP-bound form. This balance is initiated and controlled by guanine nucleotide exchange factors (GEFs) which encourage the conversion of GDP-bound molecules to unbound GTP (Van Aelst and D'Souza-Schorey, 1997). GTPase activating proteins (GAPs) promote GTP hydrolysis to GDP (Lamarche and Hall, 1994), whereas guanine nucleotide dissociation inhibitors (GDIs) block the detachment of the nucleotide from Cdc42 and result in deactivation of the protein (Olofsson, 1999; Valdés-Mora *et al*., 2009).

6.1.2.2 Rac1

The GTPase Rac1 is one of the most documented and well-studied members of the Rho family (Bishop and HALL, 2000; Etienne-Manneville and Hall, 2002). Rac1 is a member of the Rac subfamily that comprises RhoG, Rac1b, Rac2, and Rac3 (Aspenström *et al.*, 2007; Heasman and Ridley, 2008). The Rac proteins all share more than 80% sequence homology and differ principally in the C-terminal region (Wennerberg, 2004). Rac1 is known for being a master regulator of the actin cytoskeleton organisation. Rac1 participates in the initial step of pseudopodia formation (Etienne-Manneville and Hall, 2002) during phagocytsosis, but is also involved in many other cell functions including cell killing mediation via ROS, cell

differentiation, cell adhesion, apoptosis, signalling transcription factors and gene expression (Jaffe and Hall, 2005). The activity of Rac1 has been extensively studied in recent years, with findings showing active Rac1 induces the formation of membrane extensions called lamellipodium (Ridley *et al*., 1992). In addition, RAC1 is essential in moderating several signalling pathways linked with cell growth and cell division (Bid *et al*., 2013). Similar to Cdc42, the activities of Rac1 are mediated via interaction with specific effectors such as the protein kinase PAK that changes from an inactive to active state after interacting directly with GTP-Rac1 (Bid *et al*., 2013).

6.1.2.3 RhoG

RhoG is a member of the Rac subgroup of the Rho GTPase family that was reported for the first time in 1992 (Vincent *et al*., 1992). It shares 72% sequence identity with Rac1 but is characterised by its incapability to bind to proteins such as PAKs (Leung *et al*., 1996; Gauthier-Rouviere *et al*., 1998). Similar to most Rho GTPase proteins, RhoG is involved in a various cell signalling mechanisms including the regulation of actin cytoskeleton (Katoh *et al*., 2006), phagocytosis (Prieto-Sánchez *et al*., 2006), gene transcription (Vigorito *et al*., 2003) and the regulation of NADPH oxidase in neutrophils (Condliffe *et al*., 2006).

Both Rac1 and RhoG are able to signal downstream effectors after binding to GTP and become inactive when bound to GDP. Three protein families interact with Rac1 or RhoG to control the GTP/GDP balance: 1) GEFs are involved in facilitating the conversation of GDP for GTP, leading to the promotion of RhoA/RhoG-mediated signalling (Van Aelst and D'Souza-Schorey, 1997); 2) GAPs promote hydrolysis of GTP to GDP, thereby ending RhoA/RhoG-mediated signalling (Lamarche and Hall, 1994); 3) GDIs inhibit the GDP dissociation and lock the G protein in its inactive state (Olofsson, 1999).

It has been reported that the activation of RhoG provokes Rac1- and Cdc42-like activities such as lamellipodia and filopodia formation (Gauthier-Rouviere *et al*., 1998). Activated Rac1 and/or RhoG are believed to be coupled with numerous downstream effectors, leading to the control of several signalling pathways. The mechanism by which RhoG signals is not fully understood. However, a detailed

signalling pathway of RhoG leading to activation of Rac proteins, via GEFs, has been described. This mechanism of RhoG-dependent Rac signalling was reported to encourage the phagocytosis of apoptotic cells in *C. elegans* (Katoh and Negishi, 2003; Nakaya *et al*., 2006).

6.1.2.4 RhoA

RhoA is a small protein member of the Rho GTPase family. Like the other GTPases, RhoA acts as a molecular switch to regulate signalling pathways that mediate the actin cytoskeleton dynamics and organisation (Hall, 1998). RhoA was initially reported to be a key player in the regulation of the cytoskeletal dynamics, and the formation of actin stress fibres in fibroblasts stimulated with growth factors (Ridley and Hall, 1992). It was later shown by Hackam and Grinstein in 1997 that RhoA is an essential requirement in phagocytosis. Olson *et al*. (1995) also demonstrated that RhoA regulates the growth of cells and triggers cell cycle progression via the phase G1.

The majority of downstream targets of RhoA are serine-threonine kinases, including ROCK1 which binds to RhoA in a GTP-dependant manner (Leung *et al*., 1995). RhoA was also demonstrated to form actin stress fibres and focal adhesion complexes via binding to the ROCK kinase, an isoform of Rho kinase. Another Rho kinase known as Rho kinase-alpha or ROK was also shown to bind to RhoA in a GTP-dependent manner, leading to the assembly of actin filaments and the cytoskeleton organisation (Leung *et al*., 1996).

The interaction between estrogen and Rac1 in cardiovascular disease has been documented, with estrogen downregulating the expression and activity of Rac1 in monocytes and vascular smooth muscle cells and resulting in a decrease in the production of ROS in the cardiovascular system (Laufs *et al*., 2003). Estrogen enhances the phagocytic activity of rat peritoneal macrophages (Csaba, 2017) but inhibits monocyte recruitment and adhesion in the cardiovascular system (Friedrich *et al*., 2006)

6.1.3 Focal Adhesion Kinase (FAK)

The focal adhesion kinase (FAK) was first discovered in the early 1990s (Kanner *et al*., 1990; Schaller *et al*., 1992) as a new tyrosine kinase that does not possess a receptor but is expressed in several tissues (Hanks *et al*., 1992). FAK is highly conserved among different species with human FAK sharing high percentage homology with mice (97%) and zebrafish (90%) (Schaller, 2010). It has been reported that FAK plays a key role in integrin signalling which regulates phagocytosis, cell adhesion and cell survival (Schlaepfer *et al*., 2004). The gene protein tyrosine kinase 2 (*PTK2*) encodes for FAK (André and Beckerandre, 1993). It has been demonstrated that blockage of FAK in breast cancer cells reduce their metastatic ability by decreasing cell mobility (Chan *et al*., 2009).

FAK is expressed in all cell types except some blood cells, and is naturally localised in structures called focal adhesions. Focal adhesions are sites where the cytoplasmic cytoskeleton interact with the ECM (Chrzanowska-Wodnicka and Burridge, 1996). When FAK tyrosine kinase is activated, intracellular signalling pathways involved in cell migration are initiated (Hanks *et al*., 1992; Schaller *et al*., 1992). Estrogen is a known regulator of cytoskeletal constituents (Sapino *et al*., 1986), mainly actin fibres via the control of actin dynamism and organisation (Giretti *et al*., 2008; Flamini *et al*., 2009). Estrogen also regulates cell morphology and cell interaction with the ECM, a key process in phagocytosis (Giretti *et al*., 2008; Flamini *et al*., 2009; Flamini *et al*., 2011). Flamini *et al*. (2011) investigated the effect of estrogen on the remodelling of actin cytoskeleton in the Ishikawa endometrial adenocarcinoma cell line and endometrial stromal cells (ESC). This study showed remodelling estrogen promotes FAK phosphorylation in Ishikawa and ESC cells in a dose-dependent manner, resulting in an enhancement of actin and membrane remodelling, and phagocytosis.

6.2 Aim and Objectives

6.2.1 Aim

To investigate the effect of estrogen on regulating protein levels of Rac1, RhoA, Cdc42, RhoG and FAK in U937-derived M1 macrophages.

6.2.2 Objectives

To compare protein levels of Rac1, RhoA, Cdc42, RhoG and FAK in U937-derived M1 macrophages treated with/without supraphysiological levels of estrogen for 24 hours against BSA-treated negative control.

6.3 Methods

6.3.1 Generation of U937-derived M1 Macrophages

U937 monocytes were differentiated into M0 macrophages and polarised into M1-like macrophages using methods described in Section 2.2.5.

6.3.2 Immunoblotting

U937-derived M1 macrophages were treated with 1 x 10^{-7} M BSA, zero M estrogen (17β-estradiol) or 1 x 10^{-7} M estrogen for 24 hours. Macrophages were retrieved and lysed prior to extracting and quantifying proteins following methods described in Section 2.2.13.1. Immunoblotting assays were conducted as described in Section 2.2.13.2 to quantify levels of Rac1, RhoA, Cdc42, RhoG and FAK with protein normalisation conducted against the housekeeping gene β-tubulin.

6.4 Results

6.4.1 Estrogen Enhances Rac1 by U937-Derived M1 Macrophages

The effect of estrogen on levels of the GTPase Rac1 by U937-derived M1 macrophages was investigated (Figure 6.3). Estrogen had no significant effect (P>0.05) on levels of β-tubulin in U937-derived M1 macrophages compared to untreated macrophages (zero M estrogen) and the negative control (Figure 6.3.A and C). However, estrogen supplementation (Figure 6.3. B and C) significantly (P<0.01) increased relative Rac1 levels (1.9-fold change) compared to the negative control (NC). There was no significant difference (P>0.05) between Rac1 levels in the NC and untreated macrophages that lacked estrogen supplementation.

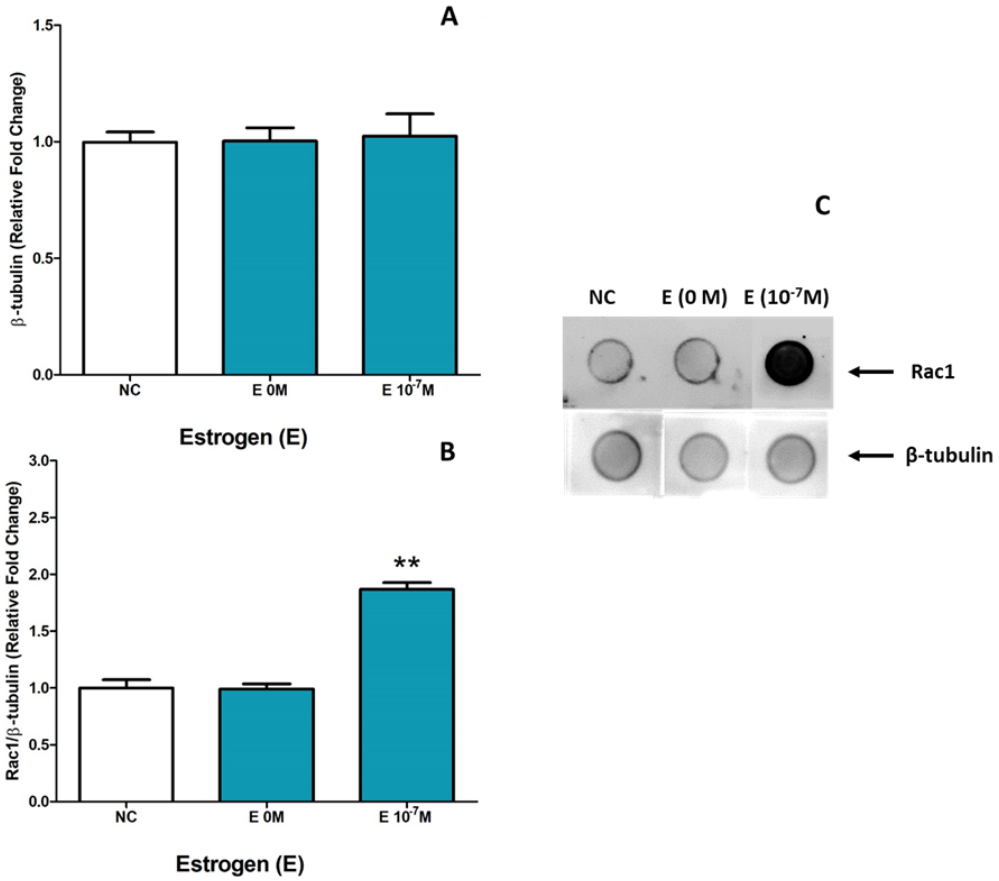

Figure 6.3. Rac1 levels in U937-derived M1 macrophages increase following exposure to supraphysiological estrogen. Rac1 levels were assessed by immunoblotting with β-tubulin used as loading control. Estrogen (E) had no effect on the β-tubulin levels in macrophages (P>0.05) (A and C), but significantly (P<0.01) increased Rac1 levels compared to the negative control (NC) and untreated (E 0M) macrophages (B and C). Results represent an average of n = 12 experimental repeats. ** Indicates significant difference in protein levels

(fold-change) compared to the NC (P<0.01). Error bars represent the standard error of the mean (StEM).

6.4.2 Estrogen Decreases RhoA in U937-Derived M1 Macrophages

Estrogen had no significant (P>0.05) effect on the housekeeping protein β-tubulin (Figure 6.4.A and C) but significantly (P<0.01) reduced relative levels of RhoA (0.7-fold change) in U937-derived M1 macrophages compared to the negative control (NC). There was no significant (P>0.05) difference in RhoA levels between the NC and untreated macrophages lacking estrogen supplementation (Figure 6.4.B and C).

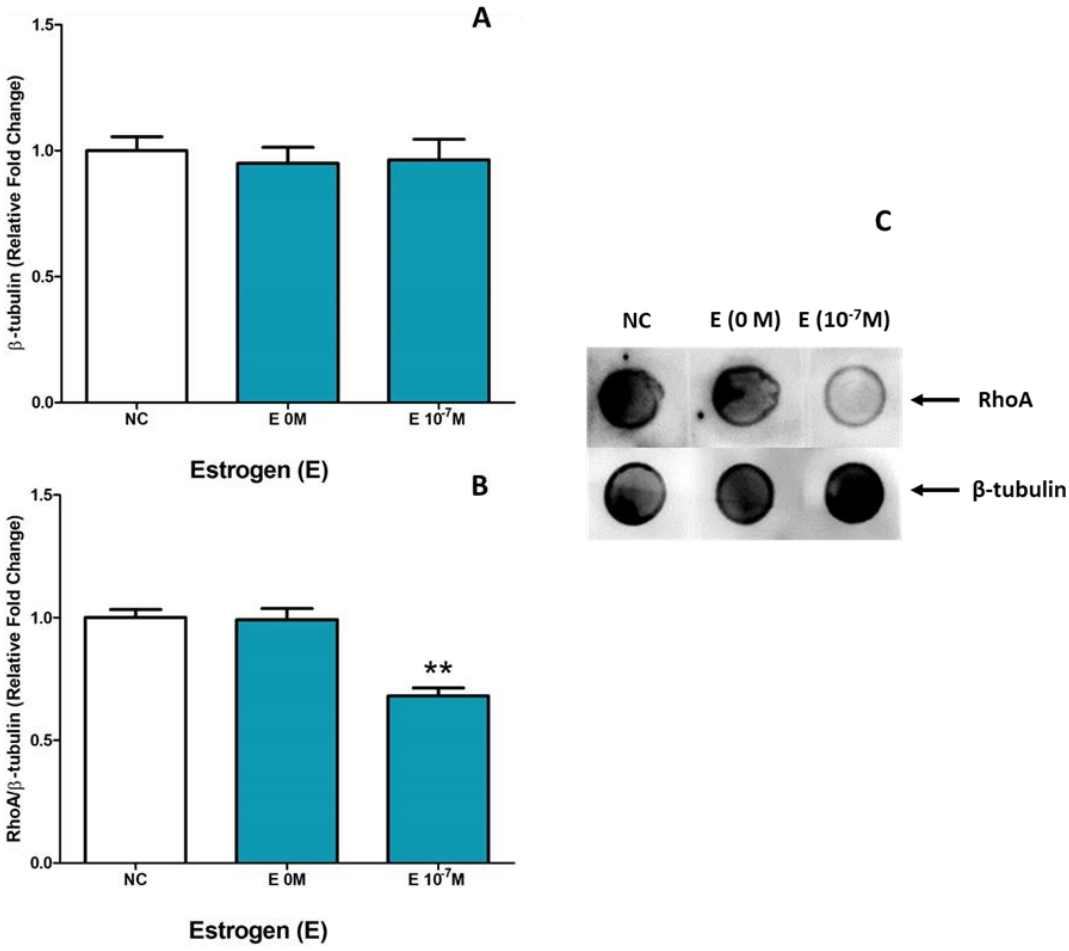

Figure 6.4. RhoA levels in U937-derived M1 macrophages reduce following treatment with supraphysiological estrogen. RhoA levels were measured by immunoblotting using β-tubulin as the housekeeping protein. Estrogen (E) had no significant (P>0.05) effect on levels of β-tubulin (A and C) but significantly (P<0.01) decreased RhoA levels compared to the negative control (NC) and untreated (E 0M) macrophages (B and C). Results represent an average of n = 12 experimental repeats. ** Indicates significant difference (P<0.01) in protein levels (fold-change) compared to the NC. Error bars represent the standard error of the mean (StEM).

6.4.3 Estrogen Increases Levels of Cdc42 in U937-Derived M1 Macrophages

Estrogen had no significant (*P*>0.05) effect on levels of the housekeeping protein β-tubulin (Figure 6.5.A and B). In comparison to the negative control (NC), the absence of estrogen in untreated macrophages had no significant (*P*>0.05) effect on Cdc42 levels (1.0-fold change). However, estrogen induced a significant (*P*< 0.01) increase in levels of Cdc42 (2.3-fold change) compared to the NC (Figure 6.5.B and C).

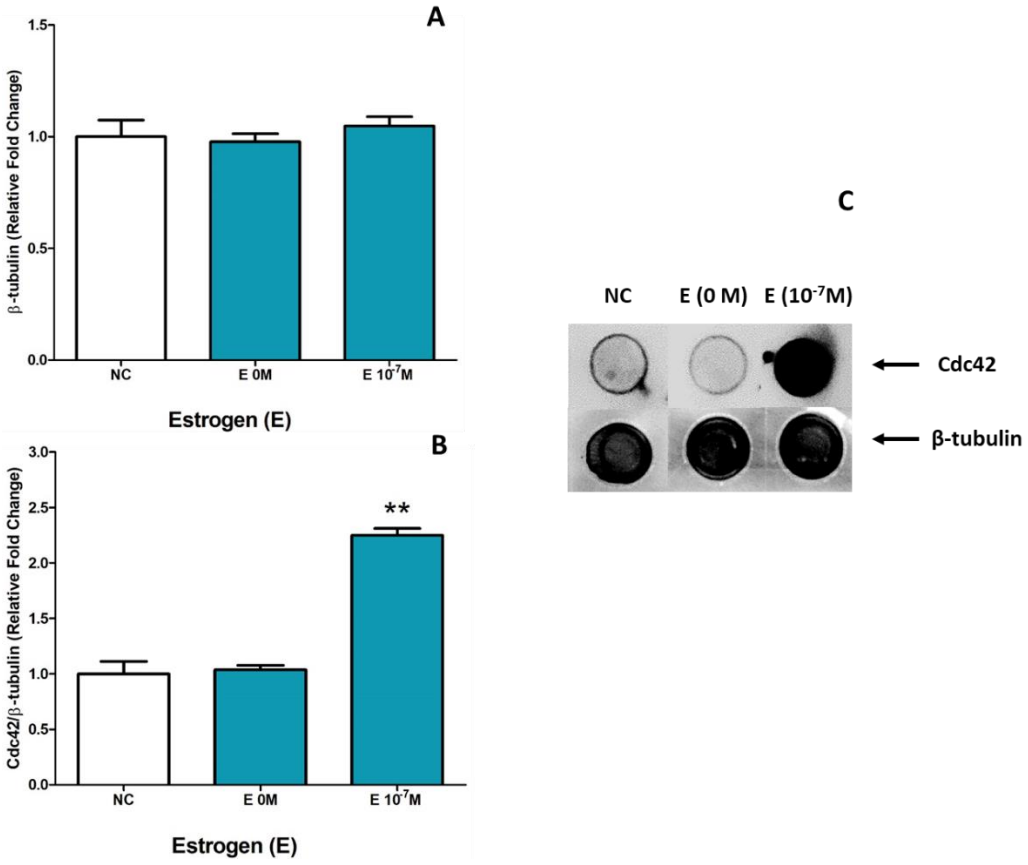

Figure 6.5. Cdc42 levels in U937-derived M1 macrophages increase following exposure to supraphysiological estrogen. Cdc42 levels were assessed by immunoblotting with β-tubulin used as loading control. Estrogen (E) had no effect on the β-tubulin levels in macrophages (*P*>0.05) (A and C), but significantly (*P*<0.01) increased Cdc42 levels compared to the negative control (NC) and untreated (E 0M) macrophages (B and C). Results represent an average of n = 12 experimental repeats. ** Indicates significant difference in protein levels (fold-change) compared to the NC (*P*<0.01). Error bars represent the standard error of the mean (StEM).

6.4.4 Estrogen Increases Levels of RhoG in U937-Derived M1 Macrophages

The effect of estrogen on levels of RhoG in U937-derived M1 macrophages was investigated (Figure 6.6). Estrogen had no significant effect on levels of β-tubulin in

U937-derived M1 macrophages, compared to negative control (NC) and untreated macrophages (E 0M) (Figure 6.6.A and C). Compared to the NC, the level of RhoG was not significantly (P>0.05) altered in untreated (E 0M) macrophages (1.1-fold change). However, estrogen significantly (P< 0.01) increased relative levels of RhoG (2.3-fold change) in comparison to the NC (Figure 6.6.B and C).

