Effect of Hormone-Driven Ageing on Inflammatory Cell Clearance of Bacteria under Hyperglycaemic Conditions

A thesis submitted in partial fulfilment of the requirements of the Manchester Metropolitan University for the degree of Doctor of Philosophy (PhD)

Amina Belboul

Department of Life Science

Faculty of Science and Engineering

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List of Abbreviations

Aβ	Amyloid β-peptide
Ab-free	Antibiotic-free
ATP	Adenosine tri phosphate
ApoE	Apolipoprotein E
BSA	Bovine serum albumin
CF	Cystic fibrosis
CFU	Colony-forming units
CD	Cluster of differentiation
CM	Complete medium
CR	Complement receptors
DAPI	4',6-diamidino-2-phenylindole
DFI	Diabetic Foot Infection
DFU	Diabetic Foot Ulcer
DHT	Dihydrotestosterone
DHEA	Dehydroepiandrosterone
DHEA-S	Dehydroepiandrosterone sulphate
DM	Diabetes Mellitus
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
ELISA	Enzyme-linked immunosorbent assay
EPS	Extracellular polymeric substance
ER	Estrogen receptor
ER-α	Estrogen receptor-alpha
ER-β	Estrogen receptor-beta
ERE	Estrogen response element
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
GCS-F	Granulocyte colony-stimulating factor
GFP	Green fluorescent protein
GPER	G Protein-coupled estrogen receptor

GM-CSF	Granulocyte-macrophage colony-stimulating factor
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HPBM	Human peripheral blood monocytes
HSCs	Hematopoietic stem cells
HRT	Hormone replacement therapy
IgG	Immunoglobulin G
IGF-1	Insulin-like growth factor-1
IKK	IκB Kinase
IFN-γ	Interferon gamma
IFN-β	Interferon-beta
IL-1β	Interleukin-1 beta
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-13	Interleukin-13
IL-17	Interleukin-17
iNOS	Inducible nitric oxide synthase
IRAK	Interleukin-1 receptor-associated kinase
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory Motif
KGF	Keratinocyte growth factor
K. pneumonia	*Klebsiella pneumoniae*
LBD	Ligand-biding domain
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
LR	Langerhans cells
M	Molar
mCD14	Membrane-bound CD14
mCD33	Membrane-bound CD33
MCS-F	Macrophage colony-stimulating factor
MCP-1	Macrophage chemoattractant protein 1
MDR	Macrophage and dendritic cells precursor
MIF	Migration inhibitory factor
MIP	Macrophage chemoattractant protein 1
MFI	Median fluorescence intensity
MMPs	Matrix metalloproteinases
mL	Millilitre
MOI	Multiplicity of Infection
MRSA	Methicillin-resistant *Staphylococcus aureus*
MRSA11	Methicillin-resistant *Staphylococcus aureus* strain 11
mTREM2	Membrane-bound TREM2
MyD88	Myeloid differentiation primary response 88
NA	Nutrient agar
NaCl	Sodium chloride

NC	Negative control
NET	Neutrophil extracellular trap
NF-κB	Nuclear transcription factor kappa B
Ng	Nanogram
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
PI3K	Phosphoinositide 3-Kinase
PKC	Protein kinase-C
PMA	Phorbol 12-myristate 13-acetate
PPT	Propyl pyrazole triol
PRRs	Pattern recognition receptors
PTK2	Protein tyrosine kinase 2
qPCR	Real-time polymerase chain reaction
ROI	Regions of interest
ROS	Reactive oxygen species
RPMI-1640	Roswell Park Memorial Institute 1640
Rpm	Revolutions per minute
RNAseq	RNA sequencing
RT	Room temperature
sCD14	Soluble CD14
sCD33	Soluble CD33
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
SERDs	Selective estrogen receptor degraders
SERMs	Selective estrogen receptor modulators
SSC	Side scattered light
sTREM2	Soluble triggering receptor expressed on myeloid cells 2
SYK	Spleen tyrosine kinase
TBS-tween	Tris-buffered saline tween
TGF β	Transforming growth factors β
THAM	Tris (hydroxymethyl) aminomethane hydrochloride
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]
TREM2	Triggering receptor expressed on myeloid cells 2

TRIF	TIR-Domain-containing adapter-inducing interferon-β
VEGF	Vascular endothelial growth factor
µg	Microgram
µm	Micrometre
µL	Microliters
°C	Degree Celsius

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Abstract

The diabetic foot ulcer (DFU) represents a substantial clinical burden, frequently complicated by infection and often necessitating amputation, particularly among elderly diabetic patients. The chronic hyperglycaemia associated with diabetes disrupts the wound healing cascade, impairing immune cell phagocytic function despite exacerbating inflammatory responses. The ageing process, driven principally through estrogen deprivation, also leads to impaired wound healing and elevated inflammation. Thus, similar underlying molecular mechanisms may mediate the detrimental effects of hyperglycaemia and hormone-driven ageing, and both factors may synergistically contribute to DFU pathology. To elucidate the effects of hyperglycaemia and hormone-driven ageing on immune cell clearance of bacteria, an *in vitro* host-pathogen model was established whereby bacterial biofilms were exposed to U937-derived macrophages cultured in increasing concentrations of glucose (11mM, 15mM, 20mM and 30mM) and treated in the presence or absence of estrogen ($1x10^{-7}$ M). Experiments conducted using the model included cell viability and growth assays, biofilm assays, phagocytosis assays, gene knockout and receptor agonist/antagonist assays, flow cytometry and/or immunoassays, and microscopy assessments of host-pathogen interactions and receptor/transcription factor localisation.

High glucose levels were found to promote bacterial proliferation and biofilm formation, compromise macrophage viability and phagocytic function, and elevate macrophage-derived inflammatory cytokine production. Notably, hyperglycaemia impaired macrophage differentiation but skewed macrophage polarization toward a proinflammatory M1 phenotype. Importantly, estrogen supplementation reversed the detrimental effects of hyperglycaemia on M1 macrophage-mediated phagocytosis and bacterial biofilm clearance in a dose-dependent manner. This phagocytic rescue was associated with suppression of tumour necrosis factor-alpha (TNF-α) secretion and nuclear transcription factor-kappa B (NF-κB) activation. Furthermore, estrogen supplementation modulated the expression of key phagocytic mediators in M1 macrophages cultured under hyperglycaemic conditions, with enhanced bacterial clearance associated with reduced CD14 but

increased TREM2/ApoE levels. Confocal and scanning electron microscopy confirmed estrogen stimulates the formation of actin cytoskeleton extensions, promotes bacterial internalization, and reduces NF-κB nuclear translocation in M1 macrophages. These findings suggest that reducing CD14 expression, NF-κB nuclear translocation and/or increasing TREM2/ApoE levels might be possible therapeutic approaches to reverse the detrimental effects of hyperglycaemia and estrogen deprivation. Moreover, the beneficial effects of estrogen were mediated through activation of estrogen receptor (ER)-alpha (ER-α) and not ER-beta (ER-β), highlighting the potential use of selective ER modulators (SERMs) in wound dressings to enhance macrophage function and dampen inflammation in DFU patients.