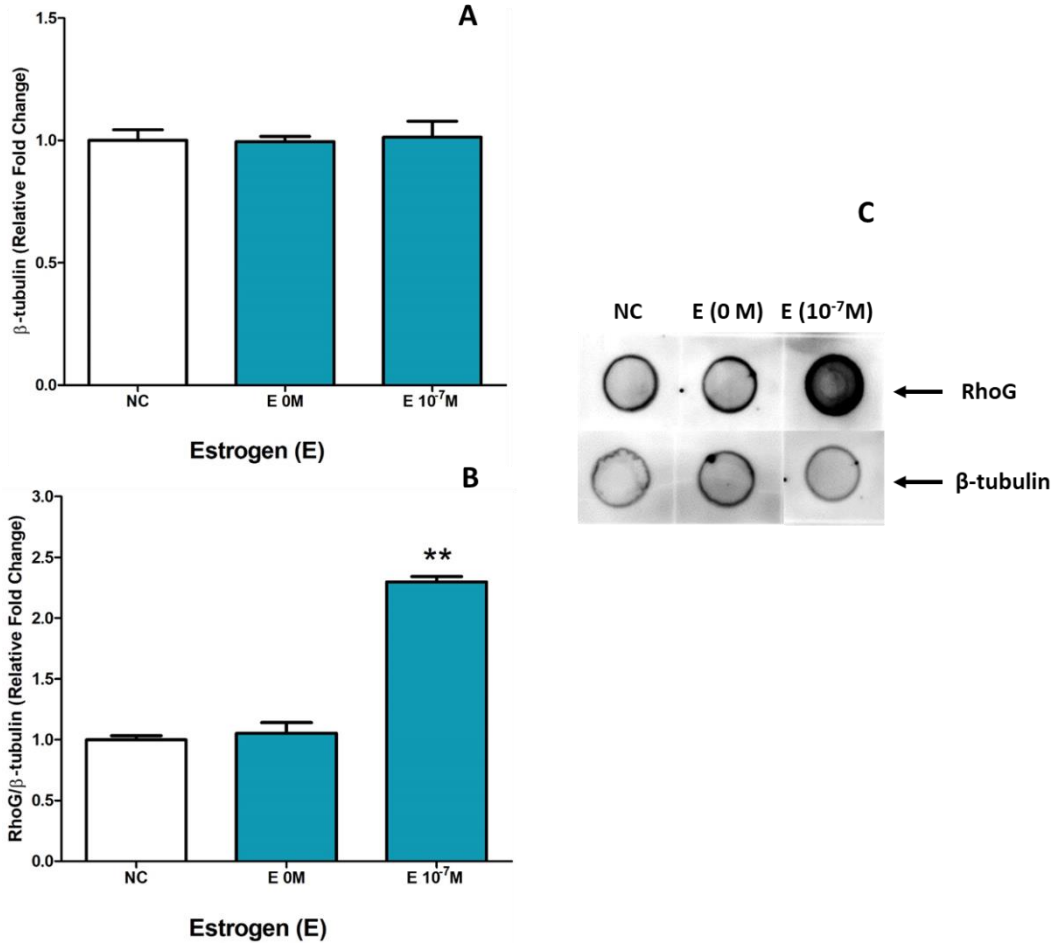

Figure 6.6. RhoG levels in U937-derived M1 macrophages increase following treatment with supraphysiological estrogen. RhoG levels were assessed by immunoblotting with β-tubulin used as loading control. Estrogen (E) had no effect on the β-tubulin levels in macrophages (P>0.05) (A and C), but significantly (P<0.01) increased RhoG levels compared to the negative control (NC) and untreated (E 0M) macrophages (B and C). Results represent an average of n = 12 experimental repeats. ** Indicates significant difference in protein levels (fold-change) compared to the NC (P<0.01). Error bars represent the standard error of the mean (StEM).

6.4.5 Estrogen Increases Levels of Focal Adhesion Kinase (FAK) in U937-Derived M1 Macrophages

Levels of the housekeeping protein β-tubulin did not significantly change (P>0.05) following treatment of U937-derived M1 macrophages with supraphysiological

estrogen (Figure 6.7.A and C). Levels of FAK in untreated macrophages (1.0-fold change) were not significantly (P>0.05) different to levels found in the negative control (NC). Intriguingly, relative levels of FAK significantly increased (P<0.01) following treatment with estrogen (2.3-fold change) compared to the NC (Figure 6.6.B and C).

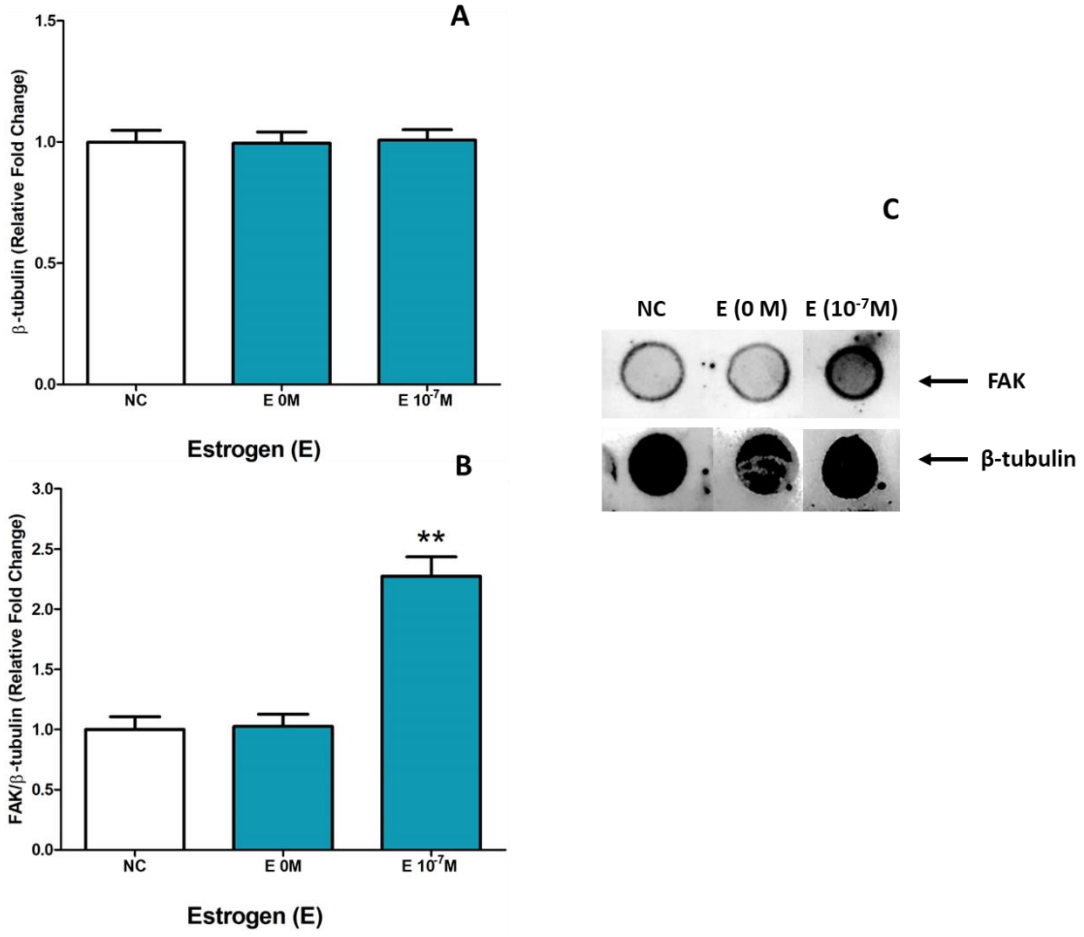

Figure 6.7. FAK levels in U937-derived M1 macrophages increase following treatment with supraphysiological estrogen. FAK levels were assessed by immunoblotting with β-tubulin used as loading control. Estrogen (E) had no effect on the β-tubulin levels in macrophages (P>0.05) (A and C), but significantly (P<0.01) increased FAK levels compared to the negative control (NC) and untreated (E 0M) macrophages (B and C). Results represent an average of n = 12 experimental repeats. ** Indicates significant difference in protein levels (fold-change) compared to the NC (P<0.01). Error bars represent the standard error of the mean (StEM).

6.5 Discussion

Estrogen promotes the phagocytosis of both MRSA and *P. aeruginosa* by human macrophages through specific binding to ER-α. (Chapter 5). Moreover, data from the SEM (Chapter 3) demonstrated major changes in the morphology of phagocytic macrophages following estrogen supplementation, with the formation of pseudopodia suggesting estrogen induces substantial changes in the actin cytoskeleton. In order to determine whether Estrogen stimulates the phagocytic activity of macrophages through changes in the actin cytoskeleton, this study measured the protein levels of four major Rho GTPase proteins (Cdc42, Rac1, RhoG and RhoA) and the focal adhesion kinase (FAK) in U937-derived M1 macrophages in the presence/absence of estrogen for 24 hours.

Estrogen significantly increased the level of FAK in M1 macrophages. FAK has been shown to be an important regulator of phagocytosis (Tzircotis *et al*., 2011). Tzircotis *et al*. (2011) transfected macrophages with RNAi against FAK and found that macrophages lacking FAK presented a 65% decrease in the number of phagocytes produced with FcγR- and CR3-mediated phagocytosis. Activation of FAK was also associated with provoking Rac1- and Cdc42-like activities, such as lamellipodia and filopodia formation (Gauthier-Rouviere *et al*., 1998), but not RhoA activities (Tzircotis *et al*., 2011). It has been reported that fibroblasts lacking FAK have impaired cell motility. Importantly, FAK is an important regulator of the pathogen-killing functions of neutrophils (Kasorn *et al*., 2009)**.** FAK signalling is linked with the organisation of integrin-based adhesion sites and the formation of lamellipodia in migrating cells, such as neutrophils and macrophages (Kasorn *et al*., 2009). Moreover, FAK deficiency in macrophages reduces cell adhesion, and is associated with failure to form lamellipodia and cell migration to sites of infection and inflammation *in vivo* (Owen *et al*., 2007).

Estrogen is a known regulator of cytoskeletal constituents, mainly actin fibres (Giretti *et al*., 2008; Flamini *et al*., 2009) via the control of actin dynamism and organisation. Estrogen also regulates cell morphology and interaction with the ECM, a key process in phagocytosis (Giretti *et al*., 2008; Flamini *et al*., 2009). Flamini *et al*.

(2011) investigated the effect of estrogen on the remodelling of actin cytoskeleton and on regulating cell mobility in an Ishikawa cell line and ESC. They showed estrogen encourages FAK phosphorylation in Ishikawa and ESC cells in a dose-dependent manner, resulting in an enhancement of cell mobility, actin and membrane remodelling (Flamini *et al*., 2011).

Importantly, estrogen has been shown to stimulate the production of membrane ruffles and pseudopodia in breast cancer cells via activation of ER-α in concordance with the findings (Chapter 3) of this study (Song *et al*., 2002). Simoncini *et al*. (2006) confirmed that estrogen encourages the migration of endothelial cells via phosphorylation and activation of FAK. Song and Santen (2006) indicated that estrogen enhances the formation of filopodia and lamellipodia with localisation of ER-α in the plasma membrane. Stimulation of cancer cells with estrogen induces cell migration and motility via interaction of FAK with ER-α and other factors including phosphatidylinositol 3 kinase and p60 Src tyrosine kinase (c-Src) (Li *et al*., 2010). Given estrogen stimulated phagocytosis via ER-α (Chapter 3) and increased levels of FAK further investigations in this area are now warranted to confirm a causal link between elevated FAK levels and enhanced phagocytosis in estrogen supplemented M1 macrophages.

In this study, macrophage levels of Rac1 significantly increased following treatment with estrogen for 24 hours compared to the negative control or macrophages lacking estrogen supplementation. Rac1 is crucial for dynamic rearrangement of the actin cytoskeleton that is essential for membrane ruffling and the development of focal complexes that are needed for filopodia and lamellipodia formation (Ridley *et al*., 1992; Allen *et al*., 1997). Rac1 regulates the organisation of actin cytoskeleton and cell adhesion in murine macrophages *in vitro* (Allen *et al*., 1997). Upregulation of active Rac1 have been detected in phagocytic cups (Hoppe and Swanson, 2004) while its inhibition was found to reduce the phagocytosis of both C3bi- and IgG-opsonised molecules (Cox *et al*., 1997). It is noteworthy that estrogen-like molecules such as daidzein have been shown to have an effect on the regulation of Rac1 during the phagocytic process. Daidzein, a soy isoflavone extract, was found to stimulate the production of estrogen in trophoblast cells (Richter *et al*., 2009).

Moreover, diadzein enhances the macrophage phagocytic capability via upregulation of the Rac1 activity *(Yen et al*., 2014).

In another report, it has been shown that the estrogen metabolite (2-ME) blocks the RhoA/ROCK1 pathway in human smooth muscle cells through downregulation of RhoA (Rigassi *et al*., 2015). It has also been reported that the upregulation of RhoA inhibits the process of phagocytosis in macrophages (Tosello-Trampont *et al*., 2003; Colucci-Guyon *et al*., 2005; Nakaya *et al*., 2006). Results in this study have demonstrated that treatment of macrophages with estrogen for 24 hours significantly decreased protein levels of RhoA compared to the negative (BSA) negative control and macrophages cultured in the absence of estrogen. Whether estrogen stimulates phagocytosis by dampening the RhoA/ROCK1 pathway warrants further investigation but accumulating evidence has indicated that upregulation of RhoA inhibits the process of phagocytosis in macrophages (Tosello-Trampont *et al*., 2003; Colucci-Guyon *et al*., 2005; Nakaya *et al*., 2006).

It is worth pointing out that many other reports support the findings of this study and show a harmonised balance of Rac1 and RhoA is essential for a successful phagocytosis. Research investigations on *Candida elegans* in mammals have reported that Rac1 and RhoA, are antagonistically implicated in the modulation of cytoskeleton reorganisation during the process of phagocytosis, as their signalling oppose each other (Ohta *et al*., 2006; Freeman and Grinstein, 2014). To date, their exact functions are not fully clear, but recent studies using mutants of Rac1 and RhoA specified that upregulation of Rac1 stimulates phagocytes to engulf apoptotic cells (Akakura *et al*., 2004; Nakaya *et al*., 2008) whereas RhoA had an opposite effect (Kim *et al*., 2017). Kim *et al.* (2017) investigated the role of Rac1 and RhoA in the clearance of apoptotic cells and showed a dynamic balance between the two proteins was needed to regulate efferocytosis, with Rac1 upregulated and RhoA downregulated during pseudopodial formation.

RhoG is associated with the regulation of actin cytoskeleton dynamics (Katoh *et al*., 2006), phagocytosis (Prieto-Sánchez *et al*., 2006), gene transcription (Vigorito *et al*., 2003) and the regulation of NADPH oxidase in neutrophils (Condliffe *et al*., 2006). Data in this chapter demonstrates for the first time that treatment of macrophages

with estrogen causes an increase in RhoG levels leading to enhanced phagocytosis of bacteria. This result supports the findings of Tzircotis *et al*. (2011) that demonstrated that RhoG stimulates phagocytosis of IgG-opsonised (for FcγR) or C3bi-opsonised (for CR3) sheep red blood cells (RBCs) in mouse macrophages. However, the findings in this chapter are the first to show that estrogen stimulates RhoG in human M1 macrophages. Therefore, elevation of RhoG levels could potentially be a novel and effective therapeutic approach for the treatment of wound. However, further investigations are needed to provide a confirmation of this hypothesis.

In this study, levels of Cdc42 in M1 macrophages following treatment with estrogen significantly increased. Resveratrol, a plant-derived polyphenol found in grapes and associated products such as wine, has both anti-estrogenic effects in breast cancer and but is believed to be estrogenic in peripheral tissues such as bone (Bhat *et al*., 2001; Tou, 2015). Azios *et al*. (2007) investigated the effect of resveratrol in estrogen-responsive breast cancer, and found that resveratrol decreased the activity of Cdc42 and Rac1 whereas a combination of resveratrol with estrogen resulted in a significant upregulation of both Cdc42 and Rac1 in breast cancer cells. Cdc42 is linked with the organisation of the actin cytoskeleton, membrane ruffling and formation of filopodia in fibroblasts and macrophages (Kozma *et al*., 1995; Cox *et al*., 1997). Research indicates Cdc42 is crucial for successful phagocytosis of *Salmonella* in COS-1 cells (L.-M. Chen *et al*., 1996; Cox *et al*., 1997). In addition, the injection of macrophages with activated Cdc42 results in the formation of long and indistinct filopodia *in vitro* (Allen *et al*., 1997). Cdc42 is an essential requirement for Fc receptor-mediated phagocytosis and for the formation of membrane ruffling mediated by diverse receptors in macrophages (Allen *et al*., 1997).

In summary, estrogen increased levels of Rac1, Cdc42, RhoG and FAK, but reduced levels of RhoA in U937-derived M1 macrophages. Further interrogation of pathways involved in the actin cytoskeleton could potentially reveal other mediators of estrogen-induced phagocytosis. Whilst results presented in this chapter provided exciting and novel links between estrogen and proteins of the cytoskeleton during the process of phagocytosis, further investigations are warranted to determine the

effect of estrogen on relative amounts of proteins in their activated (phosphorylated) state during the process of phagocytosis. Western blotting, kinase activity assays (to measure FAK activation), and intracellular flow cytometry would be very robust methods of measuring activated protein levels. Inhibitors of phagocytosis pathways could also be exploited to further understand the mechanisms by which estrogen induces phagocytosis. In addition, further work is required to determine whether the changes in protein levels are mirrored by concomitant alterations in gene expression. In order to determine the effect of ER-α activation on downstream gene expression in macrophages, RNA sequencing (RNAseq) and real time polymerase chain reaction (qPCR) analysis could be adopted. Understanding the precise mechanisms by which estrogen promotes phagocytosis might reveal key downstream targets to interrogate further and may ultimately lead to the development of novel therapies to treat wound infections in the elderly with high efficacy and minimal side effects.

Chapter 7: Discussion and Future Work

7.1 Discussion

Chronic wounds are difficult to treat and often become colonised with bacteria leading to substantial mortality and morbidity in the elderly. Impaired wounds can become colonised with a varied range of pathogens including *S. aureus* and *P. aeruginosa* (Gjødsbøl *et al*., 2006; Kirketerp-Møller *et al*., 2008; Rybtke *et al*., 2015). Treatment of bacterial infections in wounds requires aggressive medication, and the use of last-generation antibiotics (Beasley and Hirst, 2004). Thus, the treatment of wound infections in the elderly represents a big challenge for the healthcare services (Beasley and Hirst, 2004). The propensity for wound infections increases in the elderly, in part due to the delay in wound repair (Cooper *et al*., 2015). Estrogen is a key regulator of the wound healing process and estrogen deprivation in the elderly is linked with pathological wound healing (Ashcroft *et al*., 1999a; Margolis *et al*., 2002). Indeed, impaired healing in the elderly is mediated by changes in steroid hormones, particularly declining levels of estrogen with increasing age (Ashcroft *et al*., 1999a; Ashcroft *et al*., 2003; M. J. Hardman and G. S. Ashcroft, 2008). Estrogen deficiency causes a significant delay in wound repair, with topical and systemic estrogen replacement reversing this delay (Ashcroft *et al*., 1997a; Ashcroft *et al*., 1999a). Furthermore, estrogen supplementation promotes age-related wound healing in both elderly males and females (Ashcroft *et al*., 1999a; Guo and DiPietro, 2010). Delayed wound healing in the elderly is associated with a delayed, but prolonged and excessive inflammatory response, with dysregulated expression of pro-inflammatory cytokines and proteolytic mediators. The key role of estrogen in regulating inflammation has been extensively reviewed (Gilliver *et al*., 2007; Guo and DiPietro, 2010), with estrogen supplementation decreasing the inflammatory response in elderly subjects of both genders (Ashcroft *et al*., 1999a). However, despite the pronounced inflammatory response, evidence suggests declining levels of estrogen with increasing age can result in an impaired ability of inflammatory cells to eliminate bacteria from infected wounds (Emori *et al*., 1991; Thomas, 2001).

The possible role estrogen plays in macrophage-mediated bacterial clearance has not been fully characterised to date. In order to elucidate the effect of age-related changes in estrogen levels on the clearance of bacteria by human macrophages,

this study conducted host-pathogen interaction assays to investigate the phagocytosis of two major wound pathogens, MRSA and *P. aeruginosa* by *in vitro* U937-derived macrophages and *ex vivo* human peripheral blood monocyte (HPBM)-derived macrophages. Investigations were performed under conditions that model estrogen levels found in the elderly (estrogen deprivation), young adults (referred to as physiological estrogen in this thesis) and following exogenous estrogen supplementation.

The conversion of monocytes into macrophage-like cells *in vitro* was an essential requirement to conduct all the host-pathogen assays in this study. PMA is reported to induce the differentiation of monocytes into macrophages *in vitro* (Martinez *et al*., 2006; Rios de la Rosa *et al*., 2017) via upregulation of cell adhesion molecules such as beta-2integrins (CD11a, CD11c, CD18 and CD11b) (Luscinskas *et al*., 1994), enabling cells to adhere to cell culture flasks *in vitro*. The differentiation of monocytes into macrophages was assessed in this study via the detection of the CD11c surface marker by flow cytometry. The high expression of CD11c in PMA-differentiated macrophages, compared with monocytes incubated in the absence of PMA, confirmed the successful transformation of U937 monocytes and HPBMs into macrophage-like cells *in vitro*.

Macrophage stimulation with supraphysiological levels of estrogen increases the phagocytic function of human brain macrophages (microglial cells) to take up amyloid β-peptide and *E. coli* (Li *et al*., 2000). Evidence has indicated that estrogen increases bacterial clearance in an *in vitro* LPS model of *K. pneumoniae* infection in acute wounds Crompton *et al*. (2016). In concordance with these reports, novel findings presented in this thesis (Chapter 3) have demonstrated that physiological (typical of youth) and supraphysiological levels of estrogen promote the engulfment of both Gram-positive and Gram-negative live bacteria by both human U937-derived macrophages and HPBM-derived macrophages. Further, *in vitro* host-pathogen assays involving the uptake of fluorescent bacteria (GFP-*S. aureus*) by U937-derived macrophages validated these findings, with significantly increased intracellular fluorescence following treatment of phagocytes with estrogen. Due to the poor ability of the antibiotic gentamicin to permeate the macrophage

membrane, a gentamicin protection assay was performed to determine the number of viable bacteria inside the phagocytes following the killing of extracellular bacteria using gentamicin (Hamad *et al*., 2010; Rios de la Rosa *et al*., 2017). Novel findings from this assay indicated that physiological (typical of youth) and supraphysiological levels of estrogen promote not just the internalisation, but also the killing, of both MRSA and *P. aeruginosa* in a dose-dependent manner. Further evidence captured by fluorescent microscopy (epifluorescent and confocal microscopes) indicated a higher uptake of both GFP-*S. aureus* and mCherry-*P. aeruginosa* by estrogen-treated macrophages in comparison to estrogen-deprived macrophages, with Z-stack analysis confirming co-localisation of the bacteria within macrophages. Images captured by SEM provided additional evidence of enhanced host-pathogen interactions following treatment of macrophages with estrogen, with macrophages showing pathogen-specific morphological changes in response to MRSA and *P. aeruginosa*. Macrophages generated longer membrane extensions (pseudopodia) in response to *P. aeruginosa* compared to MRSA. This pathogen-specific difference is intriguing since the clearance of MRSA was consistently higher than the uptake of *P. aeruginosa* ($P<0.05$) regardless of the experimentals conditions. The lower bacterial uptake and longer pseudopodia produced by macrophages following interaction with *P. aeruginosa,* may be due to *P. aeruginosa's* being a motile bacterium (O'Toole and Kolter, 1998) and therefore phagocytes may have reduced success when stretching out further to capture a moving target due to increased demands on cytoskeleton reorganisation.