Further investigations employed a CD33 knockout (CD33KO) cell line (C4) within the model to elucidate the role of CD33, an immunoinhibitory receptor on monocytes/macrophages that is known to negatively regulate both phagocytic activity and inflammation. Interestingly, in line with observations in diabetic patients, hyperglycaemia reduced CD33 levels in CD33+ M1 macrophages but estrogen supplementation had no effect on CD33 levels. However, estrogen supplementation stimulated phagocytosis and reduced TNF-α in CD33KO-derived M1 macrophages cultured under hyperglycaemic conditions. Confocal microscopy, flow cytometry analyses and immunoassays showed estrogen and CD33KO have opposing effects, whereby estrogen increases and CD33KO inhibits TREM2 and ApoE levels in M1 macrophages. Moreover, estrogen reversed the detrimental effects of CD33KO on TREM2/ApoE expression and promoted the co-localization of membrane-bound CD33 (mCD33) and membrane-bound TREM2 (mTREM2) receptors on CD33+ M1 macrophages. These findings provide additional evidence that promoting the TREM2/ApoE axis with estrogen supplementation, ER-α activation and/or increasing local CD33 expression in wound macrophages may be a therapeutic strategy to restore macrophage function and reduce inflammation in diabetic/DFU patients.

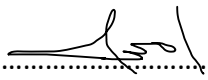
In summary, this thesis gives an insight into the combined detrimental impacts of hyperglycaemia and estrogen deprivation on CD14/NF-κB and CD33-TREM2/ApoE

axes. Results consistently indicate estrogen acts through ERα to mitigate the detrimental effects of hyperglycaemia on macrophage function by promoting TREM2/ApoE expression and blocking CD14/ NF-κB signalling to enhance the phagocytic capacity of M1 macrophages and dampen inflammatory cytokine production. Taken together, the investigations point to potential therapeutic interventions that can harness or emulate the beneficial effects of local estrogen supplementation to resolve diabetic foot infections and promote healing in DFU patients.

Declaration and Copyright Statements

Declaration

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or institute of learning.

Signed……  ……(candidate) Date…20/10/2024…….

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Publications

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Conferences

• Belboul, Amina, El Mohtadi, Mohamed, Vagg-Whitehead, Kathryn and Ashworth, Jason. Estrogen Enhances the Phagocytosis of Gram-positive and Gram-negative Bacteria by Macrophages under Hyperglycaemic Conditions". Poster Presentation at the 14th Microbial Pathogenesis and Host Response Conference hosted at the Cold Spring Harbor Laboratory in New York, USA (September 2023).

• Belboul, Amina, El Mohtadi, Mohamed and Ashworth, Jason. Poloxamer-mediated Control of Biofilms on Polycarbonate Surfaces. Oral Presentation at the SurfSAFE Biofilms Conference in Porto, Portugal (September 2023).

• Belboul, Amina, El Mohtadi, Mohamed, Vagg-Whitehead, Kathryn ORCID logo and Ashworth, Jason. Estrogen Promotes Macrophage-Mediated Clearance of Biofilms in an *in vitro* Model of Infected Diabetic Foot Ulcers". Poster Presentation at the 32nd European Congress of Clinical Microbiology & Infectious Diseases in Lisbon, Portugal (April 2022).

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Dedication

To my Almighty God who always gave me strength, to my husband Mohamed and to my precious daughter Yasmine, this thesis is for you...

Chapter 1: Introduction

1.1 Diabetes Mellitus

1.1.1 Overview of Diabetes

Diabetes mellitus (DM) is a common group of metabolic disorders characterised by the presence of high glucose concentration in the bloodstream. It is marked with a chronic and progressive hyperglycaemia, caused by either insulin deficiency or insulin resistance in cells that become unable to use the produced insulin (Chait and Bornfeldt, 2009; Hamal et al., 2022). There are two main types of DM: Type 1 and Type 2 diabetes. Type 1 diabetes, also known as insulin-dependent, represents around 10% of all cases of DM. It is characterised by autoimmune T cell-mediated destruction of pancreatic Beta-cells (β-cells) leading to a significant decrease or absence of insulin production, which results in high blood sugar levels (Ozougwu et al., 2013). Type 1 diabetes usually occurs during childhood or adolescence, but can develop at any age (Harjutsalo et al., 2008). The leading cause of type 1 diabetes is still unknown. However, both genetic and sedentary lifestyle factors are suggested to contribute to the development of the disease (Żebrowska et al., 2018).

Conversely, Type 2 diabetes involves the majority (90%) of DM cases, also known as non-insulin dependent diabetes. Although its prevalence among children and young adults has increased recently, type 2 diabetes typically occurs at a relatively advanced age (Bellary et al., 2021). Type 2 diabetes is characterised by a loss of insulin sensitivity, also called insulin resistance. The inability to maintain glucose homeostasis in type 2 diabetes is a result of either a failure to respond to insulin, or a defect in insulin production by pancreatic β-cells (Goldstein, 2002; Olokoba et al., 2012). To compensate for reduced insulin sensitivity, the β-cells undergo a compensatory mechanism of hypersecretion, producing and releasing increased amounts of insulin, which over an extended period, leads to a progressive decline in β-cell function and a reduced capacity for insulin secretion (Hudish et al., 2019). Consequently, the pancreas becomes unable to produce enough insulin, resulting in a relative insulin deficiency (Hudish et al., 2019; Sarkar et al., 2019). The insulin deficiency, coupled with the persistent insulin resistance, impair the body's ability to effectively regulate blood glucose levels resulting in chronic hyperglycaemia

(Chait and Bornfeldt, 2009). The dysregulation of glucose homeostasis is further exacerbated by the impaired suppression of hepatic glucose production and reduced glucose uptake by peripheral tissues, both of which are mediated by the actions of insulin. Several factors can lead to the development of type 2 DM. These include genetic predisposition, ageing, obesity, chronic stress, hypertension, and elevated cholesterol levels (Murea et al., 2012).

1.1.2 The Diabetes Epidemic: Prevalence and Impact

DM is one of the most common chronic diseases in humans. The incidence rates of DM cases have significantly increased in recent years. There were around 463 million estimated cases of DM in adults aged 20-79 worldwide in 2019, with projected numbers increasing to 700 million by 2045 (Karim et al., 2021; Magliano et al., 2021; World Health Organization (WHO), 2023). According to the World Health Organisation data (WHO) (2023), 9.3% of the global adult population live with diabetes, with incidence rates being higher in low and middle-income countries compared to developed countries (Shan et al., 2022). Reports indicate that more than 1.6 million deaths are directly attributed to diabetes each year (World Health Organization (WHO), 2023).

The prevalence of type 2 DM has been steadily increasing in recent years. This has been driven by several factors including obesity, physical inactivity, nutrition and previous family history (World Health Organization, 2009; Shaw et al., 2010; Olokoba et al., 2012). Research has shown a strong correlation between ageing and the prevalence of type 2 DM, with older people being more susceptible to developing the disease than young adults (Kirkman et al., 2012; Laiteerapong and Huang, 2018). Data from the US National Diabetes Statistics Report revealed that the age-adjusted prevalence of type 2 DM among adults aged 65 years or older was approximately 26.8% in 2018, compared to only 5.4% among those aged 18-44 (Sun et al., 2022). However, an increased prevalence of type 2 DM among younger populations has started to emerge in recent decades. This trend has been attributed to various lifestyle factors including unhealthy dietary patterns involving the excessive consumption of energy-dense unhealthy foods and beverages (Biddle

et al., 2015), increased rates of obesity and overweight (Hruby and Hu, 2015), widespread adoption of sedentary lifestyles with inadequate physical activity (Aune et al., 2016), and elevated levels of chronic stress (Novak et al., 2013). These lifestyle factors, often interrelated and influenced by environmental and socioeconomic factors, have been implicated in the development of insulin resistance and impaired glucose metabolism, thereby increasing the risk of type 2 DM at younger ages (Novak et al., 2013; Biddle et al., 2015; Hruby and Hu, 2015; Aune et al., 2016).

1.1.3 Complication of Diabetes

Type 2 DM is often associated with the development of several common serious health complications, which result in a deprived quality of life in humans, increased morbidity and mortality rates and elevated healthcare costs (Baena-Díez et al., 2016). The occurrence and incidence of complications vary among different populations and are influenced by a range of factors, including age, the chronicity of diabetes, glycaemic control, and the presence of other coexisting health conditions (Alzaheb and Altemani, 2018). Diabetic neuropathy is one of the most frequently occurring complications in patients with type 2 DM and affects up to 70% of the diabetic population (Amelia et al., 2019; Bodman and Varacallo, 2023). It can manifest as sensory neuropathy or autonomic neuropathy (Freeman, 2005; Koike et al., 2010). Sensory neuropathy frequently affects the lower limbs leading to symptoms such as loss of feeling, numbness, and tingling (Gomatos et al., 2024). Sensory nerve damage plays a significant role in the pathogenesis of diabetic foot wounds as it causes the patients to become unable to sense and be exposed to repeated traumas. As a result, these injuries can go unnoticed and untreated, which eventually leads to the development of ulcers (Koike et al., 2010). On the other hand, autonomic neuropathy results from nerve damage affecting the regulation of essential organ functions, such as gastric emptying, urinary bladder control, and the development of erectile dysfunction (Freeman, 2005). Diabetic retinopathy is another common complication of DM that can lead to a loss of visual acuity and, in severe cases, blindness when left untreated (Fong et al., 2004). Furthermore, the

outcome of chronic hyperglycaemia can also include long-term organ damage to the heart and kidneys when the blood vessels become damaged and ultimately lead (if left untreated) to heart/kidney failure (Sagoo and Gnudi, 2020).

DFUs and diabetic foot infections (DFIs), are frequently observed in patients with DM (Megallaa et al., 2019). These complications often arise due to diabetic neuropathy and poor blood circulation. If left untreated, DFUs can lead to serious infections and, in some cases, necessitate amputations of the lower extremities (Kumar et al., 2016). It is estimated that around 25% of diabetic patients develop foot ulcers during their lifetime (Singh et al., 2005), and approximately 85% of non-traumatic lower limb amputations result from DFUs making this severe complication a leading cause of hospitalisation among diabetic patients (Pecoraro et al., 1990). Therefore, the management of DFUs represents a challenging healthcare issue that requires the combination of efforts in research and clinical settings around the world (Falanga, 2005).

1.2 Wound Healing

1.2.1 The Normal Wound Healing Process

The wound healing process is a multifactorial dynamic mechanism, designed to repair the integrity and functionality of damaged tissue. This natural phenomenon involves several molecular and cellular events that can be divided into four major overlapping phases: haemostasis, inflammation, tissue proliferation and remodelling (Figure 1.1) (El Mohtadi *et al*., 2021).

In the event of an injury, circulating platelets rapidly migrate to the wounded area and adhere to the damaged blood vessels. Platelets play a pivotal role in initiating a haemostatic reaction, which enhances the coagulation cascade resulting in the formation of a fibrin clot (Jurk and Kehrel, 2023). This clot serves a dual purpose: stopping blood loss and providing provisional protection for the wound against foreign invaders, particularly microorganisms (Li et al., 2007). Following this initial phase, platelets within the clot and surrounding wound tissues release pro-inflammatory cytokines, chemokines and growth factors including transforming

growth factor (TGF-β), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and epidermal growth factor (EGF) (Bauer et al., 1985; Guo and DiPietro, 2010). These factors trigger the subsequent recruitment and migration of inflammatory cells such as neutrophils and macrophages, keratinocytes and fibroblasts to the wound site via a process known as chemotaxis (Singer and Clark, 1999; Guo and DiPietro, 2010).

Once bleeding is successfully controlled, neutrophils migrate to the wound site, initiating the inflammatory phase which is mainly characterised by the removal of foreign bodies, damaged matrix components, invading pathogens and dead (non-functional) cells, through the process of phagocytosis (Butterfield et al., 2006). Neutrophils play a key role in eliminating invading microorganisms including bacteria and fungi, by secreting lysosomal enzymes and reactive oxygen species (ROS), as well as destroying damaged matrix components via the release of proteinases and collagenases (Mosser and Edwards, 2010). Inflammation is also characterised by the release of growth factors such as PDGF and TGF-β which act as chemo-attractants, luring further inflammatory cells to the site, particularly macrophages (Beanes et al., 2003). As the inflammatory phase progresses, macrophages gradually replace neutrophils as the predominant inflammatory cells in the wound area, with most neutrophils being enclosed in the wound clot and are either eliminated with the eschar or by macrophages via phagocytosis, thereby paving the way for the resolution of inflammation (Newman et al., 1982).

During wound repair, macrophages undergo a phenotypic switch from pro-inflammatory (phagocytic) M1 macrophages to anti-inflammatory M2 macrophages. This transition is driven by the phagocytosis of apoptotic neutrophils that survive the inflammatory phase (Mosser and Edwards, 2008). The phenotypic change in macrophages is instrumental in stimulating tissue regeneration during the proliferative phase of acute wound healing via the release of growth factors such as insulin-like growth factor-1 (IGF-1), keratinocyte growth factor (KGF), epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF) by M2 macrophages (Meszaros et al., 2000; Mosser and Edwards, 2008). Another important function of M2 macrophages is to induce the formation of new blood

vessels, a process known as angiogenesis, through the production and secretion of Vascular endothelial growth factor (VEGF) that helps the growth, migration and proliferation of endothelial cells (Kim et al., 2019).

The proliferative phase typically begins between three to ten days post-injury as the immune response is accomplished. Proliferation encompasses three primary processes: a) re-epithelialisation which is characterised by the migration and proliferation of epidermal keratinocytes from adjacent intact skin to restore the epidermal barrier, b) the creation of a new extracellular matrix (ECM) by fibroblasts and c) revascularisation (also known as angiogenesis) of the wound area by endothelial cells (El Mohtadi, 2019).

Remodelling of a tissue scar is the final phase of wound healing. It is characterised by the remodelling of collagen and vascular maturation of newly produced capillaries, permitting vascular thickness to return to normal in the wound zone. As wound healing progresses, inflammatory cells, such as neutrophils and macrophages, are largely eliminated by apoptosis or phagocytosis (Serhan *et al*., 2008).