Known for its anti-inflammatory proprieties, estrogen reduces excessive and prolonged inflammation by directly affecting the inflammatory response of monocytes, macrophages and neutrophils (Ashcroft *et al.*, 2003; Guo and DiPietro, 2010). Estrogen dampens the inflammatory response via inhibition of pro-inflammatory cytokines production by tissue macrophages (Ashcroft and Ashworth., 2003). However, estrogen also stimulates tissue regeneration and remodelling following the resolution of inflammation (Ashcroft *et al*., 1999b; Ashcroft and Ashworth, 2003; Ashcroft *et al*., 2003). Collectively, these reports suggest that estrogen modulates both pro-inflammatory (M1) and pro-regenerative

(M2) macrophages during wound repair. The effect of estrogen on the phagocytosis of MRSA and *P. aeruginosa* by U937-derived and HPBM-derived M1/M2 macrophages was investigated in Chapter 4. High expression of CD197 confirmed the polarisation of U937-derived and HPBM-derived M0 macrophages into M1-like macrophages by IFN-γ/LPS, whilst high CD206 expression confirmed polarisation into M2-like macrophages by IL-4/IL-13 (Stossi *et al*., 2012; Rios de la Rosa *et al*., 2017). Consistent with the data in Chapter 3, physiological (typical of youth) and supraphysiological levels of estrogen were found to promote the phagocytosis of MRSA and *P. aeruginosa* in a dose-dependent manner by both M1 and M2 macrophages compared to estrogen deprivation and the absolute absence of estrogen. However, the phagocytosis of bacteria by M1 macrophages was significantly ($P<0.05$) higher than that with M2 or M0 macrophages, regardless of whether the macrophages were derived from U937s or HPBMs. This data demonstrated the enhanced phagocytosis of bacteria by M1 macrophages which is in agreement with published findings (Mantovani *et al*., 2004; Benoit *et al*., 2008; Murray and Wynn, 2011; Krzyszczyk *et al*., 2018).

Further work assessing potential mechanisms of estrogen-enhanced phagocytosis was subsequently conducted using pro-inflammatory M1-like macrophages instead of M0-like or M2-like macrophages. In order to elucidate the importance of the ERs in mediating the estrogen-enhanced phagocytosis of bacteria by human M1 macrophages, host-pathogen interaction assays were conducted using the SERM tamoxifen and the SERD fulvestrant. Tamoxifen and fulvestrant are mainly used to treat breast cancer (Cole *et al*., 1971; Cummings *et al*., 1999) owing to their antiestrogenic effects in breast tissue (Love *et al*., 1992; Black *et al*., 1994). Tamoxifen acts as an ER antagonist in the breast, but is agonist in peripheral tissues such as the bone and the uterus (Love *et al*., 1992; Black *et al*., 1994). Interestingly, stimulation of macrophages with tamoxifen mirrored the effects of estrogen by significantly ($P<0.01$) increasing the phagocytosis of both MRSA and *P. aeruginosa* by U937-derived and HPBM-derived M1 macrophages. These results are supported by the findings of Corriden *et al*. (2015) which showed tamoxifen improves the phagocytic function of human neutrophils against a range of bacteria, including

MRSA. Hardman *et al*. (2007) also reported that tamoxifen accelerates wound healing in ovariectomised mice by exhibiting estrogenic effects, principally by reducing the inflammatory response. Collectively, these findings suggest that tamoxifen could potentially be a beneficial therapeutic strategy to treat infected wounds in the elderly.

Degradation of the ER by fulvestrant blocked the beneficial effects of estrogen on the phagocytosis of MRSA and *P. aeruginosa* by U937-derived and HPBM-derived M1 macrophages. Compared with estrogen, fulvestrant has a higher binding affinity to the ER (Morris and Wakeling, 2002), and when fulvestrant is coupled with the ER, the nuclear localisation of the ER is blocked leading to its degradation (Morris and Wakeling, 2002; Osborne *et al*., 2004).

Together, these data from the use of tamoxifen and fulvestrant provide evidence that estrogen is promoting phagocytosis via the binding and activation of the ER. Consequently, the role of the two estrogen receptor subtypes, ER-α and ER-β, in this process was investigated using ER isoform-specific agonists and antagonists. In a similar manner to estrogen and tamoxifen, selective activation of ER-α with PPT increased the phagocytosis of both MRSA and *P. aeruginosa* by M1 macrophages. However, treatment with the ER-β agonist DPN failed to promote the phagocytosis of either MRSA or *P. aeruginosa*. Intriguingly, blockade of ER-α with TPBM reversed the effect of PPT on the bacterial clearance. These novel findings collectively indicate that estrogen induces the phagocytosis of bacteria through activation of ER-α. This supports published findings showing that ER-α regulates inflammation, with ER-β modulating the later phases of wound healing (Campbell *et al.*, 2010). Toutain *et al*. (2009) identified ER-α as the main isoform involved in promoting the beneficial effects of 17β-estradiol in a model of skin necrosis. ER-α has also been shown to be the predominant ER involved in regulating the thickness of epidermis during wound healing (Stumpf *et al*., 1974; Bidmon *et al*., 1990). In summary, the findings described in Chapter 5 provide an indication that ER-α is the ER isoform through which estrogen promotes phagocytosis by macrophages. Selective targeting of ER-α with agents such as PPT may potentially lead to the development of therapeutic strategies to combat wound infections in the elderly.

Images from the SEM (Figure 3.16) illustrated major morphological changes in the membrane of phagocytes (macrophages) following estrogen treatment, with the establishment of multiple pseudopodial structures suggesting estrogen induces significant alterations in the actin cytoskeleton of the phagocytes. These findings suggested that the downstream genes mediated by estrogen may include mediators of the actin cytoskeleton.

In chapter 6, protein levels of four major Rho GTPase proteins (Cdc42, Rac1, RhoG and RhoA) and the focal adhesion kinase (FAK) were measured in M1 macrophages following treatment with/without estrogen.

FAK is a key molecule involved in regulating the actin cytoskeleton during phagocytosis (Cote-Vélez *et al*., 2001; Tzircotis *et al*., 2011). Immunoblotting analysis of FAK levels in this thesis indicated that estrogen supplementation significantly induced the levels of FAK in M1 macrophages. The importance of FAK in phagocytosis was highlighted by a study showing that *in vitro* transfection of macrophages with RNAi against FAK caused a 65% fall in the number of phagocytes during FcγR- and CR3-mediated phagocytosis (Tzircotis *et al*., 2011). The pathogen-killing activities of neutrophils and macrophages were mediated by FAK through the formation of lamellipodial structures (Kasorn *et al*., 2009). In contrast, FAK deficiency in macrophages is associated with a reduction in cell adhesion, and a failure in the formation of lamellipodia (Owen *et al*., 2007). These findings suggest that FAK is a potential therapeutic target that may (at least in part) mirror the beneficial effects of estrogen on bacterial clearance by macrophages.

Whilst estrogen increased the levels of Rac1 in M1 macrophages, an opposite effect on RhoA was observed. This was interesting in light of studies showing that Rac1 enhanced the phagocytosis of apoptotic cells (Akakura *et al*., 2004; Nakaya *et al*., 2008) whereas RhoA was shown to dampen the process (Kim *et al*., 2017). These reports also highlighted the need of a dynamic balance between the two proteins to regulate the apoptosis process, with concurrent upregulation of Rac1 and downregulation of RhoA required for engulfment of apoptotic cells. Similarly, other studies have also shown the opposing nature of Rac1 and RhoA during cytoskeleton reorganisation in *Candida elegans* (Ohta *et al*., 2006; Freeman and

Grinstein, 2014). The concomitant increase of Rac1 and FAK as inducers of phagocytosis is consistent with the fact that Rac1 is downstream from FAK in the regulation of actin dynamics (Kallergi *et al*., 2003; Papakonstanti *et al*., 2003; Siesser and Hanks, 2006; Kallergi *et al*., 2007). These findings suggest that manipulating the balance of Rac1 and RhoA in phagocytes of the elderly may be an additional therapeutic strategy to increase the appetite of macrophages to engulf bacteria, particularly in individuals where hormone replacement therapy (HRT) is contraindicated.

Similar to Rac1, RhoG is linked with the regulation of the actin cytoskeleton (Katoh *et al*., 2006), phagocytosis (Prieto-Sánchez *et al*., 2006), gene transcription (Vigorito *et al*., 2003) and the regulation of NADPH oxidase in neutrophils (Condliffe *et al*., 2006). Novel data in this study demonstrate that stimulation of macrophages with estrogen results in an increase in RhoG levels and enhanced phagocytosis. This result is in concordance with the findings of Tzircotis *et al*. (2011) that showed RhoG stimulates phagocytosis of IgG-opsonised (for FcγR) or C3bi-opsonised (for CR3) sheep red blood cells (RBCs) in mouse macrophages. However, the findings in this thesis are the first to show that estrogen stimulates RhoG in human M1 macrophages. Thus, promotion of RhoG may be an effective and extremely novel therapeutic strategy for infected wounds since it may recapitulate (at least in part) the beneficial effect of estrogen on bacterial clearance. However, further investigations are required to confirm this hypothesis.

Activation of Cdc42 is required for the regulation of the actin cytoskeleton dynamics, the formation of membrane ruffling and the formation of filopodia, a crucial requirement for effective phagocytosis in macrophages (Kozma *et al*., 1995; Cox *et al*., 1997). Immunoblotting analysis in this study showed an increase in the levels of Cdc42 in M1 macrophages following supplementation with estrogen. The concurrent increased levels of both Cdc42 and FAK in M1 macrophages are consistent with Cdc42 being downstream of FAK in the signalling cascade that promotes pseudopodia formation by phagocytes (Schaller, 2010). The increased levels of Cdc42 agrees with the findings of Allen *et al*. (1997) who showed that injection of macrophages with active Cdc42 resulted in the formation of long and

indistinct filopodia *in vitro*. Thus, further investigations are warranted to determine whether the longer pseudopodia produced by macrophages in this study, following interaction with *P. aeruginosa* compared with MRSA could be associated with higher levels of Cdc42. Such work may lead to novel therapeutics designed to be more effective against problematic Gram-negative/motile bacteria such as *P. aeruginosa.*

7.2 Future Work

- In order to reinforce the findings of this study, *in vitro* and *ex vivo* investigations using other phagocytes such as neutrophils (e.g. HL-60 cell line or *ex vivo* neutrophils) are warranted. One development of this would be to determine the effect of estrogen on the formation of neutrophil extracellular trap (NET) formation during host-pathogen interaction assays. This would help build a wider picture of the effects of estrogen on the clearance of wound pathogens.

- Bacterial growth in chronic wounds has been reported to take the form of irregularly shaped micro-colonies that can comprise hundreds of bacteria known as biofilms (Bjarnsholt *et al*., 2008; Rybtke *et al*., 2015). Host pathogen assays in this study investigated the effect of estrogen on bacterial clearance by macrophages. However, only single (planktonic) bacterial pathogens were utilised throughout the study. Replicating the *in vitro* and *ex vivo* experiments using biofilms of MRSA and *P. aeruginosa* would improve our *in vitro* model of chronic wound infections.

- Unpublished research in the Ashworth laboratory has shown that androgens, such as testosterone and dihydrotestosterone (DHT) inhibit the phagocytosis of MRSA in U937-derived macrophages. Indeed, the literature suggests that testosterone has opposing effects to estrogen (Gilliver *et al*., 2007). This creates many opportunities and directions for extending the

scope of this research, linking various sex steroid hormones with phagocytosis in wounds. Future work could investigate the effect of other steroid hormones and steroid hormone precursors such as dehydroepiandrosterone (DHEA) and dehydroepiandosterone-sulfate (DHEA-S) on the phagocytosis of bacteria by various human phagocytes. Moreover, local conversion of testosterone to estrogen could be investigated using aromatase inhibitors and conversion of testosterone to DHT could be investigated using 5α-reductase inhibitors.

- The findings of this study were obtained from host-pathogen assays conducted using *in vitro* and *ex vivo* macrophages. Repeating these experiments *in vivo* would provide robust evidence to confirm the beneficial effects of estrogen on promoting bacterial clearance during wound infections. One application would be to use mouse models that have been used previously to study age-related impaired healing (Ashcroft *et al*., 1997b; Hardman *et al*., 2007). *In vivo* investigations in animal models will be needed to develop potential therapeutic approaches for the treatment of wound infections before conducting human clinical trials involving chronic wounds in the elderly.

- The SEM data in Chapter 3 (Figure 3.16) illustrated distinct morphological changes occurring in macrophages when incubated with MRSA and *P. aeruginosa*. Future research is needed to determine the physical and biochemical effects induced by estrogen on pathogen-specific interactions with host cells. Fourier Transform Infra-Red (FTIR) Spectroscopy and Raman Spectroscopy could be used to determine changes in the functional groups on the membrane of host cells and cell wall of bacteria during host-pathogen interactions.

- Tamoxifen enhanced the phagocytic activity of macrophages against Gram-positive and Gram-negative bacteria (Chapter 5). Hardman and Ashcroft (2008) indicated that estrogen, tamoxifen and raloxifen all improve wound repair in ovariectomised mice. Future work could investigate a range of SERMs, including raloxifen, to determine the most effective mediator(s) of bacterial clearance. This could help identify the most promising topical SERM(s) to treat wound infections in the elderly.

- Several proteins involved in regulating the actin cytoskeleton were found to be mediated by estrogen (Chapter 6). Protein levels were measured by simple immunoblotting due to time restrictions, but these initial findings should now be confirmed by an additional technique, such as Western blotting. Moreover, Western blot analysis of phosphorylation (activation) patterns of these proteins at different time-points before and after stimulation with estrogen would add depth to current findings and potentially reveal further details on the mechanisms of estrogen-mediated phagocytosis. Intracellular flow cytometry could be used to explore these proteins in phagocyte populations, and kinase activity assays could be adopted to measure FAK activity. In addition, further investigations are required to determine whether the changes in protein levels are mirrored by concomitant alterations in gene expression. In order to determine the effect of ER-α activation on downstream gene expression in macrophages, RNAseq and qPCR analysis could be adopted. Future work would also be to replicate the findings using agonist/antagonist treatments to stimulate/block the activity of the proteins investigated in chapter 6. Inhibitors of phagocytosis could also be exploited to further understand the mechanisms by which estrogen induces phagocytosis.

7.3 Conclusion

In conclusion, this thesis delivers a body of experimental evidence demonstrating that estrogen directly enhances the phagocytosis of the two major wound

pathogens, MRSA and *P. aeruginosa*, by human macrophages via activation of ER-α and possible transcription of genes involved in regulating the actin cytoskeleton of phagocytes. When translating the findings to the context of wound infections, the data suggest that estrogen may promote the clearance of wound infections during youth but this protection is lost as estrogen levels decline with increasing age, resulting in increased propensity and progression of wound infections in the elderly. Understanding the precise mechanisms by which estrogen promotes phagocytosis would assist in identifying downstream targets to develop effective novel therapies for the treatment of wound infections with minimal side effects.

Based on the findings in this thesis, novel dressings that provide estrogen supplementation or selective activation of ER-α and/or specific targeting of proteins that regulate the actin cytoskeleton of phagocytes may provide effective therapeutic options for infected wounds in the elderly and warrant further investigation.

Chapter 8: References

Abramson, S. and Gallin, J. (1990) 'IL-4 inhibits superoxide production by human mononuclear phagocytes.' *Journal of Immunology*, 144(2) pp. 625-630.

Adams, R. H. and Alitalo, K. (2007) 'Molecular regulation of angiogenesis and lymphangiogenesis.' *Nature Reviews Molecular Cell Biology*, 8(6) p. 464-478.

Aderem, A. and Underhill, D. M. (1999) 'Mechanisms of phagocytosis in macrophages.' *Annual Review of Immunology*, 17(1) pp. 593-623.

Adeyi, A., Muzerengi, S., Adeyi, I. G. A. and Gupta, I. (2009) 'Leg ulcers in older people: a review of management.' *British Journal of Medical Practitioners*, 2(3) pp. 21-28

Affinito, P., Palomba, S., Sorrentino, C., Di Carlo, C., Bifulco, G., Arienzo, M. P. and Nappi, C. (1999) 'Effects of postmenopausal hypoestrogenism on skin collagen.' *Maturitas*, 33(3) pp. 239-247.

Ajami, B., Bennett, J. L., Krieger, C., Tetzlaff, W. and Rossi, F. M. V. (2007) 'Local self-renewal can sustain CNS microglia maintenance and function throughout adult life.' *Nature Neuroscience*, 10, pp. 1538-1549.

Akakura, S., Singh, S., Spataro, M., Akakura, R., Kim, J.-I., Albert, M. L. and Birge, R. B. (2004) 'The opsonin MFG-E8 is a ligand for the αvβ5 integrin and triggers DOCK180-dependent Rac1 activation for the phagocytosis of apoptotic cells.' *Experimental Cell Research*, 292(2) pp. 403-416.

Akashi, K., Traver, D., Miyamoto, T. and Weissman, I. L. (2000) 'A clonogenic common myeloid progenitor that gives rise to all myeloid lineages.' *Nature*, 404(6774) pp. 193-197.

Alberts, B., Johnson, A., Walter, P., Lewis, J., Raff, M. and Roberts, K. (2008) 'Molecular Cell Biology.' *New York: Garland Science*,

Albright, F., Smith, P. H. and Richardson, A. M. (1941) 'Postmenopausal osteoporosis: its clinical features.' *Journal of the American Medical Association*, 116(22) pp. 2465-2474.

Ali, S. and Coombes, R. C. (2000) 'Estrogen receptor alpha in human breast cancer: occurrence and significance.' *Journal of Mammary Gland Biology and Neoplasia*, 5(3) pp. 271-281.

Aliprantis, A. O., Yang, R.-B., Mark, M. R., Suggett, S., Devaux, B., Radolf, J. D., Klimpel, G. R., Godowski, P. and Zychlinsky, A. (1999) 'Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2.' *Science*, 285(5428) pp. 736-739.

Allen, W. E., Jones, G. E., Pollard, J. W. and Ridley, A. J. (1997) 'Rho, Rac and Cdc42 regulate actin organization and cell adhesion in macrophages.' *Journal of Cell Science*, 110(6) pp. 707-720.

Allman, R. M., Goode, P. S., Patrick, M. M., Burst, N. and Bartolucci, A. A. (1995) 'Pressure ulcer risk factors among hospitalized patients with activity limitation.' *Journal of the American Medical Association*, 273(11) pp. 865-870.

Anderson, G. L., Limacher, M. C., Assaf, A. R., Bassford, T., Beresford, S. A., Black, H. R., Bonds, D. E., Brunner, R. L., Brzyski, R. G. and Caan, B. (2004) 'Effects of conjugated equine estrogen in postmenopausal women with hysterectomy: the Women's Health Initiative randomized controlled trial.' *Journal of the American Medical Association*, 291, pp. 1701-1712.

André, E. and Beckerandre, M. (1993) 'Expression of an N-terminally truncated form of human focal adhesion kinase in brain.' *Biochemical and Biophysical Research Communications*, 190(1) pp. 140-147.

Apelqvist, J. and Larsson, J. (2000) 'What is the most effective way to reduce incidence of amputation in the diabetic foot?' *Diabetes/Metabolism Research and Reviews*, 16(S1) pp. S75-S83.

Arandjelovic, S. and Ravichandran, K. S. (2015) 'Phagocytosis of apoptotic cells in homeostasis.' *Nature Immunology*, 16(9) pp. 907-917.

Aron-Wisnewsky, J., Tordjman, J., Poitou, C., Darakhshan, F., Hugol, D., Basdevant, A., Aissat, A., Guerre-Millo, M. and Clement, K. (2009) 'Human adipose tissue macrophages: m1 and m2 cell surface markers in subcutaneous and omental depots and after weight loss.' *The Journal of Clinical Endocrinology & Metabolism*, 94(11) pp. 4619-4623.

Ascenzi, P., Bocedi, A. and Marino, M. (2006) 'Structure–function relationship of estrogen receptor α and β: impact on human health.' *Molecular Aspects of Medicine*, 27(4) pp. 299-402.

Ashcroft, G. S. and Ashworth, J. J. (2003) 'Potential role of estrogens in wound healing.' *American Journal of Clinical Dermatology*, 4(11) pp. 737-743.

Ashcroft, Mills, S. J. and Ashworth, J. J. (2002) 'Ageing and wound healing.' *Biogerontology*, 3(6) pp. 337-345.

Ashcroft, G. S., Greenwell-Wild, T., Horan, M. A., Wahl, S. M. and Ferguson, M. W. (1999) 'Topical estrogen accelerates cutaneous wound healing in aged humans associated with an altered inflammatory response.' *American Journal of Pathology*, 155(4) pp. 1137-1146.

Ashcroft, Greenwell-Wild, T., Horan, M. A., Wahl, S. M. and Ferguson, M. W. (1999b) 'Topical estrogen accelerates cutaneous wound healing in aged humans associated with an altered inflammatory response.' *The American Journal of Pathology*, 155(4) pp. 1137-1146.

Ashcroft, Dodsworth, J., Boxtel, E. V., Tarnuzzer, R. W., Horan, M. A., Schultz, G. S. and Ferguson, M. W. J. (1997a) 'Estrogen accelerates cutaneous wound healing associated with an increase in TGF-β1 levels.' *Nature Medicine*, 3(11), 1997/11/01, pp. 1209-1215.

Ashcroft, Dodsworth, J., Van Boxtel, E., Tarnuzzer, R., Horan, M. A., Schultz, G. S. and Ferguson, M. (1997b) 'Estrogen accelerates cutaneous wound healing associated with an increase in TGF-b1 levels.' *Nature Medicine*, 3(11) pp. 1209-1215.