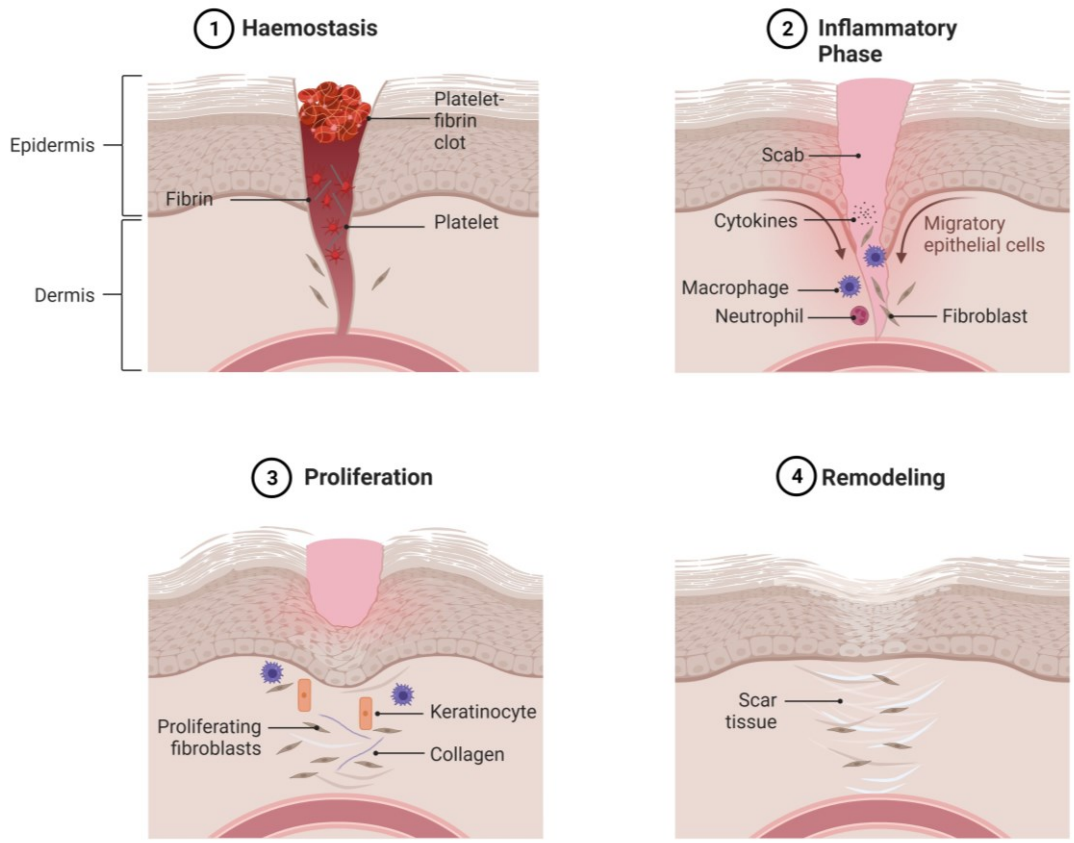

Figure 1.1 The Phases of Acute Wound Healing.
The wound healing process is divided into 4 phases: 1) Haemostasis; characterised by the formation of a fibrin clot by platelets. 2) Inflammation; characterised by the recruitment of phagocytes including neutrophils and monocytes from the bloodstream and the subsequent differentiation of monocytes to tissue macrophages at the wound site. 3) Proliferation; characterised by re-epithelialisation, ECM formation and angiogenesis. 4) Remodelling; characterised by the formation of a new mature tissue scar. Figure drawn by the author based on information in (Lisovsky et al., 2015).

1.2.2 Factors Affecting Wound Healing

For a successful wound healing process, wound repair requires successful progression through all four phases in their designated order and within the appropriate timeframe. Wound healing is a complex process that can be influenced by a variety of local and systemic factors, which play an important role in the process (Krzyszczyk et al., 2018). Local factors are those tied to the wound environment including tissue oxygenation, proximal trauma, pressure, necrosis, or infection (Regan et al., 1991). Conversely, systemic factors relate to the overall health or disease status of the patient including age, sex, diet, stress levels, medications, lifestyle habits (e.g. smoking, alcohol) and underlying pathologies such

as diabetes or cardiovascular diseases (Rajalakshmi et al., 2016). Therefore, it is crucial to meticulously identify and control the specific risk factors that are unique to each individual when attempting to treat impaired wounds. This facilitates implementing appropriate measures in promoting optimal healing outcomes.

1.2.2.1 Age-Related Impaired Wounds

Ageing is a key risk factor for the development of impaired wounds. Numerous human and animal studies have assessed the negative impact of age on wound healing processes (Holt et al., 1992; Ashcroft et al., 2002; Harding et al., 2002b). Ageing is defined by Imahori (1992) as the "regression of physiological functions with increasing age". Ageing is frequently associated with a temporary delay in wound healing, rather than an actual impairment in the quality of healing (Gosain and DiPietro, 2004; Keylock et al., 2008). This delay in wound healing among the elderly is associated with reduced immune, circulatory, and respiratory system function, and an altered hormonal profile (Holt et al., 1992; Gosain and DiPietro, 2004). Several research studies have shown that impaired wound healing in the elderly is associated with a delayed haemostasis, an excessive inflammatory phase marked by altered immune responses (Eming et al., 2007), reduced phagocytic functions in innate immune cells (Koh and DiPietro, 2011), altered expression of Toll-like receptors (TLRs) in phagocytes (Van Duin and Shaw, 2007), delayed re-epithelialisation (Landén et al., 2016), defective angiogenesis and reduced matrix deposition (Ashcroft et al., 1999a; Swift et al., 2001; El Mohtadi et al., 2021). Thus, age-related delayed wounds are commonly associated with elevated risks of developing wound infections in the elderly (Thomas, 2001).

1.2.2.2 Impaired Wound Healing in Diabetes

It is well-accepted that diabetes disrupts the wound healing process (Baltzis et al., 2014; Burgess et al., 2021). The wound healing process in diabetic patients is mainly marked with a delayed and persistent inflammatory phase (Burgess et al., 2021). Hyperglycaemia can lead to the development of chronic wounds which are defined as wounds that fail to heal through the orderly set of wound healing phases within a normal timeframe (Nunan et al., 2014). Wounds are considered chronic if they

remain non-healed for a minimum of 6 weeks or even months/years in some severe cases (Adeyi *et al.,* 2009). Diabetic wounds are frequently observed in the elderly population, with a higher prevalence in people aged over 60 years.

Chronic wounds, particularly those observed in diabetic patients, have a major physical, psychological, and social burden on people's life, and a substantial financial burden on healthcare services. Treatment of DFUs is estimated to cost the UK National Health Service (NHS) around £17 million per year (Harding et al., 2002b; 2002a; Boulton et al., 2005). At a cellular and molecular level, Hyperglycaemia induces alterations that significantly impair the wound healing process, leading to the formation of chronic wounds. Additionally, chronic wounds exhibit marked defective macrophage functions that lead to an increased propensity for bacterial infection and impaired resolution of inflammation (Mohsin et al., 2024).

1.3 Diabetic Foot Ulcers

DFUs are one of the most severe complications of DM. Reports in the UK indicate that over 15 % of patients with diabetes are likely to develop chronic DFUs (Guo and DiPietro, 2010), amongst which 85 % will need amputation of their lower extremities due to gangrene development (Blakytny and Jude, 2006). The elevated blood sugar concentration in diabetes can provoke nerve damage, leading to vasculopathy (microcirculation dysfunction) and autonomic neuropathy (Leung, 2007; Tellechea et al., 2010). This can induce hypoxia and local hypertension, resulting in tissue necrosis and ulcer development. DFUs (Figure 1.2) typically develop following application of repetitive mechanical pressure on the feet and ankle regions of patients with diabetic neuropathy, which is partly due to their incapability to sense pain and cutaneous pressure (Boulton et al., 2004). Diabetic patients with neuropathy are commonly unaware of the initial trauma which leads to further tissue damage at the wound site (Bonham, 2003). Similar to all types of chronic wounds, DFUs are associated with reduced immune functions in phagocytes, such as neutrophils and macrophages, which in turn increase the risk of developing infection and prolong the healing time (Falanga, 2005; Graves and Dasu,

2011). If DFUs are left untreated, they could potentially become infected with microbes (e.g. bacteria) which results in tissue repair failure and aggravates skin ulceration (Cavanagh *et al.,* 2005).

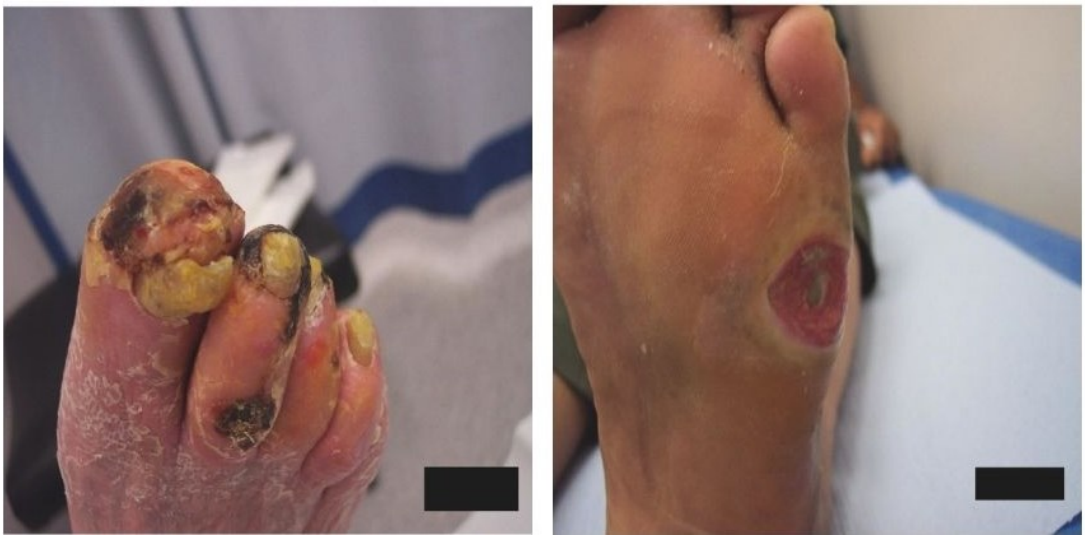

Figure 1.2 Patients with Severe Infected Diabetic Foot Ulcers (DFUs).
Images adapted from (Goyal et al., 2020).

Under healthy non-diabetic conditions, immune cells such as neutrophils and macrophages are recruited to the site of injury to quickly eliminate invading microorganisms (Daryabor et al., 2020). When chronic wounds develop, the excessive inflammatory response is marked by the prolonged presence of inflammatory cells (particularly macrophages) and continuous release of pro-inflammatory mediators/cytokines, e.g., Interleukin-1 beta (IL-1β), TNF-α, Macrophage Inflammatory Protein 1 (MIP1) and Macrophage Inflammatory Protein 2 (MIP2) which delay wound repair further (Sahay et al., 2009; Anas et al., 2010a; Fernández-Real et al., 2011b). Such cellular changes predispose diabetic patients to an increased risk of developing wound infections.

In diabetic wounds, macrophages fail to switch from a pro-inflammatory phenotype to an anti-inflammatory state, leading to an increase in the secretion of pro-inflammatory cytokines such as interleukin-6 (IL-6) and TNF-α and a decrease in the release of anti-inflammatory cytokines such as IL-10, Transforming Growth Factor-beta (TGF-β), IL-4 and IL-13 (Falanga, 2005). This significant change suggests that hyperglycaemic conditions prevent the phenotypic changes in macrophages, which maintain a pro-inflammatory phenotype that exacerbates inflammation and

contributes to the delay in wound repair and potential development of infection (Figure 1.3).

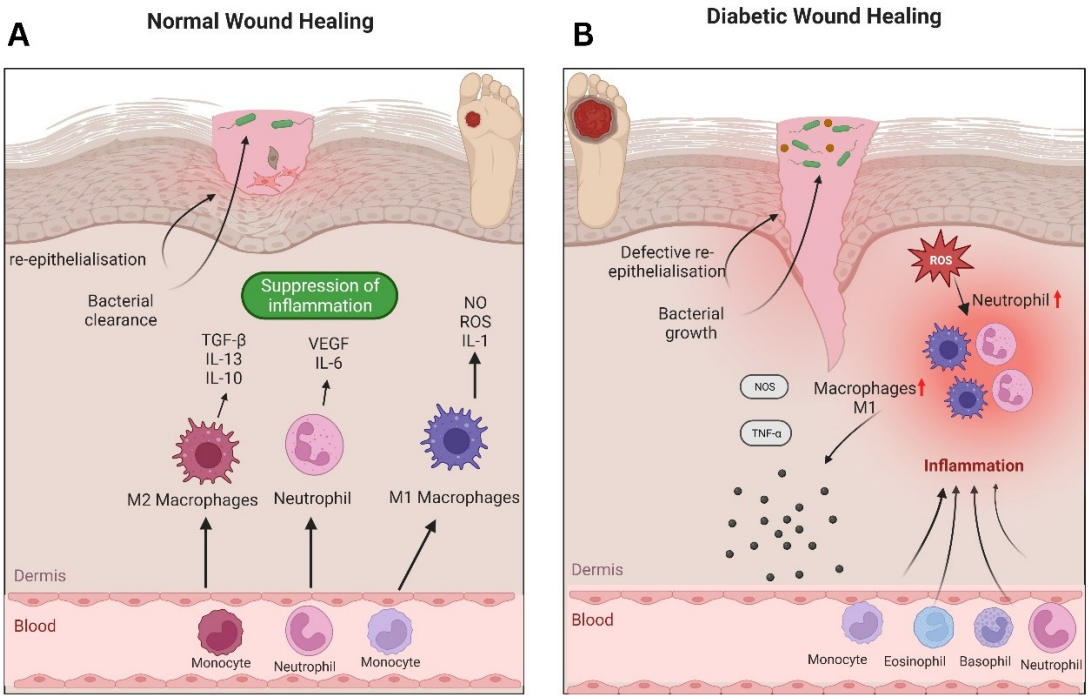

Figure 1.3 The Cellular Changes Observed in Diabetic Wounds Compared to Normal Wounds.