Ashcroft, G. S., Mills, S. J., Lei, K., Gibbons, L., Jeong, M.-J., Taniguchi, M., Burow, M., Horan, M. A., Wahl, S. M. and Nakayama, T. (2003) 'Estrogen modulates cutaneous wound healing by downregulating macrophage migration inhibitory factor.' *Journal of Clinical Investigation*, 111(9) pp. 1309-1318.

Ashworth, J. J. (2005) *Estrogen receptor polymorphisms and wound healing.* The University of Manchester (United Kingdom).

Aspenström, P., Ruusala, A. and Pacholsky, D. (2007) 'Taking Rho GTPases to the next level: the cellular functions of atypical Rho GTPases.' *Experimental Cell Research*, 313(17) pp. 3673-3679.

Azcoitia, I., Sierra, A., Veiga, S., Honda, S. i., Harada, N. and Garcia-Segura, L. M. (2001) 'Brain aromatase is neuroprotective.' *Journal of Neurobiology*, 47(4) pp. 318-329.

Azios, N. G., Krishnamoorthy, L., Harris, M., Cubano, L. A., Cammer, M. and Dharmawardhane, S. F. (2007) 'Estrogen and resveratrol regulate Rac and Cdc42

signaling to the actin cytoskeleton of metastatic breast cancer cells.' *Neoplasia*, 9(2) pp. 147-158.

Bæk, K. T., Bowman, L., Millership, C., Søgaard, M. D., Kaever, V., Siljamäki, P., Savijoki, K., Varmanen, P., Nyman, T. A. and Gründling, A. (2016) 'The cell wall polymer lipoteichoic acid becomes nonessential in *Staphylococcus aureus* cells lacking the ClpX chaperone.' *mBio*, 7(4) pp. e01228-01216.

Baker, S., Stacey, M., Jopp-McKay, A., Hoskin, S. and Thompson, P. (1991) 'Epidemiology of chronic venous ulcers.' *British Journal of Surgery*, 78(7) pp. 864-867.

Balhara, J. and Gounni, A. (2012) 'The alveolar macrophages in asthma: a double-edged sword.' *Mucosal immunology*, 5(6) p. 605.

Barkhem, T., Carlsson, B., Nilsson, Y., Enmark, E., Gustafsson, J.-Å. and Nilsson, S. (1998) 'Differential response of estrogen receptor α and estrogen receptor β to partial estrogen agonists/antagonists.' *Molecular Pharmacology*, 54(1) pp. 105-112.

Basset, C., Holton, J., O'Mahony, R. and Roitt, I. (2003) 'Innate immunity and pathogen-host interaction.' *Vaccine*, 21 Suppl 2, Jun 1, 2003/05/24, pp. S12-23.

Bauer, E., Cooper, T., Huang, J., Altman, J. and Deuel, T. (1985) 'Stimulation of *in vitro* human skin collagenase expression by platelet-derived growth factor.' *Proceedings of the National Academy of Sciences*, 82(12) pp. 4132-4136.

Beanes, S. R., Dang, C., Soo, C. and Ting, K. (2003) 'Skin repair and scar formation: the central role of TGF-[beta].' *Expert Reviews in Molecular Medicine*, 5(08) pp. 1-22.

Beasley, W. and Hirst, G. (2004) 'Making a meal of MRSA—the role of biosurgery in hospital-acquired infection.' *Journal of Hospital Infection*, 56(1) pp. 6-9.

Begam, A. J., Jubie, S. and Nanjan, M. (2017) 'Estrogen receptor agonists/antagonists in breast cancer therapy: A critical review.' *Bioorganic Chemistry*, 71 pp. 257-274.

Benoit, M., Desnues, B. and Mege, J.-L. (2008) 'Macrophage polarization in bacterial infections.' *The Journal of Immunology*, 181(6) pp. 3733-3739.

Bergman, L., Beelen, M. L., Gallee, M. P., Hollema, H., Benraadt, J. and van Leeuwen, F. E. (2000) 'Risk and prognosis of endometrial cancer after tamoxifen for breast cancer.' *Lancet*, 356(9233) pp. 881-887.

Berthois, Y., Katzenellenbogen, J. A. and Katzenellenbogen, B. S. (1986) 'Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture.' *Proceedings of the National Academy of Sciences*, 83(8) pp. 2496-2500.

Bhat, K. P., Lantvit, D., Christov, K., Mehta, R. G., Moon, R. C. and Pezzuto, J. M. (2001) 'Estrogenic and antiestrogenic properties of resveratrol in mammary tumor models.' *Cancer Research*, 61(20) pp. 7456-7463.

Bid, H. K., Roberts, R. D., Manchanda, P. K. and Houghton, P. J. (2013) 'RAC1: An Emerging Therapeutic Option for Targeting Cancer Angiogenesis and Metastasis.' *Molecular Cancer Therapeutics*, 12(10) p. 1925.

Bidmon, H., Pitts, J., Solomon, H., Bondi, J. and Stumpf, W. (1990) 'Estradiol distribution and penetration in rat skin after topical application, studied by high resolution autoradiography.' *Histochemistry*, 95(1) pp. 43-54.

Billiau, A. and Vandekerckhove, F. (1991) 'Cytokines and their interactions with other inflammatory mediators in the pathogenesis of sepsis and septic shock.' *European Journal of Clinical Investigation*, 21(6) pp. 559-573.

Bishop, A. L. and HALL. (2000) 'Rho GTPases and their effector proteins.' *Biochemical Journal*, 348(2) pp. 241-255.

Biswas, S. K. and Mantovani, A. (2010) 'Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm.' *Nature Immunology*, 11(10) pp. 889-896.

Bjarnsholt, T., Kirketerp-Møller, K., Jensen, P. Ø., Madsen, K. G., Phipps, R., Krogfelt, K., Høiby, N. and Givskov, M. (2008) 'Why chronic wounds will not heal: a novel hypothesis.' *Wound Repair and Regeneration*, 16(1) pp. 2-10.

Bjarnsholt, T., Jensen, P. Ø., Burmølle, M., Hentzer, M., Haagensen, J. A., Hougen, H. P., Calum, H., Madsen, K. G., Moser, C. and Molin, S. (2005) 'Pseudomonas aeruginosa tolerance to tobramycin, hydrogen peroxide and polymorphonuclear leukocytes is quorum-sensing dependent.' *Microbiology*, 151(2) pp. 373-383.

Black, J., Baharestani, M. M., Cuddigan, J., Dorner, B., Edsberg, L., Langemo, D., Posthauer, M. E., Ratliff, C. and Taler, G. (2007) 'National Pressure Ulcer Advisory Panel's updated pressure ulcer staging system.' *Advances in Skin & Wound Care*, 20(5) pp. 269-274.

Black, Sato, M., Rowley, E. R., Magee, D. E., Bekele, A., Williams, D. C., Cullinan, G. J., Bendele, R., Kauffman, R. F. and Bensch, W. R. (1994) 'Raloxifene (LY139481 HCl) prevents bone loss and reduces serum cholesterol without causing uterine hypertrophy in ovariectomized rats.' *The Journal of Clinical Investigation*, 93(1) pp. 63-69.

Blair, I. A. (2010) 'Analysis of estrogens in serum and plasma from postmenopausal women: past present, and future.' *Steroids*, 75(4-5) pp. 297-306.

Blin-Wakkach, C., Rouleau, M. and Wakkach, A. (2014) 'Roles of osteoclasts in the control of medullary hematopoietic niches.' *Archives of Biochemistry and Biophysics*, 561 pp. 29-37.

Boltjes, A. and Van Wijk, F. (2014) 'Human dendritic cell functional specialization in steady-state and inflammation.' *Frontiers in Immunology*, 5 p. 131.

Bone, R. C. (1992) 'Toward an epidemiology and natural history of SIRS (systemic inflammatory response syndrome).' *Journal of the Amearican Medical Assocaition*, 268(24) pp. 3452-3455.

Boulton, A. (2004) 'The diabetic foot: from art to science. The 18th Camillo Golgi lecture.' *Diabetologia*, 47(8) pp. 1343-1353.

Boulton, A. J., Vileikyte, L., Ragnarson-Tennvall, G. and Apelqvist, J. (2005) 'The global burden of diabetic foot disease.' *Lancet*, 366(9498) pp. 1719-1724.

Boulton, A. J., Hardisty, C. A., Betts, R. P., Franks, C. I., Worth, R. C., Ward, J. D. and Duckworth, T. (1983) 'Dynamic foot pressure and other studies as diagnostic and management aids in diabetic neuropathy.' *Diabetes Care*, 6(1) pp. 26-33.

Bowler, P. G. (1999) 'The microbiology of acute and chronic wounds.' *Wounds*, 11 pp. 72-78.

Bowler, P. G. and Davies, B. J. (1999) 'The microbiology of infected and noninfected leg ulcers.' *International Journal of Dermatology*, 38(8) pp. 573-578.

Brincat, M. P. (2000) 'Hormone replacement therapy and the skin.' Maturitas, 35(2) pp. 107-117.

Brincat, M., Versi, E., Moniz, C., Magos, A. and Studd, J. (1987) 'Skin collagen changes in postmenopausal women receiving different regimens of estrogen therapy.' *Obstetrics and Gynecology*, 70(1) pp. 123-127.

Brown, J. (2016) *Investigating the actin cytoskeleton in cancer.* University of Glasgow.

Broz, P. and Monack, D. M. (2013) 'Newly described pattern recognition receptors team up against intracellular pathogens.' *Nature Reviews Immunology*, 13(8) pp. 551-565.

Brufani, M., Ceccacci, F., Filocamo, L., Garofalo, B., Joudioux, R., La Bella, A., Leonelli, F., Migneco, L. M., Marini Bettolo, R. and Farina, P. M. (2009) 'Novel locally active estrogens accelerate cutaneous wound healing. A preliminary study.' *Molecular Pharmaceutics*, 6(2) pp. 543-556.

Bryant, H., Wilson, P., Adrian, M., Cole, H., Phillips, D., Dodge, J., Grese, T., Sluka, J. and Glasebrook, A. (1996) 'Selective estrogen receptor modulators: pharmacological profile in the rat uterus.' *Journal of the Society for Gynecologic Investigation*, 2(3) p. 152A.

Brzozowski, A. M., Pike, A. C., Dauter, Z., Hubbard, R. E., Bonn, T., Engström, O., Öhman, L., Greene, G. L., Gustafsson, J.-Å. and Carlquist, M. (1997) 'Molecular basis of agonism and antagonism in the oestrogen receptor.' *Nature*, 389(6652) p. 753.

Burridge, K., Fath, K., Kelly, T., Nuckolls, G. and Turner, C. (1988) 'Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton.' *Annual Review of Cell Biology*, 4(1) pp. 487-525.

Cagnacci, A., Soldani, R., Carriero, P. L., Paoletti, A. M., Fioretti, P. and Melis, G. B. (1992) 'Effects of low doses of transdermal 17 beta-estradiol on carbohydrate metabolism in postmenopausal women.' *Journal of Clinical Endocrinology & Metabolism*, 74(6) pp. 1396-1400.

Calandra, T. and Roger, T. (2003) 'Macrophage migration inhibitory factor: a regulator of innate immunity.' *Nature Reviews Immunology*, 3(10) p. 791.

Campbell, L., Emmerson, E., Davies, F., Gilliver, S. C., Krust, A., Chambon, P., Ashcroft, G. S. and Hardman, M. J. (2010) 'Estrogen promotes cutaneous wound healing via estrogen receptor β independent of its antiinflammatory activities.' *Journal of Experimental Medicine*, 207(9) pp. 1825-1833.

Campbell, L., Emmerson, E., Williams, H., Saville, C. R., Krust, A., Chambon, P., Mace, K. A. and Hardman, M. J. (2014) 'Estrogen receptor-alpha promotes alternative macrophage activation during cutaneous repair.' *Journal of Investigative Dermatology*, 134(9) pp. 2447-2457.

Cardona, A. F. and Wilson, S. E. (2015) 'Skin and soft-tissue infections: a critical review and the role of telavancin in their treatment.' *Clinical Infectious Diseases*, 61(suppl 2) pp. S69-S78.

Carlier, M. F., Clainche, C. L., Wiesner, S. and Pantaloni, D. (2003) 'Actin-based motility: from molecules to movement.' *Bioessays*, 25(4) pp. 336-345.

Casas-Campillo, C., Balandrano, D. and Galarza, A. (1961) 'STEROIDS CLIX: Antimicrobial Properties of 21, 22-Dimethoxy Progesterone and Other Progesterone Analogues1.' *Journal of Bacteriology*, 81(3) p. 366.

Chambers, H. F. (2001) 'The changing epidemiology of *Staphylococcus aureus*?' *Emerging Infectious Diseases*, 7(2) p. 178-182.

Chan, K. T., Cortesio, C. L. and Huttenlocher, A. (2009) 'FAK alters invadopodia and focal adhesion composition and dynamics to regulate breast cancer invasion.' *The Journal of Cell Biology*, 185(2) pp. 357-370.

Chang, K. C., Wang, Y., Oh, I. G., Jenkins, S., Freedman, L. P., Thompson, C. C., Chung, J. H. and Nagpal, S. (2010) 'Estrogen receptor β is a novel therapeutic target for photoaging.' *Molecular Pharmacology*, 77(5) pp. 744-750.

Chen, G., Li, J., Ochani, M., Rendon-Mitchell, B., Qiang, X., Susarla, S., Ulloa, L., Yang, H., Fan, S. and Goyert, S. M. (2004) 'Bacterial endotoxin stimulates macrophages to release HMGB1 partly through CD14-and TNF-dependent mechanisms.' *Journal of Leukocyte Biology*, 76(5) pp. 994-1001.

Chen, L.-M., Hobbie, S. and Galan, J. E. (1996) 'Requirement of CDC42 for Salmonella-induced cytoskeletal and nuclear responses.' *Science*, 274(5295) pp. 2115-2118.

Chenu, C., Adlanmerini, M., Boudou, F., Chantalat, E., Guihot, A.-L., Toutain, C., Raymond-Letron, I., Vicendo, P., Gadeau, A.-P. and Henrion, D. (2017) 'Testosterone prevents cutaneous ischemia and necrosis in males through complementary estrogenic and androgenic actions.' *Arteriosclerosis, Thrombosis, and Vascular Biology*, 37, pp. 919-919.

Cho, C. H. and Nuttall, M. E. (2001) 'Therapeutic potential of oestrogen receptor ligands in development for osteoporosis.' *Emerging Drugs*, 6(1) pp. 137-154.

Chrzanowska-Wodnicka, M. and Burridge, K. (1996) 'Rho-stimulated contractility drives the formation of stress fibers and focal adhesions.' *The Journal of Cell Biology*, 133(6) pp. 1403-1415.

Clark, R. A., Lanigan, J. M., DellaPelle, P., Manseau, E., Dvorak, H. F. and Colvin, R. B. (1982) 'Fibronectin and fibrin provide a provisional matrix for epidermal cell migration during wound reepithelialization.' *Journal of Investigative Dermatology*, 79(5) pp. 264-269.

Clinton, A. and Carter, T. (2015) 'Chronic Wound Biofilms: Pathogenesis and Potential Therapies.' *Laboratory Medicine*, 46(4) pp. 277-284.

Cocchiara, R., Albeggiani, G., Di Trapani, G., Azzolina, A., Lampiasi, N., Rizzo, F. and Geraci, D. (1990) 'Modulation of rat peritoneal mast cell and human basophil histamine release by estrogens.' *International Archives of Allergy and Immunology*, 93(2-3) pp. 192-197.

Cockett, F. B. (1955) 'The pathology and treatment of venous ulcers of the leg.' *British Journal of Surgery*, 43(179) pp. 260-278.

Cole, M. P., Jones, C. and Todd, I. (1971) 'A new anti-oestrogenic agent in late breast cancer: an early clinical appraisal of ICI46474.' *British journal of cancer*, 25(2) p. 270.

Colucci-Guyon, E., Niedergang, F., Wallar, B. J., Peng, J., Alberts, A. S. and Chavrier, P. (2005) 'A role for mammalian diaphanous-related formins in complement receptor (CR3)-mediated phagocytosis in macrophages.' *Current Biology*, 15(22) pp. 2007-2012.

Condliffe, A. M., Webb, L. M., Ferguson, G. J., Davidson, K., Turner, M., Vigorito, E., Manifava, M., Chilvers, E. R., Stephens, L. R. and Hawkins, P. T. (2006) 'RhoG regulates the neutrophil NADPH oxidase.' *The Journal of Immunology*, 176(9) pp. 5314-5320.

Cooper, R. L., Segal, R. A., Diegelmann, R. F. and Reynolds, A. M. (2015) 'Modeling the effects of systemic mediators on the inflammatory phase of wound healing.' *Journal of Theoretical Biology*, 367 pp. 86-99.

Corriden, R., Hollands, A., Olson, J., Derieux, J., Lopez, J., Chang, J. T., Gonzalez, D. J. and Nizet, V. (2015) 'Tamoxifen augments the innate immune function of neutrophils through modulation of intracellular ceramide.' *Nature Communications*, 6 p. 8369.

Cossart, P. and Sansonetti, P. J. (2004) 'Bacterial Invasion: The Paradigms of Enteroinvasive Pathogens.' *Science*, 304(5668) pp. 242-248.

Costerton, J. W., Stewart, P. S. and Greenberg, E. P. (1999) 'Bacterial biofilms: a common cause of persistent infections.' *Science*, 284(5418) pp. 1318-1322.

Cote-Vélez, M. A., Ortega, E. and Ortega, A. (2001) 'Involvement of pp125FAK and p60SRC in the signaling through FcγRII–FcγRIII in murine macrophages.' *Immunology Letters*, 78(3) pp. 189-194.

Cox, D., Chang, P., Zhang, Q., Reddy, P. G., Bokoch, G. M. and Greenberg, S. (1997) 'Requirements for both Rac1 and Cdc42 in membrane ruffling and phagocytosis in leukocytes.' *Journal of Experimental Medicine*, 186(9) pp. 1487-1494.

Crompton, R., Williams, H., Ansell, D., Campbell, L., Holden, K., Cruickshank, S. and Hardman, M. J. (2016) 'Oestrogen promotes healing in a bacterial LPS model of delayed cutaneous wound repair.' *Laboratory Investigation*, 96(4) p. 439.

Cross, A., Allen, J. R., Burke, J., Ducel, G., Harris, A., John, J., Johnson, D., Lew, M., MacMillan, B. and Meers, P. (1983) 'Nosocomial infections due to Pseudomonas aeruginosa: review of recent trends.' *Review of Infectious Diseases*, 5(Supplement 5) pp. S837-S845.

Csaba, G. (2017) 'Is there a hormonal regulation of phagocytosis at unicellular and multicellular levels? A critical review.' *Acta Microbiologica et Immunologica Hungarica*, 64(4) pp. 357-372.

Cummings, S. R., Eckert, S., Krueger, K. A., Grady, D., Powles, T. J., Cauley, J. A., Norton, L., Nickelsen, T., Bjarnason, N. H. and Morrow, M. (1999) 'The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial.' *Jama*, 281(23) pp. 2189-2197.

Cutolo, M., Sulli, A., Capellino, S., Villaggio, B., Montagna, P., Seriolo, B. and Straub, R. (2004) 'Sex hormones influence on the immune system: basic and clinical aspects in autoimmunity.' *Lupus*, 13(9) pp. 635-638.

Daigneault, M., Preston, J. A., Marriott, H. M., Whyte, M. K. and Dockrell, D. H. (2010) 'The identification of markers of macrophage differentiation in PMA-stimulated THP-1 cells and monocyte-derived macrophages.' *PloS one*, 5(1) p. e8668.

Daniell, H. W. (1997) 'Osteoporosis after orchiectomy for prostate cancer.' *Journal of Urology*, 157(2) pp. 439-444.

Davies, L. C., Jenkins, S. J., Allen, J. E. and Taylor, P. R. (2013) 'Tissue-resident macrophages.' *Nature Immunology*, 14(10) pp. 986-995.

Davies, C. E., Hill, K. E., Wilson, M. J., Stephens, P., Hill, C. M., Harding, K. G. and Thomas, D. W. (2004) 'Use of 16S ribosomal DNA PCR and denaturing gradient gel electrophoresis for analysis of the microfloras of healing and nonhealing chronic venous leg ulcers.' *Journal of Clinical Microbiology*, 42(8) pp. 3549-3557.

Davis, S. C., Ricotti, C., Cazzaniga, A., Welsh, E., Eaglstein, W. H. and Mertz, P. M. (2008) 'Microscopic and physiologic evidence for biofilm-associated wound colonization in vivo.' *Wound Repair and Regeneration*, 16(1) pp. 23-29.

DeMichele, A., Troxel, A. B., Berlin, J. A., Weber, A. L., Bunin, G. R., Turzo, E., Schinnar, R., Burgh, D., Berlin, M. and Rubin, S. C. (2008) 'Impact of raloxifene or tamoxifen use on endometrial cancer risk: a population-based case-control study.' *Journal of Clinical Oncology*, 26(25) p. 4151.

den Haan, J. M. and Kraal, G. (2012) 'Innate immune functions of macrophage subpopulations in the spleen.' *Journal of Innate Immunity*, 4(5-6) pp. 437-445.

Deshpande, R., Khalili, H., Pergolizzi, R. G., Michael, S. D. and Chang, M. D. Y. (1997) 'Estradiol down-regulates LPS-induced cytokine production and NFkB activation in murine macrophages.' *American Journal of Reproductive Immunology*, 38(1) pp. 46-54.

Desjardins, M., Houde, M. and Gagnon, E. (2005) 'Phagocytosis: the convoluted way from nutrition to adaptive immunity.' *Immunological Reviews*, 207(1) pp. 158-165.

Devreotes, P. and Horwitz, A. R. (2015) 'Signaling networks that regulate cell migration.' *Cold Spring Harbor Perspectives in Biology*, 7(8) p. a005959.

Diekema, D., Pfaller, M., Schmitz, F., Smayevsky, J., Bell, J., Jones, R. and Beach, M. (2001) 'Survey of infections due to Staphylococcus species: frequency of occurrence and antimicrobial susceptibility of isolates collected in the United States, Canada, Latin America, Europe, and the Western Pacific region for the SENTRY Antimicrobial Surveillance Program, 1997–1999.' *Clinical Infectious Diseases*, 32(Supplement 2) pp. S114-S132.