Diabetic wounds (B) are characterised by a prolonged inflammatory response, marked with an increased presence of inflammatory cells and elevated secretion of pro-inflammatory cytokines. This persistent inflammation disrupts the normal wound healing process (A) resulting in defective re-epithelisation and reduced angiogenesis. Diabetic wounds are also associated with unpaired phagocytic responses leading to higher bacterial loads within the wound site. Figure drawn by the author based on information in (Raziyeva et al., 2021).

1.3.1 Infection in DFUs

It is well documented that low bacterial numbers in wounds could have a beneficial impact on wound repair through moderate stimulation of inflammatory responses (Weiss and Schaible, 2015). However, microbial colonisation in chronic wounds such as DFUs, that take several weeks or months to heal, is detrimental to the healing process (White and Cutting, 2008; Sachdeva et al., 2022). It has been reported that chronic wounds are often colonised by a wide range of microbial species including bacteria (Bowler and Davies, 1999). When the bacterial colonisation is not reduced effectively by the host immune system, the result is infection (Friedman and Su, 1984). Interestingly, research indicates that the environment of diabetic wounds favours the growth of bacteria, which leads to

bacterial infections, especially when the wound site is dry and ischaemic (Rubinstein et al., 1983; Whiston et al., 1994; Hasan et al., 2015).

1.3.2 Common Bacterial Species in DFUs

The aetiology of DFUs usually involves trauma and is often associated with peripheral neuropathy and ischemia (Bowler and Davies, 1999). These ulcers can frequently become infected leading to severe life-threatening complications such as osteomyelitis and sepsis which may ultimately result in amputation in some cases (Leung, 2007). The management and treatment of these infections can be clinically challenging, particularly with the increased risk of developing antimicrobial resistance due to repeated antibiotic exposure (Cavanagh et al., 2005). Therefore, a good understanding of the bacterial wound population is crucial for the development of new therapeutic approaches that rely less on antibiotics.

The microbiology of DFU infections has been globally studied. Research indicates that *Staphylococcus aureus* is the main pathogen isolated from DFIs in most studies, along with *Pseudomonas aeruginosa*, *Streptococcus* spp*, Escherichia coli, Staphylococcus epidermidis* and *Enterococcus* spp (Rybtke et al., 2015; Dunyach-Remy et al., 2016; Du et al., 2022). This aligns with the findings of a recent meta-analysis study where a total of 112 studies were analysed with a total of 16,159 patients, from which 22,198 microbial isolates were obtained (Table 1.1). Another clinical study conducted on 433 diabetic patients with DFIs in the United States indicated that several bacterial species were present: *S. aureus* was the most common isolate, being recovered from 38.4% of cases, methicillin-resistant *S. aureus* (MRSA) was found in 20% of wounds*,* followed by *P. aeruginosa, Streptococcus* spp*, Enterococcus* spp *and Corynebacterium* spp (Citron et al., 2007). In summary, the most predominant gram-positive aerobes isolated from infected DFUs are *Staphylococcus* spp, while the most predominant gram-negative aerobes are *Pseudomonas* spp.

Table 1-1 Bacterial Species Commonly Associated with DFUs
The distribution of bacterial species detected in 8418 microbial isolates from 6736 clinical samples across 57 studies (Macdonald et al., 2021).

Isolates	Frequency (%)
S. aureus (inc. MRSA)	1952 (23.18)
Pseudomonas spp	1008 (11.97)
E. coli	1002 (11.90)
Proteus	801 (9.51)
Klebsiella	666 (7.91)
Enterococcus	633 (7.52)
Staphylococcus (coagulase negative)	585 (6.95)
Streptococcus	491 (5.83)
Enterobacter	251 (2.98)
Acinetobacter	208 (2.47)
Other	821 (9.75)

It is commonly accepted that patients with type 2 diabetes are more susceptible to infection (Hine et al., 2017; Mor et al., 2017). Numerous studies have demonstrated that hyperglycaemia in diabetic patients can cause defects in immune responses, including dysfunction of neutrophils and macrophages, which increases the prevalence of microbial infections and related comorbidities (Bessman and Sapico, 1992; Joshi et al., 1999; Lecube et al., 2011; Hodgson et al., 2015; Nagendra et al., 2022). Mechanisms that impair host defence mechanisms against microbial invasion can be linked with the suppression of cytokine production by inflammatory cells (Geerlings and Hoepelman, 1999; Peleg *et al.*, 2007), or significant impairment of the phagocytosis process (Joshi et al., 1999; Peleg et al., 2007; Vardakas et al., 2007). Furthermore, a high glucose environment can increase the virulence of certain pathogens (Casqueiro *et al.*, 2012).

1.3.2.1 *Staphylococcus aureus* in DFUs

S. aureus is a gram-positive coccus bacterium. It is a commensal organism that resides in the skin, mucosa, nose, respiratory tract and intestines of around 50% of people, who are normally identified as healthy carriers, of which up to 20% maintain chronic colonisation without causing infection (Yang et al., 2018; Howden et al., 2023). However, *S. aureus* is known to be an opportunistic nosocomial pathogen that is heavily linked with hospital-acquired wound infections, particularly in patients with DFUs (Parlet et al., 2019; Howden et al., 2023).

S. aureus has the ability to develop resistance to a wide range of antibiotics, including penicillin and methicillin. MRSA is one of the most aggressive antibiotic-resistant strains that has widely emerged in recent decades. The danger of MRSA comes from its capacity to evade the immune system and gain access to the blood circulation, which could result in the development of infection, leading to severe complications such as sepsis, meningitis and endocarditis (Stryjewski and Corey, 2014).

MRSA is commonly associated with chronic wound infections, particularly DFUs. Several studies have reported high prevalence rates of MRSA among *S. aureus* isolates from chronic wounds. In Nepal, 60.6% of *S. aureus* isolates from 182 wound specimens were MRSA strains (Upreti et al., 2018). This high prevalence is mirrored in a retrospective study conducted in China, where 53.4% of wounds in 815 patients were colonised by MRSA (Guan et al., 2021). The severity of the issue is further highlighted by a hospital-based study in Texas, USA, which reported MRSA isolation from 63% of patients with skin and soft-tissue infections (Forcade et al., 2011). In addition, the first two vancomycin-resistant MRSA strains were found in patients with DFUs (Chang *et al.*, 2003). On a broader scale, a study across 11 major USA cities indicated that MRSA was responsible for more than 50% of skin and soft-tissue infections presented to emergency rooms (Moran *et al.,* 2006). Even in specialised settings like the Manchester Foot Hospital, MRSA represented 30.2% of *S. aureus* cases isolated from infected DFUs (Dang et al., 2003). Therefore, MRSA wound infection has emerged as a serious healthcare issue that could lead to life-threatening complications, which necessitate urgent attention from healthcare

researchers and clinicians worldwide (Krishna and Miller, 2012). Another significant virulence factor contributing to the pathogenesis of MRSA in chronic wound infections is its ability to form biofilms (Cascioferro et al., 2021). MRSA strains are particularly proficient at producing strong biofilms, which can stay on wound surfaces and medical equipment, resulting in persistent and resistant infections.