Doebel, T., Voisin, B. and Nagao, K. (2017) 'Langerhans cells–the macrophage in dendritic cell clothing.' *Trends in Immunology*, 38(11) pp. 817-828.

Dowd, S. E., Sun, Y., Secor, P. R., Rhoads, D. D., Wolcott, B. M., James, G. A. and Wolcott, R. D. (2008) 'Survey of bacterial diversity in chronic wounds using pyrosequencing, DGGE, and full ribosome shotgun sequencing.' *BMC Microbiology*, 8(1) p. 43.

Dumitru, C. D., Ceci, J. D., Tsatsanis, C., Kontoyiannis, D., Stamatakis, K., Lin, J. H., Patriotis, C., Jenkins, N. A., Copeland, N. G., Kollias, G. and Tsichlis, P. N. (2000) 'TNF-alpha induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway.' *Cell*, 103(7), Dec 22, 2001/02/13, pp. 1071-1083.

Dunkelberger, J. R. and Song, W.-C. (2010) 'Complement and its role in innate and adaptive immune responses.' *Cell Research*, 20(1) p. 34-50.

Duque, G. A. and Descoteaux, A. (2015) 'Macrophage cytokines: involvement in immunity and infectious diseases.' *Secretion of Cytokines and Chemokines by Innate Immune Cells*, p. 6.

Edwards, J. V., Howley, P. and Cohen, I. K. (2004) '*In vitro* inhibition of human neutrophil elastase by oleic acid albumin formulations from derivatized cotton wound dressings.' *International Journal of Pharmaceutics*, 284(1) pp. 1-12.

El Ghalbzouri, A. and Ponec, M. (2004) 'Diffusible factors released by fibroblasts support epidermal morphogenesis and deposition of basement membrane components.' *Wound Repair and Regeneration*, 12(3) pp. 359-367.

El Ghalbzouri, A., Hensbergen, P., Gibbs, S., Kempenaar, J., van der Schors, R. and Ponec, M. (2004) 'Fibroblasts facilitate re-epithelialization in wounded human skin equivalents.' *Laboratory Investigation*, 84(1) p. 102.

Elsinghorst, E. A. (1994) 'Measurement of invasion by gentamicin resistance.' *In Methods in Enzymology*. Vol. 236. Academic Press, pp. 405-420.

Emmerson, E., Campbell, L., Ashcroft, G. S. and Hardman, M. J. (2009) 'Unique and synergistic roles for 17β-estradiol and macrophage migration inhibitory factor during cutaneous wound closure are cell type specific.' *Endocrinology*, 150(6) pp. 2749-2757.

Emori, T. G., Banerjee, S. N., Culver, D. H., Gaynes, R. P., Horan, T. C., Edwards, J. R., Jarvis, W. R., Tolson, J. S., Henderson, T. S. and Martone, W. J. (1991) 'Nosocomial infections in elderly patients in the United States, 1986–1990.' *American Journal of Medicine*, 91(3) pp. S289-S293.

Engelich, G., Wright, D. G. and Hartshorn, K. L. (2001) 'Acquired Disorders of Phagocyte function Complicating Medical and Surgical Illnesses.' *Clinical Infectious Diseases*, 33(12) pp. 2040-2048.

Enright, M. C., Robinson, D. A., Randle, G., Feil, E. J., Grundmann, H. and Spratt, B. G. (2002) 'The evolutionary history of methicillin-resistant Staphylococcus aureus (MRSA).' *Proceedings of the National Academy of Sciences*, 99(11) pp. 7687-7692.

Eriksson, M., Berglund, L., Rudling, M., Henriksson, P. and Angelin, B. (1989) 'Effects of estrogen on low density lipoprotein metabolism in males. Short-term and long-term studies during hormonal treatment of prostatic carcinoma.' *Journal of Clinical Investigation*, 84(3) pp. 802-810.

Erwig, L.-P., Kluth, D. C., Walsh, G. M. and Rees, A. J. (1998) 'Initial cytokine exposure determines function of macrophages and renders them unresponsive to other cytokines.' *The Journal of Immunology*, 161(4) pp. 1983-1988.

Etienne-Manneville, S. and Hall, A. (2002) 'Rho GTPases in cell biology.' *Nature*, 420(6916), 2002/12/01, pp. 629-635.

Falanga, V. (2005) 'Wound healing and its impairment in the diabetic foot.' *Lancet*, 366(9498) pp. 1736-1743.

Fang, J., Hodivala-Dilke, K., Johnson, B. D., Du, L. M., Hynes, R. O., White, G. C. and Wilcox, D. A. (2005) 'Therapeutic expression of the platelet-specific integrin, αIIbβ3, in a murine model for Glanzmann thrombasthenia.' *Blood*, 106(8) pp. 2671-2679.

Fauvart, M., De Groote, V. N. and Michiels, J. (2011) 'Role of persister cells in chronic infections: clinical relevance and perspectives on anti-persister therapies.' *Journal of Medical Microbiology*, 60(6) pp. 699-709.

Fazli, M., Bjarnsholt, T., Kirketerp-Møller, K., Jørgensen, A., Andersen, C. B., Givskov, M. and Tolker-Nielsen, T. (2011) 'Quantitative analysis of the cellular inflammatory response against biofilm bacteria in chronic wounds.' *Wound Repair and Regeneration*, 19(3) pp. 387-391.

Fazli, M., Bjarnsholt, T., Kirketerp-Møller, K., Jørgensen, B., Andersen, A. S., Krogfelt, K. A., Givskov, M. and Tolker-Nielsen, T. (2009) 'Nonrandom distribution of Pseudomonas aeruginosa and Staphylococcus aureus in chronic wounds.' *Journal of Clinical Microbiology*, 47(12) pp. 4084-4089.

Finlay, B. B. and Falkow, S. (1989) 'Common themes in microbial pathogenicity.' *Microbiological Reviews*, 53(2) pp. 210-230.

Fisher, B., Costantino, J. P., Wickerham, D. L., Cecchini, R. S., Cronin, W. M., Robidoux, A., Bevers, T. B., Kavanah, M. T., Atkins, J. N. and Margolese, R. G. (2005) 'Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study.' *Journal of the National Cancer Institute*, 97(22) pp. 1652-1662.

Flamini, Sanchez, A. M., Genazzani, A. R. and Simoncini, T. (2011) 'Estrogen regulates endometrial cell cytoskeletal remodeling and motility via focal adhesion kinase.' *Fertility and Sterility*, 95(2) pp. 722-726.

Flamini, Sanchez, A., Goglia, L., Tosi, V., Genazzani, A. and Simoncini, T. (2009) 'Differential actions of estrogen and SERMs in regulation of the actin cytoskeleton of endometrial cells.' *Molecular Human Reproduction*, 15(10) pp. 675-685.

Fogg, D. K., Sibon, C., Miled, C., Jung, S., Aucouturier, P., Littman, D. R., Cumano, A. and Geissmann, F. (2006) 'A clonogenic bone marrow progenitor specific for macrophages and dendritic cells.' *Science*, 311(5757) pp. 83-87.

Freeman, S. A. and Grinstein, S. (2014) 'Phagocytosis: receptors, signal integration, and the cytoskeleton.' *Immunological Reviews*, 262(1) pp. 193-215.

Freeman, S. A. and Grinstein, S. (2016) 'Phagocytosis: How Macrophages Tune Their Non-professional Counterparts.' *Current Biology*, 26(24) pp. R1279-R1282.

Friedman, S. J. and Su, W. D. (1984) 'Management of leg ulcers with hydrocolloid occlusive dressing.' *Archives of Dermatology*, 120(10) pp. 1329-1336.

Friedrich, E. B., Clever, Y. P., Wassmann, S., Hess, C. and Nickenig, G. (2006) '17β-Estradiol inhibits monocyte adhesion via down-regulation of Rac1 GTPase.' *Journal of Molecular and Cellular Cardiology*, 40(1), 2006/01/01/, pp. 87-95.

Fukata, M. and Arditi, M. (2013) 'The role of pattern recognition receptors in intestinal inflammation.' *Mucosal Immunology*, 6(3) pp. 451-463.

Furr, B. and Jordan, V. (1984) 'The pharmacology and clinical uses of tamoxifen.' *Pharmacology & Therapeutics*, 25(2) pp. 127-205.

Gabbiani, G. (1999) 'Some historical and philosophical reflections on the myofibroblast concept.' *In Tissue Repair and Fibrosis*. Springer, pp. 1-5.

Gál, P., Novotný, M., Vasilenko, T., Depta, F., Šulla, I. and Tomori, Z. (2010) 'Decrease in wound tensile strength following post-surgical estrogen replacement therapy in ovariectomized rats during the early phase of healing is mediated via ER-α rather than ER-β: a preliminary report.' *Journal of Surgical Research*, 159(1) pp. e25-e28.

Galan, J. E. (1994) 'Interactions of bacteria with non-phagocytic cells.' *Current Opinion in Immunology*, 6(4) pp. 590-595.

Galinari, É., Sabry, D. A., Sassaki, G. L., Macedo, G. R., Passos, F. M. L., Mantovani, H. C. and Rocha, H. A. O. (2017) 'Chemical structure, antiproliferative and antioxidant activities of a cell wall α-d-mannan from yeast Kluyveromyces marxianus.' *Carbohydrate Polymers*, 157 pp. 1298-1305.

Ganz, T. (2012) 'Macrophages and systemic iron homeostasis.' *Journal of Innate Immunity*, 4(5-6) pp. 446-453.

García-Gómez, E., González-Pedrajo, B. and Camacho-Arroyo, I. (2012) 'Role of sex steroid hormones in bacterial-host interactions.' *BioMed Research International*, 2013

Garcia-Gomez, S., Doforno, R. A., Martinez-Barricarte, R., Torres, J. M., Cerdan, A. F., Davila, M., Hernández-Jiménez, E., Toledano, V., Cubillos-Zapata, C. and Vallejo-Cremades, M. T. (2016) 'Actin polymerisation after FCγR stimulation of human fibroblasts is BCL10 independent.' *Clinical Immunology*, 163 pp. 120-122.

Gauthier-Rouviere, C., Vignal, E., Mériane, M., Roux, P., Montcourier, P. and Fort, P. (1998) 'RhoG GTPase controls a pathway that independently activates Rac1 and Cdc42Hs.' *Molecular Biology of The Cell*, 9(6) pp. 1379-1394.

Gautier, E. L., Shay, T., Miller, J., Greter, M., Jakubzick, C., Ivanov, S., Helft, J., Chow, A., Elpek, K. G. and Gordonov, S. (2012) 'Gene-expression profiles and transcriptional regulatory pathways that underlie the identity and diversity of mouse tissue macrophages.' *Nature Immunology*, 13(11) p. 1118.

Geissmann, F., Manz, M. G., Jung, S., Sieweke, M. H., Merad, M. and Ley, K. (2010) 'Development of Monocytes, Macrophages, and Dendritic Cells.' *Science*, 327(5966) pp. 656-661.

Ghisletti, S., Meda, C., Maggi, A. and Vegeto, E. (2005) '17β-estradiol inhibits inflammatory gene expression by controlling NF-κB intracellular localization.' *Molecular and Cellular Biology*, 25(8) pp. 2957-2968.

Giacometti, A., Cirioni, O., Schimizzi, A., Del Prete, M., Barchiesi, F., D'errico, M., Petrelli, E. and Scalise, G. (2000) 'Epidemiology and microbiology of surgical wound infections.' *Journal of Clinical Microbiology*, 38(2) pp. 918-922.

Gidlund, M., Örn, A., Pattengale, P. K., Jansson, M., Wigzell, H. and Nilsson, K. (1981) 'Natural killer cells kill tumour cells at a given stage of differentiation.' *Nature*, 292(5826) p. 848.

Gilliver, S. C., Ashworth, J. J. and Ashcroft, G. S. (2007) 'The hormonal regulation of cutaneous wound healing.' *Clinics in Dermatology*, 25(1) pp. 56-62.

Gilliver, S. C., Emmerson, E., Campbell, L., Chambon, P., Hardman, M. J. and Ashcroft, G. S. (2010) '17β-Estradiol inhibits wound healing in male mice via estrogen receptor-α.' *The American Journal of Pathology*, 176(6) pp. 2707-2721.

Ginhoux, F. and Guilliams, M. (2016) 'Tissue-resident macrophage ontogeny and homeostasis.' *Immunity*, 44(3) pp. 439-449.

Giretti, M. S., Fu, X.-D., De Rosa, G., Sarotto, I., Baldacci, C., Garibaldi, S., Mannella, P., Biglia, N., Sismondi, P. and Genazzani, A. R. (2008) 'Extra-nuclear signalling of estrogen receptor to breast cancer cytoskeletal remodelling, migration and invasion.' *PloS one*, 3(5) p. e2238.

Gjødsbøl, K., Christensen, J. J., Karlsmark, T., Jørgensen, B., Klein, B. M. and Krogfelt, K. A. (2006) 'Multiple bacterial species reside in chronic wounds: a longitudinal study.' *International Wound Journal*, 3(3) pp. 225-231.

Gladen, B. C., Lubahn, D. B., Bunch, D. O., Goulding, E. H., Eddy, E. M., Korach, K. S. and Washburn, T. F. (1996) 'Targeted disruption of the estrogen receptor gene in male mice causes alteration of spermatogenesis and infertility.' *Endocrinology*, 137(11) pp. 4796-4805.

Gordon, S. and Martinez, F. O. (2010) 'Alternative activation of macrophages: mechanism and functions.' *Immunity*, 32(5) pp. 593-604.

Gosain, A. and DiPietro, L. A. (2004) 'Aging and Wound Healing.' *World Journal of Surgery*, 28(3), March 01, pp. 321-326.

Gottenbos, B., Grijpma, D. W., van der Mei, H. C., Feijen, J. and Busscher, H. J. (2001) 'Antimicrobial effects of positively charged surfaces on adhering Gram-positive and Gram-negative bacteria.' *Journal of Antimicrobial Chemotherapy*, 48(1) pp. 7-13.

Greenhalgh, D. G. (1998) 'The role of apoptosis in wound healing.' *The international Journal of Biochemistry & Cell Biology*, 30(9) pp. 1019-1030.

Grey, J. E., Harding, K. G. and Enoch, S. (2006) 'Venous and arterial leg ulcers.' *British Medicla Journal*, 332(7537) pp. 347-350.

Grinnell, F., Ho, C.-H. and Wysocki, A. (1992) 'Degradation of fibronectin and vitronectin and vitronectin in chronic wound fluid: analysis by cell blotting, immunoblotting, and cell adhesion assays.' *Journal of Investigative Dermatology*, 98(4) pp. 410-416.

Group, C. D. P. R. (1973) 'The Coronary Drug Project: findings leading to discontinuation of the 2.5 mg/day estrogen group.' *Journal of the American Medical Assiciation*, 226 pp. 652-657.

Groves, E., Dart, A., Covarelli, V. and Caron, E. (2008) 'Molecular mechanisms of phagocytic uptake in mammalian cells.' *Cellular and Molecular Life Sciences*, 65(13) pp. 1957-1976.

Gruber, C. J., Tschugguel, W., Schneeberger, C. and Huber, J. C. (2002) 'Production and actions of estrogens.' *New England Journal of Medicine*, 346(5) pp. 340-352.

Guest, J. F., Ayoub, N., McIlwraith, T., Uchegbu, I., Gerrish, A., Weidlich, D., Vowden, K. and Vowden, P. (2015) 'Health economic burden that wounds impose on the National Health Service in the UK.' *British Medical Journal Open*, 5(12) p. e009283.

Gulshan, S., McCruden, A. and Stimson, W. (1990) 'Oestrogen receptors in macrophages.' *Scandinavian Journal of Immunology*, 31(6) pp. 691-697.

Guo, S. a. and DiPietro, L. A. (2010) 'Factors affecting wound healing.' *Journal of Dental Research*, 89(3) pp. 219-229.

Hachem, R. Y., Chemaly, R. F., Ahmar, C. A., Jiang, Y., Boktour, M. R., Rjaili, G. A., Bodey, G. P. and Raad, I. I. (2007) 'Colistin is effective in treatment of infections caused by multidrug-resistant *Pseudomonas aeruginosa* in cancer patients.' *Antimicrobial Agents and Chemotherapy*, 51(6) pp. 1905-1911.

Hall. (1998) 'Rho GTPases and the Actin Cytoskeleton.' *Science*, 279(5350) p. 509.

Hall, A. (1998) 'Rho GTPases and the actin cytoskeleton.' *Science*, 279(5350) pp. 509-514.

Hall, N. (2017) *The effects of c-reactive protein (CRP) isoforms on inflammation and wound healing processes.* Manchester Metropolitan University.

Hamad, M. A., Skeldon, A. M. and Valvano, M. A. (2010) 'Construction of aminoglycoside-sensitive Burkholderia cenocepacia strains for use in studies of intracellular bacteria with the gentamicin protection assay.' *Applied and Environmental Microbiology*, 76(10) pp. 3170-3176.

Hanks, S. K., Calalb, M. B., Harper, M. C. and Patel, S. K. (1992) 'Focal adhesion protein-tyrosine kinase phosphorylated in response to cell attachment to fibronectin.' *Proceedings of the National Academy of Sciences*, 89(18) pp. 8487-8491.

Hansson, C., Hoborn, J., Möller, A. and Swanbeck, G. (1995) 'The microbial flora in venous leg ulcers without clinical signs of infection. Repeated culture using a validated standardised microbiological technique.' *Acta Dermato-Venereologica*, 75(1) pp. 24-30.

Harding, K., Morris, H. and Patel, G. (2002) 'Healing chronic wounds.' *British Medical Journal*, 324(7330) p. 160.

Hardman, M. and Ashcroft, G. (2005) 'Hormonal influences on wound healing: a review of current experimental data.' *Wounds* , 17(11) pp. 313-320.

Hardman, M. J. and Ashcroft, G. S. (2008) 'Estrogen, not intrinsic aging, is the major regulator of delayed human wound healing in the elderly.' *Genome Biology*, 9(5) p. R80.

Hardman, M. J., Emmerson, E., Campbell, L. and Ashcroft, G. S. (2007) 'Selective estrogen receptor modulators accelerate cutaneous wound healing in ovariectomized female mice.' *Endocrinology*, 149(2) pp. 551-557.

Hardman, M.J., Waite, A., Zeef, L., Burow, M., Nakayama, T. and Ashcroft, G. S. (2005) 'Macrophage migration inhibitory factor: a central regulator of wound healing.' *American Journal of Pathology*, 167(6), Dec, 2005/11/30, pp. 1561-1574.

Hardman, M. J. and Ashcroft, G. S. (2008) 'Estrogen, not intrinsic aging, is the major regulator of delayed human wound healing in the elderly.' *Genome Biology*, 9(5) p. R80.

Hardman, M. J., Waite, A., Zeef, L., Burow, M., Nakayama, T. and Ashcroft, G. S. (2005) 'Macrophage migration inhibitory factor: a central regulator of wound healing.' *American Journal of Pathology*, 167(6) pp. 1561-1574.

Heaney, R. P. and Draper, M. W. (1997) 'Raloxifene and estrogen: comparative bone-remodeling kinetics.' *The Journal of Clinical Endocrinology & Metabolism*, 82(10) pp. 3425-3429.

Heasman, S. J. and Ridley, A. J. (2008) 'Mammalian Rho GTPases: new insights into their functions from *in vivo* studies.' *Nature reviews Molecular Cell Biology*, 9(9) p. 690.

Hesketh, M., Sahin, K. B., West, Z. E. and Murray, R. Z. (2017) 'Macrophage phenotypes regulate scar formation and chronic wound healing.' *International journal of Molecular Sciences*, 18(7) p. 1545.

Hess, P., Altenhöfer, A., Khan, A. S., Daryab, N., Kim, K. S., Hacker, J. and Oelschlaeger, T. A. (2004) 'A Salmonella fim homologue in Citrobacter freundii mediates invasion *in vitro* and crossing of the blood-brain barrier in the rat pup model.' *Infection and Immunity*, 72(9) pp. 5298-5307.

Hesse, M., Modolell, M., La Flamme, A. C., Schito, M., Fuentes, J. M., Cheever, A. W., Pearce, E. J. and Wynn, T. A. (2001) 'Differential regulation of nitric oxide synthase-2 and arginase-1 by type 1/type 2 cytokines in vivo: granulomatous pathology is shaped by the pattern of L-arginine metabolism.' *The Journal of Immunology*, 167(11) pp. 6533-6544.

Hettinger, J., Richards, D. M., Hansson, J., Barra, M. M., Joschko, A.-C., Krijgsveld, J. and Feuerer, M. (2013) 'Origin of monocytes and macrophages in a committed progenitor.' *Nature Immunology*, 14, 06/30/online, p. 821-830.

Hinz, B. (2007) 'Formation and function of the myofibroblast during tissue repair.' *Journal of Investigative Dermatology*, 127(3) pp. 526-537.

Hiramatsu, K., Aritaka, N., Hanaki, H., Kawasaki, S., Hosoda, Y., Hori, S., Fukuchi, Y. and Kobayashi, I. (1997) 'Dissemination in Japanese hospitals of strains of Staphylococcus aureus heterogeneously resistant to vancomycin.' *Lancet*, 350(9092) pp. 1670-1673.

Hockenberry, A. M., Hutchens, D. M., Agellon, A. and So, M. (2016) 'Attenuation of the Type IV Pilus Retraction Motor Influences Neisseria gonorrhoeae Social and Infection Behavior.' *mBio*, 7(6) pp. e01994-01916.

Hoffmann, J. A., Kafatos, F. C., Janeway, C. A. and Ezekowitz, R. (1999) 'Phylogenetic perspectives in innate immunity.' *Science*, 284(5418) pp. 1313-1318.

Hofmann, C., Shepelev, M. and Chernoff, J. (2004) 'The genetics of Pak.' *Journal of Cell Science*, 117(19) pp. 4343-4354.

Hohn, D., MacKay, R., Halliday, B. and Hunt, T. (1976) 'Effect of O2 tension on microbicidal function of leukocytes in wounds and *in vitro*.' *Surgical Forum*, Vol. 27(62),pp. 18-20.

Holmes, K. C., Popp, D., Gebhard, W. and Kabsch, W. (1990) 'Atomic model of the actin filament.' *Nature*, 347(6288) p. 44.

Hoppe, A. D. and Swanson, J. A. (2004) 'Cdc42, Rac1, and Rac2 display distinct patterns of activation during phagocytosis.' *Molecular Biology of the Cell*, 15(8) pp. 3509-3519.

Horng, H. C., Chang, W. H., Yeh, C. C., Huang, B. S., Chang, C. P., Chen, Y. J., Tsui, K. H. and Wang, P. H. (2017) 'Estrogen Effects on Wound Healing.' International Journal of Molecular Sciences, 18(11), p. 2325.

Insall, R. H. and Machesky, L. M. (2009) 'Actin dynamics at the leading edge: from simple machinery to complex networks.' *Developmental Cell*, 17(3) pp. 310-322.

Isenberg, G., Aebi, U. and Pollard, T. D. (1980) 'An actin-binding protein from Acanthamoeba regulates actin filament polymerization and interactions.' *Nature*, 288(5790) p. 455.

Italiani, P. and Boraschi, D. (2014) 'From Monocytes to M1/M2 Macrophages: Phenotypical vs. Functional Differentiation.' *Frontiers in Immunology*, 5, p.514.

Iyer, V., Klebba, I., McCready, J., Arendt, L. M., Betancur-Boissel, M., Wu, M.-F., Zhang, X., Lewis, M. T. and Kuperwasser, C. (2012) 'Estrogen promotes ER-Negative tumor growth and angiogenesis through mobilization of bone marrow–derived monocytes.' *Cancer Research*, 72(11) pp. 2705-2713.

Jacinto, A., Martinez-Arias, A. and Martin, P. (2001) 'Mechanisms of epithelial fusion and repair.' *Nature Cell Biology*, 3(5) p. E117.

Jaffe, A. B. and Hall, A. (2005) 'Rho GTPases: biochemistry and biology.' *Annu. Rev. Cell Dev. Biol.*, 21 pp. 247-269.

James, G. A., Swogger, E., Wolcott, R., Secor, P., Sestrich, J., Costerton, J. W. and Stewart, P. S. (2008) 'Biofilms in chronic wounds.' *Wound Repair and Rregeneration*, 16(1) pp. 37-44.

Jameson, J., Ugarte, K., Chen, N., Yachi, P., Fuchs, E., Boismenu, R. and Havran, W. L. (2002) 'A Role for Skin γδ T Cells in Wound Repair.' *Science*, 296(5568) pp. 747-749.

Janeway Jr, C. A. and Medzhitov, R. (2002) 'Innate immune recognition.' *Annual Review of Immunology*, 20(1) pp. 197-216.

Jeffcoate, W. J. and Harding, K. G. (2003) 'Diabetic foot ulcers.' *The lancet*, 361(9368) pp. 1545-1551.

Jensen, E. V. (1962) 'On the mechanism of estrogen action.' *Perspectives in Biology and Medicine*, 6(1) pp. 47-60.

Jensen, E. V. and Jacobson, H. I. (1960) 'Fate of steroid estrogens in target tissues.' In: *Biological activities of steroids in relation to cancer*. Elsevier, pp. 161-178.

Jensen, P. Ø., Bjarnsholt, T., Phipps, R., Rasmussen, T. B., Calum, H., Christoffersen, L., Moser, C., Williams, P., Pressler, T. and Givskov, M. (2007) 'Rapid necrotic killing of polymorphonuclear leukocytes is caused by quorum-sensing-controlled

production of rhamnolipid by *Pseudomonas aeruginosa*.' *Microbiology*, 153(5) pp. 1329-1338.

Jessen, O., Rosendal, K., Bülow, P., Faber, V. and Eriksen, K. R. (1969) 'Changing staphylococci and staphylococcal infections: a ten-year study of bacteria and cases of bacteremia.' *New England Journal of Medicine*, 281(12) pp. 627-635.

Jevons. (1961) 'To-day's Drugs.' British Medical Journal, 1(5219) pp. 124-125.

Jilkine, A. (2009) *A wave-pinning mechanism for eukaryotic cell polarization based on Rho GTPase dynamics.* University of British Columbia.

Johnson, D. I. (1999) 'Cdc42: an essential Rho-type GTPase controlling eukaryotic cell polarity.' *Microbiol. Mol. Biol. Rev.*, 63(1) pp. 54-105.

Jordan, V. C. (2006) 'Tamoxifen (ICI46, 474) as a targeted therapy to treat and prevent breast cancer.' *British Journal of Pharmacology*, 147(S1) pp. S269-S276.

Jordan, V. C. and Morrow, M. (1999) 'Tamoxifen, raloxifene, and the prevention of breast cancer.' *Endocrine Reviews*, 20(3) pp. 253-278.

Jordan, V. C., Phelps, E. and Lindgren, J. U. (1987) 'Effects of anti-estrogens on bone in castrated and intact female rats.' *Breast Cancer Research and Treatment*, 10(1) pp. 31-35.

Kallergi, G., Agelaki, S., Markomanolaki, H., Georgoulias, V. and Stournaras, C. (2007) 'Activation of FAK/PI3K/Rac1 signaling controls actin reorganization and inhibits cell motility in human cancer cells.' *Cellular Physiology and Biochemistry*, 20(6) pp. 977-986.

Kallergi, G., Tsapara, A., Kampa, M., Papakonstanti, E. A., Krasagakis, K., Castanas, E. and Stournaras, C. (2003) 'Distinct signaling pathways regulate differential opioid effects on actin cytoskeleton in malignant MCF7 and nonmalignant MCF12A human breast epithelial cells.' *Experimental Cell Research*, 288(1) pp. 94-109.

Kanner, S. B., Reynolds, A. B., Vines, R. R. and Parsons, J. T. (1990) 'Monoclonal antibodies to individual tyrosine-phosphorylated protein substrates of oncogene-encoded tyrosine kinases.' *Proceedings of the National Academy of Sciences*, 87(9) pp. 3328-3332.

Kasorn, A., Alcaide, P., Jia, Y., Subramanian, K. K., Sarraj, B., Li, Y., Loison, F., Hattori, H., Silberstein, L. E. and Luscinskas, W. F. (2009) 'Focal adhesion kinase regulates pathogen-killing capability and life span of neutrophils via mediating both adhesion-dependent and-independent cellular signals.' *The Journal of Immunology*, 183(2) pp. 1032-1043.

Katoh, H. and Negishi, M. (2003) 'RhoG activates Rac1 by direct interaction with the Dock180-binding protein Elmo.' *Nature*, 424(6947), 2003/07/01, pp. 461-464.

Katoh, H., Hiramoto, K. and Negishi, M. (2006) 'Activation of Rac1 by RhoG regulates cell migration.' *Journal of Cell Science*, 119(1) pp. 56-65.

Kedar, R., Bourne, T. H., Collins, W., Campbell, S., Powles, T., Ashley, S. and Cosgrove, D. (1994) 'Effects of tamoxifen on uterus and ovaries of postmenopausal women in a randomised breast cancer prevention trial.' *Lancet*, 343(8909) pp. 1318-1321.

Kiley, S. C. and Parker, P. J. (1995) 'Differential localization of protein kinase C isozymes in U937 cells: evidence for distinct isozyme functions during monocyte differentiation.' *Journal of Cell Science*, 108(3) pp. 1003-1016.

Kim, S.-Y., Kim, S., Bae, D.-J., Park, S.-Y., Lee, G.-Y., Park, G.-M. and Kim, I.-S. (2017) 'Coordinated balance of Rac1 and RhoA plays key roles in determining phagocytic appetite.' *PloS one*, 12(4) p. e0174603.

Kirketerp-Møller, K., Jensen, P. Ø., Fazli, M., Madsen, K. G., Pedersen, J., Moser, C., Tolker-Nielsen, T., Høiby, N., Givskov, M. and Bjarnsholt, T. (2008) 'Distribution, organization, and ecology of bacteria in chronic wounds.' *Journal of Clinical Microbiology*, 46(8) pp. 2717-2722.

Kirsner, R. S. and Eaglstein, W. (1993) 'The wound healing process.' *Dermatologic Clinics*, 11(4) pp. 629-640.

Klein and Roberts, C. W. (2010) *Sex hormones and Immunity to Infection.* Springer.

Klein, Cornejo, J. C., Polakos, N. K., John, B., Wuensch, S. A., Topham, D. J., Pierce, R. H. and Crispe, I. N. (2007) 'Kupffer cell heterogeneity: functional properties of bone marrow derived and sessile hepatic macrophages.' *Blood*, 110(12), Dec 1, 2007/08/11, pp. 4077-4085.

Klinge, C. M. (2000) 'Estrogen receptor interaction with co-activators and co-repressors.' *Steroids*, 65(5) pp. 227-251.

Koeffler, H. P. (1983) 'Induction of differentiation of human acute myelogenous leukemia cells: therapeutic implications.' *Blood*, 62(4) pp. 709-721.

Koh, T. J. and DiPietro, L. A. (2011) 'Inflammation and wound healing: the role of the macrophage.' *Expert Reviews in Molecular Medicine*, 13

Köhler, T., Michea-Hamzehpour, M., Plesiat, P., Kahr, A.-L. and Pechere, J.-C. (1997) 'Differential selection of multidrug efflux systems by quinolones in Pseudomonas aeruginosa.' *Antimicrobial Agents and Chemotherapy*, 41(11) pp. 2540-2543.

Kopp, E. B. and Medzhitov, R. (1999) 'The Toll-receptor family and control of innate immunity.' *Current Opinion in Immunology*, 11(1) pp. 13-18.

Kozma, R., Ahmed, S., Best, A. and Lim, L. (1995) 'The Ras-related protein Cdc42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts.' *Molecular and Cellular Biology*, 15(4) pp. 1942-1952.

Krzyszczyk, P., Schloss, R., Palmer, A. and Berthiaume, F. (2018) 'The role of macrophages in acute and chronic wound healing and interventions to promote pro-wound healing phenotypes.' *Frontiers in Physiology*, 9

Kuiper, G. G., Shughrue, P. J., Merchenthaler, I. and Gustafsson, J.-Å. (1998) 'The estrogen receptor β subtype: a novel mediator of estrogen action in neuroendocrine systems.' *Frontiers in Neuroendocrinology*, 19(4) pp. 253-286.

Kuiper, G. G., Carlsson, B., Grandien, K., Enmark, E., Häggblad, J., Nilsson, S. and Gustafsson, J.-A. k. (1997) 'Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β.' *Endocrinology*, 138(3) pp. 863-870.

Kzhyshkowska, J., Gratchev, A. and Goerdt, S. (2006) 'Stabilin-1, a homeostatic scavenger receptor with multiple functions.' *Journal of Cellular and Molecular Medicine*, 10(3) pp. 635-649.

Labonte, A. C., Tosello-Trampont, A.-C. and Hahn, Y. S. (2014) 'The role of macrophage polarization in infectious and inflammatory diseases.' *Molecules and Cells*, 37(4) pp. 275-285.

Labrie, F., Belanger, A., Simard, J., LUU-THE, V. and Labrie, C. (1995) 'DHEA and peripheral androgen and estrogen formation: intracrinology.' *Annals of the New York Academy of Sciences*, 774(1) pp. 16-28.

Labrie, F., Bélanger, A., Luu-The, V., Labrie, C., Simard, J., Cusan, L., Gomez, J.-L. and Candas, B. (1998) 'DHEA and the intracrine formation of androgens and estrogens in peripheral target tissues: its role during aging.' *Steroids*, 63(5) pp. 322-328.

Lai, A. C. and Crews, C. M. (2017) 'Induced protein degradation: an emerging drug discovery paradigm.' *Nature Reviews Drug Discovery*, 16(2) p. 101.

Lam, E. K., Tai, E. K., Wu, W. K., Yu, L., Wong, H. P., Shin, V. Y. and Cho, C. (2017) 'Differential effects of lipoteichoic acid and lipopolysaccharides on gastric cancer cell proliferation via distinctive involvement of TLR2 signaling mediators.' Archives of Digestive Disorders, 1(1) pp. 22-27.

Lamarche, N. and Hall, A. (1994) 'GAPs for rho-related GTPases.' *Trends in Genetics*, 10(12), 1994/12/01/, pp. 436-440.

Laufs, U., Adam, O., Strehlow, K., Wassmann, S., Konkol, C., Laufs, K., Schmidt, W., Böhm, M. and Nickenig, G. (2003) 'Down-regulation of Rac-1 GTPase by estrogen.' *Journal of Biological Chemistry*, 278(8) pp. 5956-5962.

Leblanc, D., Schneider, M., Angele, P., Vollmer, G. and Docheva, D. (2017) 'The effect of estrogen on tendon and ligament metabolism and function.' *The Journal of Steroid Biochemistry and Molecular Biology*, 172 pp. 106-116.

Lee, Goodwin, A. and Wilcken, N. (2017) 'Fulvestrant for hormone-sensitive metastatic breast cancer.' *Cochrane Database of Systematic Reviews*, (1)

Lee, W.-L., Cheng, M.-H., Tarng, D.-C., Yang, W.-C., Lee, F.-K. and Wang, P.-H. (2013) 'The benefits of estrogen or selective estrogen receptor modulator on kidney and its related disease—chronic kidney disease—mineral and bone disorder: Osteoporosis.' *Journal of the Chinese Medical Association*, 76(7) pp. 365-371.

Leung, Manser, E., Tan, L. and Lim, L. (1995) 'A novel serine/threonine kinase binding the Ras-related RhoA GTPase which translocates the kinase to peripheral membranes.' *Journal of Biological Chemistry*, 270(49), Dec 8, 1995/12/08, pp. 29051-29054.

Leung, Chen, X.-Q., Manser, E. and Lim, L. (1996) 'The p160 RhoA-binding kinase ROK alpha is a member of a kinase family and is involved in the reorganization of the cytoskeleton.' *Molecular and Cellular Biology*, 16(10) pp. 5313-5327.

Lev, M. (1959) 'The growth-promoting activity of compounds of the vitamin K group and analogues for a rumen strain of Fusiformis nigrescens.' *J Gen Microbiol*, 20(3), Jun, 1959/06/01, pp. 697-703.

Ley, K., Laudanna, C., Cybulsky, M. I. and Nourshargh, S. (2007) 'Getting to the site of inflammation: the leukocyte adhesion cascade updated.' *Nature Reviews Immunology*, 7, 09/01/online, p. 678.

Li, J., Chen, J. and Kirsner, R. (2007) 'Pathophysiology of acute wound healing.' Clinics in Dermatology, 25(1) pp. 9-18.

Li, R., Shen, Y., Yang, L. B., Lue, L. F., Finch, C. and Rogers, J. (2000) 'Estrogen enhances uptake of amyloid β-protein by microglia derived from the human cortex.' *Journal of Neurochemistry*, 75(4) pp. 1447-1454.

Li, Wang, J.-P., Santen, R. J., Kim, T.-H., Park, H., Fan, P. and Yue, W. (2010) 'Estrogen stimulation of cell migration involves multiple signaling pathway interactions.' *Endocrinology*, 151(11) pp. 5146-5156.

Lin, H., Lee, E., Hestir, K., Leo, C., Huang, M., Bosch, E., Halenbeck, R., Wu, G., Zhou, A. and Behrens, D. (2008) 'Discovery of a cytokine and its receptor by functional screening of the extracellular proteome.' *Science*, 320(5877) pp. 807-811.

Ling, S., Dai, A., Dilley, R. J., Jones, M., Simpson, E., Komesaroff, P. A. and Sudhir, K. (2004) 'Endogenous estrogen deficiency reduces proliferation and enhances apoptosis-related death in vascular smooth muscle cells: insights from the aromatase-knockout mouse.' *Circulation*, 109(4) pp. 537-543.

Liu, K., Victora, G. D., Schwickert, T. A., Guermonprez, P., Meredith, M. M., Yao, K., Chu, F.-F., Randolph, G. J., Rudensky, A. Y. and Nussenzweig, M. (2009) '*In vivo* analysis of dendritic cell development and homeostasis.' *Science*, 324(5925) pp. 392-397.

London, A., Cohen, M. and Schwartz, M. (2013) 'Microglia and monocyte-derived macrophages: functionally distinct populations that act in concert in CNS plasticity and repair.' *Frontiers in Cellular Neuroscience*, 7 article 34.

Long, X. and Nephew, K. P. (2006) 'Fulvestrant (ICI 182,780)-dependent interacting proteins mediate immobilization and degradation of estrogen receptor-α.' *Journal of Biological Chemistry*, 281(14) pp. 9607-9615.

Lookingbill, D. P., Miller, S. H. and Knowles, R. C. (1978) 'Bacteriology of chronic leg ulcers.' *Archives of Dermatology*, 114(12) pp. 1765-1768.

Lorenz, H. P. and Longaker, M. T. (2003) 'Wounds: biology, pathology, and management.' *In Essential Practice of Surgery*. Springer, pp. 77-88.

Louie, T. J., Bartlett, J. G., Tally, F. P. and Gorbach, S. L. (1976) 'Aerobic and anaerobic bacteria in diabetic foot ulcers.' *Annals of Internal Medicine*, 85(4) pp. 461-463.

Love, R. R., Mazess, R. B., Barden, H. S., Epstein, S., Newcomb, P. A., Jordan, V. C., Carbone, P. P. and DeMets, D. L. (1992) 'Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer.' *New England Journal of Medicine*, 326(13) pp. 852-856.

Luscinskas, F. W., Kansas, G. S., Ding, H., Pizcueta, P., Schleiffenbaum, B. E., Tedder, T. F. and Gimbrone, M. A. (1994) 'Monocyte rolling, arrest and spreading on IL-4-activated vascular endothelium under flow is mediated via sequential action of L-selectin, beta 1-integrins, and beta 2-integrins.' *The Journal of Cell Biology*, 125(6) pp. 1417-1427.

MacCorquodale, D., Thayer, S. A. and Doisy, E. A. (1936) 'The isolation of the principal estrogenic substance of liquor folliculi.' *Journal of Biological Chemistry*, 115(2) pp. 435-448.

Machesky, L. M. and Hall, A. (1996) 'Rho: a connection between membrane receptor signalling and the cytoskeleton.' *Trends in Cell Biology*, 6(8) pp. 304-310.

Machesky, L. M. and Insall, R. H. (1999) 'Signaling to actin dynamics.' *The Journal of Cell Biology*, 146(2) pp. 267-272.

Mackaness, G. (1964) 'The immunological basis of acquired cellular resistance.' *Journal of Experimental Medicine*, 120(1) pp. 105-120.

Mah, T.-F., Pitts, B., Pellock, B., Walker, G. C., Stewart, P. S. and O'Toole, G. A. (2003) 'A genetic basis for Pseudomonas aeruginosa biofilm antibiotic resistance.' *Nature*, 426(6964) pp. 306-310.

Mahla, R. S., Reddy, C. M., Prasad, D. and Kumar, H. (2013) 'Sweeten PAMPs: role of sugar complexed PAMPs in innate immunity and vaccine biology.' *Frontiers in Immunology*, 4 p. 248.

Malissen, B., Tamoutounour, S. and Henri, S. (2014) 'The origins and functions of dendritic cells and macrophages in the skin.' *Nature reviews Immunology*, 14(6) p. 417.

Mann, M. K. (2008) *Synthesis of non-steroidal estrogen receptor proteolysis targeting chimeric molecules (PROTACS).* University of Illinois at Urbana-Champaign.

Mantovani, A., Sica, A. and Locati, M. (2005) 'Macrophage polarization comes of age.' *Immunity*, 23(4) pp. 344-346.

Mantovani, A., Biswas, S. K., Galdiero, M. R., Sica, A. and Locati, M. (2013) 'Macrophage plasticity and polarization in tissue repair and remodelling.' Journal of Pathology, 229(2) pp. 176-185.

Mantovani, A., Sica, A., Sozzani, S., Allavena, P., Vecchi, A. and Locati, M. (2004) 'The chemokine system in diverse forms of macrophage activation and polarization.' *Trends in Immunology*, 25(12) pp. 677-686.

Man-Kupisinska, A., Michalski, M., Maciejewska, A., Swierzko, A. S., Cedzynski, M., Lugowski, C. and Lukasiewicz, J. (2016) 'A new ligand-based method for purifying active human plasma-derived ficolin-3 complexes supports the phenomenon of crosstalk between pattern-recognition molecules and immunoglobulins.' *PloS One*, 11(5) p. e0156691.

Margolis, D. J., Knauss, J. and Bilker, W. (2002) 'Hormone replacement therapy and prevention of pressure ulcers and venous leg ulcers.' *Lancet*, 359(9307) pp. 675-677.

Marikovsky, M., Breuing, K., Liu, P. Y., Eriksson, E., Higashiyama, S., Farber, P., Abraham, J. and Klagsbrun, M. (1993) 'Appearance of heparin-binding EGF-like growth factor in wound fluid as a response to injury.' *Proceedings of the National Academy of Sciences*, 90(9) pp. 3889-3893.

Marino, M., Galluzzo, P. and Ascenzi, P. (2006) 'Estrogen signaling multiple pathways to impact gene transcription.' *Current Genomics*, 7(8) pp. 497-508.

Martinez and Gordon, S. (2014) 'The M1 and M2 paradigm of macrophage activation: time for reassessment.' *F1000prime Reports*, 6

Martinez, Gordon, S., Locati, M. and Mantovani, A. (2006) 'Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression.' *The Journal of Immunology*, 177(10) pp. 7303-7311.

Martinez, Sica, A., Mantovani, A. and Locati, M. (2008) 'Macrophage activation and polarization.' *Front Biosci*, 13(1) pp. 453-461.

Matsukawa, M. and Greenberg, E. (2004) 'Putative exopolysaccharide synthesis genes influence Pseudomonas aeruginosa biofilm development.' *Journal of Bacteriology*, 186(14) pp. 4449-4456.

Matthews, J. and Gustafsson, J.-Å. (2003) 'Estrogen signaling: a subtle balance between ER-α and ER-β.' *Molecular Interventions*, 3(5) p. 281.

Maus, U. A., Koay, M. A., Delbeck, T., Mack, M., Ermert, M., Ermert, L., Blackwell, T. S., Christman, J. W., Schlondorff, D. and Seeger, W. (2002) 'Role of resident alveolar macrophages in leukocyte traffic into the alveolar air space of intact mice.' *American Journal of Physiology-Lung Cellular and Molecular Physiology*, 26(6) p. L1245.