1.3.2.2 *Pseudomonas aeruginosa* in DFUs

P. aeruginosa is a common, Gram-negative rod-shaped bacterium. It is a nosocomial pathogen known for its advanced antibiotic resistance mechanisms (Gill et al., 2016). *P. aeruginosa* is commonly found in wounds and is widely associated with major hospital-acquired infections. Amongst all the Gram-negative bacterial species, *P. aeruginosa* is the most frequently isolated bacterium from DFUs (Bowler and Davies, 1999; Sharma et al., 2006; Alavi et al., 2007; Bansal et al., 2008; Rybtke et al., 2015). Infection with *P. aeruginosa* can cause serious health problems in immunocompromised elderly people, particularly those who suffer from chronic conditions such as diabetes, cystic fibrosis and burns (Hachem *et al.,* 2007).

P. aeruginosa is capable of forming biofilms, which are surface-attached bacterial communities, as part of its survival strategy in various environments (Yoon *et al.,* 2002). The formation of these biofilm communities allows *P. aeruginosa* colonies to clump together and develop complex structures that confer enhanced resistance against antibiotics and host immune responses, contributing to the persistence and resistance of infections (Mah *et al.,* 2003). Similar to infections with MRSA, *P. aeruginosa* infections can lead to severe complications and represent a serious threat to elderly immunocompromised people.

Chronic infection with nosocomial bacteria (e.g. *P. aeruginosa* and MRSA) is strongly associated with biofilm formation (Høiby, 2014). In addition, chronic wounds such as DFUs are commonly invaded by biofilms of several bacterial communities including *S. aureus* and *P. aeruginosa* (Clinton and Carter, 2015). Biofilms have been shown to affect 60% of chronic wounds and 10% of acute wounds, which suggests that biofilms have a significant impact on the development and/or aggravation of chronic wounds including DFUs (James *et al*., 2008). When

established, wound biofilms become more tenacious, possessing a robust extracellular matrix that allows them to develop additional resistance mechanisms to evade the host immune responses and traditional antibiotic therapies, which makes them extremely difficult to eradicate (Clinton and Carter, 2015).

1.3.2.3 Biofilm Formation in DFUs

Although bacteria have traditionally been studied in their free-floating, planktonic state, there is now widespread recognition that biofilm formation represents a more prevalent and alternative mode of microbial growth and survival (Edwards and Harding, 2004; Mottola et al., 2016; Pouget et al., 2020). Biofilms have been implicated in many recurrent clinical infections including DFUs, urinary tract infections, cystic fibrosis, otitis, dental plaque, and periodontal disease (Bjarnsholt, 2013).

Due to their heterogeneous nature, there is no single definition that describes all biofilms. Overall, they can be defined as a structured and persistent two-dimensional community of microbes embedded in an extracellular polymeric substance (EPS), forming multicellular communities (Vasudevan, 2014). These EPS contain proteins, lipids, DNA from lysed microorganisms and polysaccharides which provide a physical protection to the microbes within the biofilm structure and allow them to resist host immune defences and different treatments. This results in the development of chronic and persistent infections (Bjarnsholt, 2013; Kostakioti et al., 2013).

A research study carried out by James et al. in 2008, which involved the examination of wound tissue biopsies using electron microscopy, suggested that 60% of chronic wounds exhibited bacterial biofilms. Furthermore, the nature of necrotic tissue and cell debris found in DFUs facilitate bacterial adhesion and biofilm formation (Zhao et al., 2013). Therefore, the role of biofilm formation in the chronicity of DFUs is an important area of ongoing research (James et al., 2008; Jamal et al., 2018).

1.3.2.4 Common Biofilm-Producing Bacteria in DFUs

Research indicates that DFUs are often characterised by the presence of bacterial biofilms. These biofilms involve both gram-positive and gram-negative bacterial species, with *P. aeruginosa* and *S. aureus* being among the most commonly associated bacteria with biofilm formation in DFUs (Bowler et al., 2012; Devasia et al., 2022; Mamdoh et al., 2023; Xu et al., 2023). Despite the prevalence of these bacteria in DFUs, relatively few studies have investigated the specific microbial profiles and mechanisms underlying biofilm formation in DFUs. Understanding these mechanisms, such as how bacteria adhere to surfaces, produce extracellular matrices, and evade immune responses can lead to the identification of potential targets for immunomodulatory or anti-inflammatory therapies that can enhance the body's ability to combat DFIs.

Recent research has shed the light on some of these mechanisms. For instance, Xie et al. (2020) found that *S. aureus* can form robust biofilms under the influence of advanced glycation end products (AGEs). AGEs facilitate bacterial adhesion by modifying the surface properties of both the bacteria and host tissues, promoting stronger attachment and biofilm formation in different environments. Additionally, AGEs increase the release of extracellular DNA (eDNA) by *S. aureus*, which serves as a structural component within the biofilm matrix, enhancing its stability (Xie et al., 2020). In a study by Mamdoh et al. (2023), phenotypic detection methods revealed that all *Staphylococci* strains isolated from DFUs were capable of forming biofilms. Furthermore, molecular detection of biofilm-encoding genes identified key proteins involved in this process, including those associated with biofilm polysaccharide intercellular adhesin synthesis and cell surface proteins facilitating adhesion and accumulation within biofilm structures (Mamdoh et al., 2023). Similarly, Yakout and Abdelwahab (2022) used molecular detection methods to investigate biofilm formation in *P. aeruginosa* isolates from DFUs and found that 93% of the isolates were classified as biofilm-producing bacteria (Yakout and Abdelwahab, 2022). This suggests that hyperglycaemia not only impairs the healing process but also aggravates the biofilm-related challenges in wound healing (G. Zhao et al., 2012; Nguyen and Oglesby-Sherrouse, 2016).

1.3.2.5 Current Therapeutic Options for DFUs

Numerous therapeutic approaches exist for the treatment of DFUs such as wound dressings, antibiotics, tissue engineering, growth factors, stem cells, gene therapy, and topical oxygen therapy. However, even with optimal management, only 50% to 60 % of therapeutic can heal DFUs (Sen et al., 2009; Hingorani et al., 2016). There is still a significant room for more research and exploration of novel treatment options that could potentially heal DFUs.

Rapid diagnosis is crucial for clinicians to promptly plan an effective wound treatment strategy. In the case of DFUs, the removal of infected or necrotic tissue through debridement is imperative to facilitate the overall healing process and to reduce bacterial infection. Without debridement, wounds often fail to heal properly (Roghmann et al., 2001; McGuckin et al., 2003). Subsequently, the wound can be treated using different advanced topical dressings such as hydrogels and hydrocolloids to prevent further infection and promote healing (Shi et al., 2020).

While antibiotics may be administered, their usage requires careful consideration of the potential antibiotic resistance risks (Martinez et al., 2008). As a result, the use of topical antiseptics is increasingly established to prevent infection or inhibit the growth of both planktonic and biofilm-related bacteria. This trend is attributed to their nonspecific bactericidal mechanisms of action. Unlike antibiotics, bacteria are unlikely to develop resistance to antiseptics (Alves et al., 2021).