Mayor, R. and Carmona-Fontaine, C. (2010) 'Keeping in touch with contact inhibition of locomotion.' *Trends in Cell Biology*, 20(6) pp. 319-328.

McGowan, J. E., Barnes, M. W. and Finland, M. (1975) 'Bacteremia at Boston City Hospital: occurrence and mortality during 12 selected years (1935-1972), with special reference to hospital-acquired cases.' *Journal of Infectious Diseases*, 132(3) pp. 316-335.

Mendelsohn, M. E. and Karas, R. H. (1999) 'The protective effects of estrogen on the cardiovascular system.' *New England Journal of Medicine*, 340(23) pp. 1801-1811.

Meyers, M. J., Sun, J., Carlson, K. E., Marriner, G. A., Katzenellenbogen, B. S. and Katzenellenbogen, J. A. (2001) 'Estrogen receptor-β potency-selective ligands: structure− activity relationship studies of diarylpropionitriles and their acetylene and polar analogues.' *Journal of Medicinal Chemistry*, 44(24) pp. 4230-4251.

Midgley, A. C., Morris, G., Phillips, A. O. and Steadman, R. (2016) '17β-estradiol ameliorates age-associated loss of fibroblast function by attenuating IFN-γ/STAT 1-dependent miR-7 upregulation.' *Aging Cell*, 15(3) pp. 531-541.

Mills, C. (2012) 'M1 and M2 macrophages: oracles of health and disease.' Critical Reviews in Immunology, 32(6) pp. 463-488.

Mills, S. J., Ashworth, J. J., Gilliver, S. C., Hardman, M. J. and Ashcroft, G. S. (2005) 'The sex steroid precursor DHEA accelerates cutaneous wound healing via the estrogen receptors.' *Journal of Investigative Dermatology*, 125(5) pp. 1053-1062.

Minafra, L., Di Cara, G., Albanese, N. N. and Cancemi, P. (2011) 'Proteomic differentiation pattern in the U937 cell line.' *Leukemia Research*, 35(2) pp. 226-236.

Minutolo, F., Macchia, M., Katzenellenbogen, B. S. and Katzenellenbogen, J. A. (2011) 'Estrogen receptor β ligands: recent advances and biomedical applications.' *Medicinal Research Reviews*, 31(3) pp. 364-442.

Minutti, C. M., Knipper, J. A., Allen, J. E. and Zaiss, D. M. (2017) *Tissue-specific contribution of macrophages to wound healing.* Vol. 61: Elsevier.

Mir, L. M., Belehradek, M., Domenge, C., Orlowski, S., Poddevin, B., Belehradek, J. J., Schwaab, G., Luboinski, B. and Paoletti, C. (1991) 'Electrochemotherapy, a new antitumor treatment: first clinical trial.' *Comptes Rendus de l'Academie des Sciences. Serie III, Sciences de laVvie*, 313(13) pp. 613-618.

Mirkin, S. and Pickar, J. H. (2015) 'Selective estrogen receptor modulators (SERMs): a review of clinical data.' *Maturitas*, 80(1) pp. 52-57.

Missiakas, D. and Schneewind, O. (2016) 'Staphylococcus aureus vaccines: Deviating from the carol.' *The Journal of Experimental Medicine*, 213(9) pp. 1645-1653.

Miura, T., Miura, C., Ohta, T., Nader, M. R., Todo, T. and Yamauchi, K. (1999) 'Estradiol-17β stimulates the renewal of spermatogonial stem cells in males.' *Biochemical and Biophysical Research Communications*, 264(1) pp. 230-234.

Moore, K., Ruge, F. and Harding, K. (1997) 'T lymphocytes and the lack of activated macrophages in wound margin biopsies from chronic leg ulcers.' *British Journal of Dermatology*, 137(2) pp. 188-194.

Morales, D. E., McGowan, K. A., Grant, D. S., Maheshwari, S., Bhartiya, D., Cid, M. C., Kleinman, H. K. and Schnaper, H. W. (1995) 'Estrogen promotes angiogenic activity in human umbilical vein endothelial cells *in vitro* and in a murine model.' *Circulation*, 91(3) pp. 755-763.

Morris, C. and Wakeling, A. (2002) 'Fulvestrant ('Faslodex')--a new treatment option for patients progressing on prior endocrine therapy.' *Endocrine-related cancer*, 9(4) pp. 267-276.

Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A. and Coffman, R. L. (1986) 'Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins.' *The Journal of Immunology*, 136(7) pp. 2348-2357.

Mosselman, S., Polman, J. and Dijkema, R. (1996) 'ERβ: identification and characterization of a novel human estrogen receptor.' *FEBS Letters*, 392(1) pp. 49-53.

Mosser, D. M. and Edwards, J. P. (2008) 'Exploring the full spectrum of macrophage activation.' Nature Reviews Immunology, 8(12) pp. 958-969.

Mosser, D. M. and Edwards, J. P. (2008) 'Exploring the full spectrum of macrophage activation.' *Nature Reviews Immunology*, 8(12) pp. 958-969.

Mosser, D. M. and Edwards, J. P. (2010) 'Exploring the full spectrum of macrophage activation.' *Nature Reviews Immunology*, 10(6) pp. 460-460.

Moyses, C., Cederholm-Williams, S. and Michel, C. (1987) 'Haemoconcentration and accumulation of white cells in the feet during venous stasis.' *International Journal of Microcirculation, Clinical and Experimental*, 5(4) pp. 311-320.

Mukai, K., Urai, T., Asano, K., Nakajima, Y. and Nakatani, T. (2016) 'Evaluation of effects of topical estradiol benzoate application on cutaneous wound healing in ovariectomized female mice.' *PloS one*, 11(9) p. e0163560.

Mukherjee, S., Ghosh, R. N. and Maxfield, F. R. (1997) 'Endocytosis.' *Physiological Reviews*, 77(3) pp. 759-803.

Murray, P. J. and Wynn, T. A. (2011) 'Protective and pathogenic functions of macrophage subsets.' *Nature Reviews Immunology*, 11(11) p. 723.

Murray, P. J., Allen, J. E., Biswas, S. K., Fisher, E. A., Gilroy, D. W., Goerdt, S., Gordon, S., Hamilton, J. A., Ivashkiv, L. B. and Lawrence, T. (2014) 'Macrophage activation and polarization: nomenclature and experimental guidelines.' *Immunity*, 41(1) pp. 14-20.

Mustoe. (2005) 'Dermal ulcer healing: Advances in understanding.' *Tissue repair and ulcer/wound healing: molecular mechanisms, therapeutic targets and future directions. Paris, France: Euroconferences.*

Naimi, T. S., LeDell, K. H., Boxrud, D. J., Groom, A. V., Steward, C. D., Johnson, S. K., Besser, J. M., O'Boyle, C., Danila, R. N. and Cheek, J. E. (2001) 'Epidemiology and clonality of community-acquired methicillin-resistant Staphylococcus aureus in Minnesota, 1996–1998.' *Clinical infectious diseases*, 33(7) pp. 990-996.

Nakaya, M., Kitano, M., Matsuda, M. and Nagata, S. (2008) 'Spatiotemporal activation of Rac1 for engulfment of apoptotic cells.' *Proceedings of the National Academy of Sciences*, 105(27) pp. 9198-9203.

Nakaya, M., Tanaka, M., Okabe, Y., Hanayama, R. and Nagata, S. (2006) 'Opposite effects of rho family GTPases on engulfment of apoptotic cells by macrophages.' *Journal of Biological Chemistry*, 281(13) pp. 8836-8842.

Nathan, C. F., Murray, H. W., Wiebe, M. and Rubin, B. Y. (1983) 'Identification of interferon-gamma as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity.' *Journal of Experimental Medicine*, 158(3) pp. 670-689.

Nawata, H., Tanaka, S., Tanaka, S., Takayanagi, R., Sakai, Y., Yanase, T., Ikuyama, S. and Haji, M. (1995) 'Aromatase in bone cell: association with osteoporosis in postmenopausal women.' *Journal of Steroid Biochemistry and Molecular Biology*, 53(1-6) pp. 165-174.

Nelson, L. R. and Bulun, S. E. (2001) 'Estrogen production and action.' *Journal of the American Academy of Dermatology*, 45(3) pp. S116-S124.

Neven, P., Goldstein, S. R., Ciaccia, A. V., Zhou, L., Silfen, S. L. and Muram, D. (2002) 'The effect of raloxifene on the incidence of ovarian cancer in postmenopausal women.' *Gynecologic Oncology*, 85(2) pp. 388-390.

Newman, S. L., Henson, J. E. and Henson, P. M. (1982) 'Phagocytosis of senescent neutrophils by human monocyte-derived macrophages and rabbit inflammatory macrophages.' *Journal of Experimental Medicine*, 156(2) pp. 430-442.

Nguyen, K. D., Qiu, Y., Cui, X., Goh, Y. S., Mwangi, J., David, T., Mukundan, L., Brombacher, F., Locksley, R. M. and Chawla, A. (2011) 'Alternatively activated macrophages produce catecholamines to sustain adaptive thermogenesis.' *Nature*, 480(7375) p. 104.

Nobes, C. D. and Hall, A. (1995) 'Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia.' *Cell*, 81(1) pp. 53-62.

Novak, M. L. and Koh, T. J. (2013) 'Macrophage phenotypes during tissue repair.' *Journal of Leukocyte Biology*, 93(6) pp. 875-881.

O'Toole, G. A. and Kolter, R. (1998) 'Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development.' *Molecular microbiology*, 30(2) pp. 295-304.

O'Donnell, L., Robertson, K. M., Jones, M. E. and Simpson, E. R. (2001) 'Estrogen and spermatogenesis.' Endocrine Reviews, 22(3) pp. 289-318.

Oelschlaeger, T. A. (2010) 'Mechanisms of probiotic actions–a review.' *International Journal of Medical Microbiology*, 300(1) pp. 57-62.

Ofek, I. and Doyle, R. J. (2012) *Bacterial adhesion to cells and tissues.* Springer Science & Business Media.

Ogle, M. E., Segar, C. E., Sridhar, S. and Botchwey, E. A. (2016) 'Monocytes and macrophages in tissue repair: implications for immunoregenerative biomaterial design.' *Experimental Biology and Medicine*, 241(10) pp. 1084-1097.

Ohta, Y., Hartwig, J. H. and Stossel, T. P. (2006) 'FilGAP, a Rho-and ROCK-regulated GAP for Rac binds filamin A to control actin remodelling.' *Nature Cell Biology*, 8(8) p. 803.

Olczyk, P., Mencner, Ł. and Komosinska-Vassev, K. (2014) 'The role of the extracellular matrix components in cutaneous wound healing.' *BioMed Research International*, 2014 2014/03/17, pp. 747584-747584.

Oliver, A., Cantón, R., Campo, P., Baquero, F. and Blázquez, J. (2000) 'High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection.' *Science*, 288(5469) pp. 1251-1253.

Olofsson, B. (1999) 'Rho Guanine Dissociation Inhibitors: Pivotal Molecules in Cellular Signalling.' *Cellular Signalling*, 11(8), 1999/08/01/, pp. 545-554.

Olson, M., Ashworth, A. and Hall, A. (1995) 'An essential role for Rho, Rac, and Cdc42 GTPases in cell cycle progression through G1.' *Science*, 269(5228) pp. 1270-1272.

Osborne, C., Wakeling, A. and Nicholson, R. (2004) 'Fulvestrant: an oestrogen receptor antagonist with a novel mechanism of action.' *British Journal of Cancer*, 90(S1) p. S2.

Owen, K. A., Pixley, F. J., Thomas, K. S., Vicente-Manzanares, M., Ray, B. J., Horwitz, A. F., Parsons, J. T., Beggs, H. E., Stanley, E. R. and Bouton, A. H. (2007) 'Regulation of lamellipodial persistence, adhesion turnover, and motility in macrophages by focal adhesion kinase.' *The Journal of Cell Biology*, 179(6) pp. 1275-1287.

Paige, L. A., Christensen, D. J., Grøn, H., Norris, J. D., Gottlin, E. B., Padilla, K. M., Chang, C.-y., Ballas, L. M., Hamilton, P. T. and McDonnell, D. P. (1999) 'Estrogen receptor (ER) modulators each induce distinct conformational changes in ER α and ER β.' *Proceedings of the National Academy of Sciences*, 96(7) pp. 3999-4004.

Paolicelli, R. C., Bolasco, G., Pagani, F., Maggi, L., Scianni, M., Panzanelli, P., Giustetto, M., Ferreira, T. A., Guiducci, E. and Dumas, L. (2011) 'Synaptic pruning by microglia is necessary for normal brain development.' *science*, 333(6048) pp. 1456-1458.

Papakonstanti, E. A., Kampa, M., Castanas, E. and Stournaras, C. (2003) 'A rapid, nongenomic, signaling pathway regulates the actin reorganization induced by activation of membrane testosterone receptors.' *Molecular Endocrinology*, 17(5) pp. 870-881.

Park, W.-C. and Jordan, V. C. (2002) 'Selective estrogen receptor modulators (SERMS) and their roles in breast cancer prevention.' *Trends in Molecular Medicine*, 8(2) pp. 82-88.

Passador, L., Cook, J. M., Gambello, M. J., Rust, L. and Iglewski, B. H. (1993) 'Expression of Pseudomonas aeruginosa virulence genes requires cell-to-cell communication.' *Science*, 260(5111) pp. 1127-1130.

Passlick, B., Flieger, D. and Ziegler-Heitbrock, H. (1989) 'Identification and characterization of a novel monocyte subpopulation in human peripheral blood.' *Blood*, 74(7) pp. 2527-2534.

Pauwels, A.-M., Trost, M., Beyaert, R. and Hoffmann, E. (2017) 'Patterns, Receptors, and Signals: Regulation of Phagosome Maturation.' *Trends in Immunology*, 83(6) pp. 407-422.

Payne, A. H. and Hales, D. B. (2004) 'Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones.' *Endocrine Reviews*, 25(6) pp. 947-970.

Pepe, G., Braga, D., Renzi, T. A., Villa, A., Bolego, C., D'Avila, F., Barlassina, C., Maggi, A., Locati, M. and Vegete, E. (2017) 'Self-renewal and phenotypic conversion are the main physiological responses of macrophages to the endogenous estrogen surge.' *Scientific Reports*, 7 p. 44270.

Peschel, A. (2002) 'How do bacteria resist human antimicrobial peptides?' *Trends in Microbiology*, 10(4) pp. 179-186.

Pesci, E. C., Milbank, J. B., Pearson, J. P., McKnight, S., Kende, A. S., Greenberg, E. P. and Iglewski, B. H. (1999) 'Quinolone signaling in the cell-to-cell communication system of Pseudomonas aeruginosa.' *Proceedings of the National Academy of Sciences*, 96(20) pp. 11229-11234.

Peskin, C. S., Odell, G. M. and Oster, G. F. (1993) 'Cellular motions and thermal fluctuations: the Brownian ratchet.' *Biophysical Journal*, 65(1) pp. 316-324.

Peyssonnaux, C., Cejudo-Martin, P., Doedens, A., Zinkernagel, A. S., Johnson, R. S. and Nizet, V. (2007) 'Cutting edge: essential role of hypoxia inducible factor-1α in development of lipopolysaccharide-induced sepsis.' *The Journal of Immunology*, 178(12) pp. 7516-7519.

Picard, F., Deshaies, Y., Lalonde, J., Samson, P., Labrie, C., Bélanger, A., Labrie, F. and Richard, D. (2000) 'Effects of the estrogen antagonist EM-652. HCl on energy balance and lipid metabolism in ovariectomized rats.' *International Journal of Obesity*, 24(7) p. 830.

Pickar, J. H., MacNeil, T. and Ohleth, K. (2010) 'SERMs: progress and future perspectives.' *Maturitas*, 67(2) pp. 129-138.

Plant, T. M. and Zeleznik, A. J. (2014) *Knobil and Neill's physiology of reproduction.* Academic Press.

Pollard. (2009) 'Trophic macrophages in development and disease.' *Nat Rev Immunol*, 9(4), Apr, 2009/03/14, pp. 259-270.

Pollard and Borisy. (2003) 'Cellular motility driven by assembly and disassembly of actin filaments.' *Cell*, 112(4), Feb 21, 2003/02/26, pp. 453-465.

Pollard, T. D. and Borisy, G. G. (2003) 'Cellular motility driven by assembly and disassembly of actin filaments.' *Cell*, 112(4) pp. 453-465.

Pollard, T. D., Earnshaw, W. C., Lippincott-Schwartz, J. and Johnson, G. (2016) *Cell Biology E-Book.* Elsevier Health Sciences.

Prieto-Sánchez, R. M., Berenjeno, I. M. and Bustelo, X. R. (2006) 'Involvement of the Rho/Rac family member RhoG in caveolar endocytosis.' *Oncogene*, 25(21) p. 2961.

Puig-Kröger, A., Serrano-Gómez, D., Caparrós, E., Domínguez-Soto, A., Relloso, M., Colmenares, M., Martínez-Muñoz, L., Longo, N., Sánchez-Sánchez, N. and Rincon, M. (2004) 'Regulated expression of the pathogen receptor dendritic cell-specific intercellular adhesion molecule 3 (ICAM-3)-grabbing nonintegrin in THP-1 human leukemic cells, monocytes, and macrophages.' *Journal of Biological Chemistry*, 279(24) pp. 25680-25688.

Qadir, M. I., Parveen, A. and Ali, M. (2015) 'Cdc42: role in cancer management.' *Chemical Biology & Drug Design*, 86(4) pp. 432-439.

Qu, H., Ricklin, D. and Lambris, J. D. (2009) 'Recent developments in low molecular weight complement inhibitors.' *Molecular Immunology*, 47(2-3) pp. 185-195.

Quirke, V. M. (2017) 'Tamoxifen from failed contraceptive pill to best-selling breast cancer medicine: a case-study in pharmaceutical innovation.' *Frontiers in Pharmacology*, 8 p. 620.

Rabinovitch, M. (1995) 'Professional and non-professional phagocytes: an introduction.' *Trends in Cell Biology*, 5(3) pp. 85-87.

Raes, G., De Baetselier, P., Noël, W., Beschin, A., Brombacher, F. and Gh, G. H. (2002) 'Differential expression of FIZZ1 and Ym1 in alternatively versus classically activated macrophages.' *Journal of Leukocyte Biology*, 71(4) pp. 597-602.

Raftopoulou, M. and Hall, A. (2004) 'Cell migration: Rho GTPases lead the way.' *Developmental Biology*, 265(1), 2004/01/01/, pp. 23-32.

Raja, S. K., Garcia, M. S. and Isseroff, R. R. (2007) 'Wound re-epithelialization: modulating keratinocyte migration in wound healing.' *Frontiers in Bioscience*, 12(3) pp. 2849-2868.

Randow, F., MacMicking, J. D. and James, L. C. (2013) 'Cellular self-defense: how cell-autonomous immunity protects against pathogens.' *Science*, 340(6133) pp. 701-706.

Richter, D. U., Mylonas, I., Toth, B., Scholz, C., Briese, V., Friese, K. and Jeschke, U. (2009) 'Effects of phytoestrogens genistein and daidzein on progesterone and estrogen (estradiol) production of human term trophoblast cells in vitro.' *Gynecological Endocrinology*, 25(1) pp. 32-38.

Ridley, A. J. and Hall, A. (1992) 'The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors.' *Cell*, 70(3) pp. 389-399.

Ridley, A. J., Paterson, H. F., Johnston, C. L., Diekmann, D. and Hall, A. (1992) 'The small GTP-binding protein rac regulates growth factor-induced membrane ruffling.' *Cell*, 70(3) pp. 401-410.

Rigassi, L., Barchiesi Bozzolo, F., Lucchinetti, E., Zaugg, M., Fingerle, J., Rosselli, M., Imthurn, B., Jackson, E. K. and Dubey, R. K. (2015) '2-Methoxyestradiol blocks the RhoA/ROCK1 pathway in human aortic smooth muscle cells.' *American Journal of Physiology-Endocrinology and Metabolism*, 309(12) pp. E995-E1007.

Rios de la Rosa, J. M., Tirella, A., Gennari, A., Stratford, I. J. and Tirelli, N. (2017) 'The CD44-Mediated Uptake of Hyaluronic Acid-Based Carriers in Macrophages.' *Advanced Healthcare Materials*, 6(4)

Robson, M. C. (1997) 'Wound infection: a failure of wound healing caused by an imbalance of bacteria.' *Surgical Clinics of North America*, 77(3) pp. 637-650.

Rohatgi, R., Ma, L., Miki, H., Lopez, M., Kirchhausen, T., Takenawa, T. and Kirschner, M. W. (1999) 'The interaction between N-WASP and the Arp2/3 complex links Cdc42-dependent signals to actin assembly.' *Cell*, 97(2) pp. 221-231.

Rubanyi, G. M., Johns, A. and Kauser, K. (2002) 'Effect of estrogen on endothelial function and angiogenesis.' *Vascular Pharmacology*, 38(2) pp. 89-98.

Rubinstein, A., Pierce Jr, C. E. and Bloomgarden, Z. (1983) 'Rapid healing of diabetic foot ulcers with continuous subcutaneous insulin infusion.' *The American Journal of Medicine*, 75(1) pp. 161-165.

Ryan, K. J. and Ray, C. G. (2004) *Medical microbiology: An introduction to infectious diseases.* Mcgraw-Hill.

Rybtke, M., Hultqvist, L. D., Givskov, M. and Tolker-Nielsen, T. (2015) 'Pseudomonas aeruginosa biofilm infections: community structure, antimicrobial tolerance and immune response.' *Journal of Molecular Biology*, 427(23) pp. 3628-3645.

Santoni, G., Cardinali, C., Morelli, M. B., Santoni, M., Nabissi, M. and Amantini, C. (2015) 'Danger-and pathogen-associated molecular patterns recognition by pattern-recognition receptors and ion channels of the transient receptor potential family triggers the inflammasome activation in immune cells and sensory neurons.' *Journal of Neuroinflammation*, 12(1) p. 21.

Sapino, A., Pietribiasi, F., Bussolati, G. and Marchisio, P. C. (1986) 'Estrogen-and tamoxifen-induced rearrangement of cytoskeletal and adhesion structures in breast cancer MCF-7 cells.' *Cancer Research*, 46(5) pp. 2526-2531.

Sarma, J. V. and Ward, P. A. (2011) 'The complement system.' *Cell and Tissue Research*, 343(1) pp. 227-235.

Sator, P.-G., Schmidt, J., Sator, M., Huber, J. and Hönigsmann, H. (2001) 'The influence of hormone replacement therapy on skin ageing: a pilot study.' *Maturitas*, 39(1) pp. 43-55.

Sauerbronn, A., Fonseca, A., Bagnoli, V., Saldiva, P. and Pinotti, J. (2000) 'The effects of systemic hormonal replacement therapy on the skin of postmenopausal women.' *International Journal of Gynecology & Obstetrics*, 68(1) pp. 35-41.

Savvas, M., Bishop, J., Laurent, G., Watson, N. and Studd, J. (1993) 'Type III collagen content in the skin of postmenopausal women receiving oestradiol and testosterone implants.' *British Journal of Obstetrics & Gynaecology*, 100(2) pp. 154-156.

Schaller, M. D. (2010) 'Cellular functions of FAK kinases: insight into molecular mechanisms and novel functions.' *Journal of Cell Science*, 123(7) pp. 1007-1013.

Schaller, M. D., Borgman, C. A., Cobb, B. S., Vines, R. R., Reynolds, A. B. and Parsons, J. T. (1992) 'pp125FAK a structurally distinctive protein-tyrosine kinase associated with focal adhesions.' *Proceedings of the National Academy of Sciences*, 89(11) pp. 5192-5196.

Schlaepfer, D. D., Mitra, S. K. and Ilic, D. (2004) 'Control of motile and invasive cell phenotypes by focal adhesion kinase.' *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, 1692(2-3) pp. 77-102.

Schönfelder, U., Abel, M., Wiegand, C., Klemm, D., Elsner, P. and Hipler, U.-C. (2005) 'Influence of selected wound dressings on PMN elastase in chronic wound fluid and their antioxidative potential in vitro.' *Biomaterials*, 26(33) pp. 6664-6673.

Sen, C. K. and Roy, S. (2008) 'Redox signals in wound healing.' *Biochimica et Biophysica Acta (BBA)-General Subjects*, 1780(11) pp. 1348-1361.

Seppä, H., Grotendorst, G., Seppä, S., Schiffmann, E. and Martin, G. R. (1982) 'Platelet-derived growth factor in chemotactic for fibroblasts.' *The Journal of Cell Biology*, 92(2) pp. 584-588.

Serhan, C. N., Chiang, N. and Van Dyke, T. E. (2008) 'Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators.' *Nature Reviews Immunology*, 8(5) pp. 349-361.

Serini, G. and Gabbiani, G. (1999) 'Mechanisms of myofibroblast activity and phenotypic modulation.' *Experimental cell research*, 250(2) pp. 273-283.

Shah, M. G. and Maibach, H. I. (2001) 'Estrogen and skin.' *American Journal of Clinical Dermatology*, 2(3) pp. 143-150.

Shaw, L. M., Messier, J. M. and Mercurio, A. M. (1990) 'The activation dependent adhesion of macrophages to laminin involves cytoskeletal anchoring and phosphorylation of the alpha 6 beta 1 integrin.' *The Journal of Cell Biology*, 110(6) pp. 2167-2174.

Shenep, J. L. and Mogan, K. A. (1984) 'Kinetics of endotoxin release during antibiotic therapy for experimental gram-negative bacterial sepsis.' *Journal of Infectious Diseases*, 150(3) pp. 380-388.

Shepherd, V. L. (1986) 'The role of the respiratory burst of phagocytes in host defense.' *Seminars in Respiratory Infection*, 1(2) pp. 99-106.

Sica, A. and Mantovani, A. (2012) 'Macrophage plasticity and polarization: *in vivo* veritas.' *The Journal of Clinical Investigation*, 122(3) pp. 787-795.

Siesser, P. M. and Hanks, S. K. (2006) 'The signaling and biological implications of FAK overexpression in cancer.' *Clinical Cancer Research*, 12(11) pp. 3233-3237.

Silva-Gomes, S., Decout, A. and Nigou, J. (2016) 'Pathogen-associated molecular patterns (PAMPs).' *Compendium of Inflammatory Diseases*, pp. 1055-1069.

Simoncini, T., Scorticati, C., Mannella, P., Fadiel, A., Giretti, M. S., Fu, X.-D., Baldacci, C., Garibaldi, S., Caruso, A. and Fornari, L. (2006) 'Estrogen receptor α interacts with Gα13 to drive actin remodeling and endothelial cell migration via the RhoA/Rho kinase/moesin pathway.' *Molecular Endocrinology*, 20(8) pp. 1756-1771.

Simpson, E. (2000) 'Role of aromatase in sex steroid action.' *Journal of Molecular Endocrinology*, 25(2) pp. 149-156.

Singer, A. J. and Clark, R. A. (1999) 'Cutaneous wound healing.' *New England Journal of Medicine*, 341(10) pp. 738-746.

Singh, P. K., Schaefer, A. L., Parsek, M. R., Moninger, T. O., Welsh, M. J. and Greenberg, E. (2000) 'Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms.' *Nature*, 407(6805) pp. 762-764.

Sintiprungrat, K., Singhto, N., Sinchaikul, S., Chen, S.-T. and Thongboonkerd, V. (2010) 'Alterations in cellular proteome and secretome upon differentiation from monocyte to macrophage by treatment with phorbol myristate acetate: insights into biological processes.' *Journal of Proteomics*, 73(3) pp. 602-618.

Sjöstedt, S. (1953) 'The effect of diethylstilbenediol on the healing of wounds in the human vagina.' *Acta Endocrinologica*, 12(3) pp. 260-263.

Sjövall, A. (1947) 'The influence of oestrogen upon the healing of vaginal wounds in rats.' *Acta Obstetricia et Gynecologica Scandinavica*, 27(1) pp. 1-10.

Snyder, R. J. (2005) 'Treatment of nonhealing ulcers with allografts.' *Clinics in Dermatology*, 23(4) pp. 388-395.

Song, R. X.-D. and Santen, R. J. (2006) 'Membrane initiated estrogen signaling in breast cancer.' *Biology of Reproduction*, 75(1) pp. 9-16.

Song, R. X.-D., McPherson, R. A., Adam, L., Bao, Y., Shupnik, M., Kumar, R. and Santen, R. J. (2002) 'Linkage of rapid estrogen action to MAPK activation by ER-α-Shc association and Shc pathway activation.' *Molecular Endocrinology*, 16(1) pp. 116-127.

Sproston, N., El Mohtadi, M., Slevin, M., Gilmore, W. and Ashworth, J. J. (2018) 'The effect of c-reactive Protein isoforms on nitric Oxide Production by U937 Monocytes/Macrophages.' *Frontiers in Immunology*, 9 p. 1500.

Srivastava, S., Weitzmann, M. N., Cenci, S., Ross, F. P., Adler, S. and Pacifici, R. (1999) 'Estrogen decreases TNF gene expression by blocking JNK activity and the resulting production of c-Jun and JunD.' *The Journal of Clinical Investigation*, 104(4) pp. 503-513.

Stashak, T. S., Farstvedt, E. and Othic, A. (2004) 'Update on wound dressings: Indications and best use.' *Clinical Techniques in Equine Practice*, 3(2) pp. 148-163.

Stein, M., Keshav, S., Harris, N. and Gordon, S. (1992) 'Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation.' *Journal of Experimental Medicine*, 176(1) pp. 287-292.

Štěpán, J. J., Lachman, M., Zvěřina, J., Pacovský, V. and Baylink, D. J. (1989) 'Castrated men exhibit bone loss: effect of calcitonin treatment on biochemical indices of bone remodeling.' *Journal of Clinical Endocrinology & Metabolism*, 69(3) pp. 523-527.

Stephens, P., Wall, I., Wilson, M., Hill, K., Davies, C., Hill, C., Harding, K. and Thomas, D. (2003) 'Anaerobic cocci populating the deep tissues of chronic wounds impair cellular wound healing responses in vitro.' *British Journal of Dermatology*, 148(3) pp. 456-466.

Stimson, W. (1988) 'Oestrogen and human T lymphocytes: presence of specific receptors in the T-suppressor/cytotoxic subset.' *Scandinavian Journal of Immunology*, 28(3) pp. 345-350.

Stojadinovic, O., Yin, N., Lehmann, J., Pastar, I., Kirsner, R. S. and Tomic-Canic, M. (2013) 'Increased number of Langerhans cells in the epidermis of diabetic foot ulcers correlates with healing outcome.' *Immunologic Research*, 57(1-3) pp. 222-228.

Stossi, F., Madak-Erdoğan, Z. and Katzenellenbogen, B. S. (2012) 'Macrophage-elicited loss of estrogen receptor-α in breast cancer cells via involvement of MAPK and c-Jun at the ESR1 genomic locus.' *Oncogene*, 31(14) p. 1825.

Stover, C. K., Pham, X. Q., Erwin, A., Mizoguchi, S., Warrener, P., Hickey, M., Brinkman, F., Hufnagle, W., Kowalik, D. and Lagrou, M. (2000) 'Complete genome sequence of Pseudomonas aeruginosa PAO1, an opportunistic pathogen.' *Nature*, 406(6799) pp. 959-964.

Strateva, T. and Yordanov, D. (2009) 'Pseudomonas aeruginosa–a phenomenon of bacterial resistance.' *Journal of Medical Microbiology*, 58(9) pp. 1133-1148.

Straub, R. H. (2007) 'The complex role of estrogens in inflammation.' *Endocrine Reviews*, 28(5) pp. 521-574.

Stryjewski, M. E. and Corey, G. R. (2014) 'Methicillin-resistant Staphylococcus aureus: an evolving pathogen.' *Clinical infectious diseases*, 58(suppl 1) pp. S10-S19.

Stumpf, W., Sar, M. and Joshi, S. (1974) 'Estrogen target cells in the skin.' *Experientia*, 30(2) pp. 196-198.

Suenaga, R., Mitamura, K., Evans, M. and Abdou, N. (1996) 'Binding affinity and quantity of estrogen receptor in peripheral blood monocytes of patients with systemic lupus erythematosus.' *Lupus*, 5(3) pp. 227-231.

Suenaga, R., Evans, M., Mitamura, K., Rider, V. and Abdou, N. (1998) 'Peripheral blood T cells and monocytes and B cell lines derived from patients with lupus express estrogen receptor transcripts similar to those of normal cells.' *The Journal of Rheumatology*, 25(7) pp. 1305-1312.

Sun, J., Meyers, M. J., Fink, B. E., Rajendran, R., Katzenellenbogen, J. A. and Katzenellenbogen, B. S. (1999) 'Novel ligands that function as selective estrogens or antiestrogens for estrogen receptor-α or estrogen receptor-β.' *Endocrinology*, 140(2) pp. 800-804.

Taylor, J. E., Laity, P. R., Hicks, J., Wong, S. S., Norris, K., Khunkamchoo, P., Johnson, A. F. and Cameron, R. E. (2005) 'Extent of iron pick-up in deforoxamine-coupled polyurethane materials for therapy of chronic wounds.' *Biomaterials*, 26(30) pp. 6024-6033.

Thammacharoen, S., Geary, N., Lutz, T. A., Ogawa, S. and Asarian, L. (2009) 'Divergent effects of estradiol and the estrogen receptor-α agonist PPT on eating

and activation of PVN CRH neurons in ovariectomized rats and mice.' *Brain research*, 1268 pp. 88-96.

Thomas. (2001) 'Age-related changes in wound healing.' *Drugs & Aging*, 18(8) pp. 607-620.

Thomas, Ko, Y. H. and Pedersen, P. L. (1992) 'Altered protein folding may be the molecular basis of most cases of cystic fibrosis.' *FEBS letters*, 312(1) pp. 7-9.

Thornton, M., Taylor, A. H., Mulligan, K., Al-Azzawi, F., Lyon, C. C., O'driscoll, J. and Messenger, A. G. (2003) The distribution of estrogen receptor β is distinct to that of estrogen receptor α and the androgen receptor in human skin and the pilosebaceous unit. *Journal of Investigative Dermatology Symposium Proceedings* 8(1), pp. 100-103.

Tokumaru, S., Higashiyama, S., Endo, T., Nakagawa, T., Miyagawa, J.-i., Yamamori, K., Hanakawa, Y., Ohmoto, H., Yoshino, K., Shirakata, Y., Matsuzawa, Y., Hashimoto, K. and Taniguchi, N. (2000) 'Ectodomain Shedding of Epidermal Growth Factor Receptor Ligands Is Required for Keratinocyte Migration in Cutaneous Wound Healing.' *The Journal of Cell Biology*, 151(2) pp. 209-220.

Toniolo, A., Tedesco, S., Bolego, C., Cignarella, A., Avogaro, A., Fadini, G. P., Cappellari, R., Maggi, A. and Vegeto, E. (2015) 'Alternative Activation of Human Macrophages Is Rescued by Estrogen Treatment *In vitro* and Impaired by Menopausal Status.' *The Journal of Clinical Endocrinology & Metabolism*, 100(1) pp. E50-E58.

Tosello-Trampont, A.-C., Nakada-Tsukui, K. and Ravichandran, K. S. (2003) 'Engulfment of apoptotic cells is negatively regulated by Rho-mediated signaling.' *Journal of Biological Chemistry*, 278(50) pp. 49911-49919.

Tou, J. C. (2015) 'Resveratrol supplementation affects bone acquisition and osteoporosis: pre-clinical evidence toward translational diet therapy.' *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, 1852(6) pp. 1186-1194.

Toutain, C. E., Brouchet, L., Raymond-Letron, I., Vicendo, P., Berges, H., Favre, J., Fouque, M.-J., Krust, A., Schmitt, A.-M. and Chambon, P. (2009) 'Prevention of skin flap necrosis by estradiol involves reperfusion of a protected vascular network.' *Circulation Research*, 104(2) pp. 245-254.

Tzircotis, G., Braga, V. M. and Caron, E. (2011) 'RhoG is required for both FcγR-and CR3-mediated phagocytosis.' *Journal of Cell Science*, 124(17) pp. 2897-2902.

Ulevitch, R. J. and Tobias, P. S. (1999) 'Recognition of gram-negative bacteria and endotoxin by the innate immune system.' *Current Opinion in Immunology*, 11(1) pp. 19-22.

Valdés-Mora, F., Gómez, d. P. T. and Lacal, J. C. (2009) 'CDC42 (cell division cycle 42 (GTP binding protein, 25kDa)).' *Atlas of Genetics and Cytogenetics in Oncology and Haematology*,

Van Aelst, L. and D'Souza-Schorey, C. (1997) 'Rho GTPases and signaling networks.' *Genes & Development*, 11(18) pp. 2295-2322.

Vannella, K. M. and Wynn, T. A. (2017) 'Mechanisms of organ injury and repair by macrophages.' *Annual Review of Physiology*, 79 pp. 593-617.

Varila, E., Rantala, I., Oikarinen, A., Risteli, J., Reunala, T., Oksanen, H. and Punnonen, R. (1995) 'The effect of topical oestradiol on skin collagen of postmenopausal women.' *British Journal of Obstetrics & Gynaecology*, 102(12) pp. 985-989.

Vaughan, M. B., Howard, E. W. and Tomasek, J. J. (2000) 'Transforming growth factor-β1 promotes the morphological and functional differentiation of the myofibroblast.' *Experimental Cell Research*, 257(1) pp. 180-189.

Vigorito, E., Billadeu, D. D., Savoy, D., McAdam, S., Doody, G., Fort, P. and Turner, M. (2003) 'RhoG regulates gene expression and the actin cytoskeleton in lymphocytes.' *Oncogene*, 22(3) p. 330.

Vincent, S., Jeanteur, P. and Fort, P. (1992) 'Growth-regulated expression of rhoG, a new member of the ras homolog gene family.' *Molecular and cellular biology*, 12(7) pp. 3138-3148.

Vural, P., Akgul, C. and Canbaz, M. (2006) 'Effects of hormone replacement therapy on plasma pro-inflammatory and anti-inflammatory cytokines and some bone turnover markers in postmenopausal women.' *Pharmacological Research*, 54(4) pp. 298-302.

Wakeling, A. E., Dukes, M. and Bowler, J. (1991) 'A potent specific pure antiestrogen with clinical potential.' *Cancer Research*, 51(15) pp. 3867-3873.

Waldbeser, L. S., Ajioka, R. S., Merz, A. J., Puaoi, D., Lin, L., Thomas, M. and So, M. (1994) 'The opaH locus of Neisseria gonorrhoeae MS11A is involved in epithelial cell invasion.' *Molecular Microbiology*, 13(5) pp. 919-928.

Walport, M. J. (2001) 'Complement.' *New England Journal of Medicine*, 344(14) pp. 1058-1066.

Webb, P., Nguyen, P., Valentine, C., Lopez, G. N., Kwok, G. R., McInerney, E., Katzenellenbogen, B. S., Enmark, E., Gustafsson, J.-A. k. and Nilsson, S. (1999) 'The estrogen receptor enhances AP-1 activity by two distinct mechanisms with different requirements for receptor transactivation functions.' *Molecular Endocrinology*, 13(10) pp. 1672-1685.

Wegner, A. (1976) 'Head to tail polymerization of actin.' *Journal of molecular biology*, 108(1) pp. 139-150.

Welshons, W. V., Wolf, M. F., Murphy, C. S. and Jordan, V. C. (1988) 'Estrogenic activity of phenol red.' *Molecular and Cellular Endocrinology*, 57(3) pp. 169-178.

Werner, S. and Grose, R. (2003) 'Regulation of wound healing by growth factors and cytokines.' *Physiological reviews*, 83(3) pp. 835-870.

Weusten, J., Blankenstein, M. A., Gmelig-Meyling, F., Schuurman, H., Kater, L. and Thijssen, J. (1986) 'Presence of oestrogen receptors in human blood mononuclear cells and thymocytes.' *European Journal of Endocrinology*, 112(3) pp. 409-414.

Weyrich, A. S. and Zimmerman, G. A. (2004) 'Platelets: signaling cells in the immune continuum.' *Trends in immunology*, 25(9) pp. 489-495.

Whiston, R., Hallett, M. B., Davies, E., Harding, K. and Lane, I. (1994) 'Inappropriate neutrophil activation in venous disease.' *British Journal of Surgery*, 81(5) pp. 695-698.

Whitchurch, C. B., Tolker-Nielsen, T., Ragas, P. C. and Mattick, J. S. (2002) 'Extracellular DNA required for bacterial biofilm formation.' *Science*, 295(5559) pp. 1487-1487.

White, J. V. and Ryjewski, C. (2005) 'Chronic venous insufficiency.' *Perspectives in Vascular Surgery and Endovascular Therapy*, 17(4), pp. 319-327.

Wichmann, M. W., Zellweger, R., DeMaso, C. M., Ayala, A. and Chaudry, I. H. (1996) 'Enhanced immune responses in females, as opposed to decreased responses in males following haemorrhagic shock and resuscitation.' *Cytokine*, 8(11) pp. 853-863.

Wilkinson, H. N. and Hardman, M. J. (2017) 'The role of estrogen in cutaneous ageing and repair.' *Maturitas*, 103 pp. 60-64.

Wira, C. R., Rodriguez-Garcia, M. and Patel, M. V. (2015) 'The role of sex hormones in immune protection of the female reproductive tract.' *Nature Reviews Immunology*, 15 (4), p. 217.

Wolcott, R. D., Rhoads, D. D. and Dowd, S. E. (2008) 'Biofilms and chronic wound inflammation.' Journal of Wound Care, 17(8) pp. 333-341.

Wynn. (2004) 'Fibrotic disease and the T H 1/T H 2 paradigm.' *Nature Reviews Immunology*, 4(8) p. 583.

Wynn, Chawla, A. and Pollard, J. W. (2013) 'Macrophage biology in development, homeostasis and disease.' *Nature*, 496(7446) p. 445.

Wysocki, A. B. and Grinnell, F. (1990) 'Fibronectin profiles in normal and chronic wound fluid.' *Laboratory Investigation*, 63(6) pp. 825-831.

Yamamoto, E. (1999) 'Studies on sex-manipulation and production of cloned populations in hirame, Paralichthys olivaceus (Temminck et Schlegel).' *Aquaculture*, 173(1) pp. 235-246.

Yen, J.-H., Yang, D.-J., Chen, M.-C., Yi-Ying, W., Hsieh, Y.-F., Cheng, Y.-M., Huang, W.-N., Szondy, Z. and Tsay, G. J. (2014) 'Daidzein enhances efferocytosis via transglutaminase 2 and augmentation of Rac1 activity.' *Molecular Immunology*, 60(2) pp. 135-142.

Yona, S., Kim, K.-W., Wolf, Y., Mildner, A., Varol, D., Breker, M., Strauss-Ayali, D., Viukov, S., Guilliams, M. and Misharin, A. (2013) 'Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis.' *Immunity*, 38(1) pp. 79-91.

Yoon, S. S., Hennigan, R. F., Hilliard, G. M., Ochsner, U. A., Parvatiyar, K., Kamani, M. C., Allen, H. L., DeKievit, T. R., Gardner, P. R. and Schwab, U. (2002) 'Pseudomonas aeruginosa anaerobic respiration in biofilms: relationships to cystic fibrosis pathogenesis.' *Developmental Cell*, 3(4) pp. 593-603.

Yotis, W. W. (1967) '*In vivo* and *in vitro* action of norethindrone on staphylococci.' *Journal of Bacteriology*, 94(5) pp. 1353-1358.

Zandman-Goddard, G., Peeva, E. and Shoenfeld, Y. (2007) 'Gender and autoimmunity.' *Autoimmunity Reviews*, 6(6) pp. 366-372.

Zeyda, M., Farmer, D., Todoric, J., Aszmann, O., Speiser, M., Györi, G., Zlabinger, G. and Stulnig, T. (2007) 'Human adipose tissue macrophages are of an anti-inflammatory phenotype but capable of excessive pro-inflammatory mediator production.' *International Journal of Obesity*, 31(9) pp. 1420-1428.

Zidan, J., Keidar, Z., Basher, W. and Israel, O. (2004) 'Effects of tamoxifen on bone mineral density and metabolism in postmenopausal women with early-stage breast cancer.' *Medical Oncology*, 21(2) pp. 117-121.

Zigmond, E. and Jung, S. (2013) 'Intestinal macrophages: well educated exceptions from the rule.' *Trends in Immunology*, 34(4) pp. 162-168.