Growth factors are one of the first advanced therapeutic approaches used for the treatment of DFUs with platelet-derived growth factor (PDGF) being the first growth factor approved by the FDA to treat DFUs (Wieman, 1998; Falanga, 2005). While the introduction of PDGF marked a significant milestone, current evidence suggests that there are limitations to the topical application of PDGF for DFUs (Yamakawa and Hayashida, 2019). Ongoing research aims to further refine and improve the efficacy of growth factor-based therapies in the treatment of DFUs.

Around the same time as growth factors were gaining interest, tissue engineering emerged as another promising therapeutic option to treat DFUs (MacNeil, 2007; Yamada et al., 2008; Harding et al., 2013; Kirsner and Vivas, 2015). Although, skin

replacement can accelerate the wound healing process up to 20% compared to other standard treatments, its application faces challenges related to: maintaining long-term stability within the wound, increased cost, and lack of availability to all patient populations in need (Bishop et al., 2017; Hassanzadeh et al., 2018).

Despite the current advanced treatments and technologies used to treat DFUs, there is still an urgent need to develop innovative alternative therapeutic options that can heal DFUs with less reliance on antibiotics and invasive technologies. This particularly due to the complex nature of necrotic tissue and cell debris found in DFUs, and their susceptibility for the development of bacterial infection and biofilm formation (Zhao et al., 2013).

1.4 Host Defence Mechanisms

Research investigating the host-pathogen interactions in DFUs has significantly expanded in recent years. Understanding the cellular and molecular processes involved in the cell-mediated innate immune responses against microbial invasion in chronic wounds (i.e. DFUs) is vital to the global public health services.

1.4.1 The Innate Immune System

The innate immune system, while non-specific compared to the adaptive immune system, plays a pivotal role in pathogenesis of chronic wounds (Akira et al., 2006).
Cells of the innate immune system, including monocytes, macrophages and neutrophils provide constant control and coordination of immune responses to pathogens and damaged cell. Innate immune cells constitute the primary line of defence against bacterial infections and facilitate adaptive immune responses through antigen presentation. Their role is tightly regulated by a complex network involving signalling molecules, receptors, and effector mechanisms (Spirig et al., 2012).

Cytokines such as TNF-α, IFN-γ, IL-1, IL-6 and IL-8 stimulate and amplify the inflammatory response, immune cell recruitment, phagocytosis, and modulate the wound microenvironment (Barrientos et al., 2014). Pattern recognition receptors

(PRRs) such as TLRs are critical for innate immune activation. They are a class of proteins, expressed on innate immune cells, that recognise specific pathogen-associated molecular patterns (PAMPs) like lipopolysaccharide (LPS). PRRs recognition triggers various signalling cascades that leads to NF-κB activation and secretion of pro-inflammatory cytokines that contribute to the initiation and modulation of immune responses during inflammation (Gordon, 2002; Spirig et al., 2012).

The combination of different signalling pathways and receptor-ligand interactions leads to the activation of robust mechanisms within the innate immune system, resulting in the clearance of bacterial colonisation and/or infection. These mechanisms include the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), and stimulation of phagocytosis (Gordon, 2002; MacLeod and Mansbridge, 2016).

In summary, a comprehensive understanding of the complex relationship between the innate immune system and its receptors in DFUs is imperative to identify potential therapeutic targets that modulate inflammation and restore normal wound repair processes.

1.4.2 Pattern Recognition Receptors

Immediately after injury, neutrophils and subsequently monocytes/macrophages are systemically recruited to the site of damage to protect the human body against microbial invasion via a process called phagocytosis (Minutti *et al.,* 2017). This recruitment process is prompted by the recognition of PAMPs by PRRs expressed on the cell surface of innate immune cells (Wada and Makino, 2016).

Following the recognition and binding of PAMPs to PRRs, a series of biochemical signalling pathways is initiated leading to phagocytosis, antigen presentation and secretion of various pro-inflammatory cytokines including IFN-γ, TNF-α, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-1β, IL-6 and IL-8. These cytokines help regulate the recruitment of neutrophils and macrophages as well as regulating their phagocytic functions

(Takeuchi and Akira, 2002; Rhoads et al., 2008). As part of the initial immune response, pathogens are labelled with complement proteins, making them easy to identify by neutrophils and macrophages, which can then bind, engulf and destroy them via phagocytosis (Medzhitov and Janeway Jr, 1997).

There are four subfamilies of PRRs: a) TLRs, b) Nucleotide-binding oligomerisation domain like receptors (NLRs), c) Retinoic acid-inducible gene 1-like receptors (RIG-1), and d) C-type lectin receptors (CLRs) (Walsh *et al.*, 2013). TLRs are the most extensively studied class of PRRs. They are integral glycoproteins found in most innate immune cells at different anatomical tissue locations (Akira *et al*., 2006). TLRs play an important role in host defence mechanisms through the recognition of microbial membrane components such as lipids, lipoproteins, and proteins. For example, TLR4 identifies the Gram-negative bacterial LPS. TLR2 along with TLR1 and TLR6 recognise a wide variety of Gram-positive PAMPs including lipoproteins, peptidoglycans and lipoteichoic acids (Mogensen, 2009).

Research studies using diabetic mouse models have shown that diabetic mice exhibit an upregulated expression of TLR2 and TLR4 at both the mRNA and protein levels in comparison to non-diabetic models (Dasu et al., 2010a; Chen et al., 2013). This was asscociated with increased levels of downstream signaling proteins such as MyD88, IRAK and NF-κB, indicating increased TLR activation (Aghamiri et al., 2022). Diabetic mice were also shown to display higher levels of inflammatory cytokines such as TNF-α and IL-6 compared to healthy mice. The reported TLR2/4-driven excessive inflammatory state was shown to impair diabetic wound healing in the studied mice models (Dasu et al., 2010a; Chen et al., 2013). However, TLR2/4 knockout was shown to reduce inflammatory signaling and was linked with improved healing in diabetic TLR2/4KO mice (Dasu et al., 2010b; Dasu and Jialal, 2013).

1.4.3 The Role of CD14 in Phagocytosis and Inflammation

Cluster of Differentiation 14 (CD14) is one of the first PRRs to be identified in human cells (Pugin et al., 1994). It is primarily expressed on myeloid cells such as

monocytes, macrophages and dendritic cells (Pugin et al., 1994). Recent studies exploring CD14 biology have further confirmed its function as a key PRR involved in initiating innate immune responses through recognition of PAMPs (Zanoni et al., 2009; Zanoni et al., 2011). CD14 is involved in the detection of various components from gram-negative and gram-positive bacteria, as well as mycobacteria. While the precise mechanisms involved in the CD14-PAMPs binding remain unclear, CD14 is known to be implicated in triggering immune responses to pathogens (Jiang et al., 2005; Zanoni and Granucci, 2013). CD14 exists in two forms, a membrane-bound glycosylphosphatidylinositol (GPI)-anchored protein (mCD14) and a soluble protein (sCD14) found in serum. The sCD14 is normally generated through cleavage of mCD14 (Figure 1.4) (Bufler et al., 1995; Ciesielska et al., 2022).

Due to the absence of a cytosolic signalling domain, it was initially considered that CD14's main function was to transfer LPS to the TLR4 protein (Pugin et al., 1994). Subsequent investigations provided additional insights into these findings revealing the precise interactions between CD14 and TLR4 and the molecular mechanisms promoting LPS downstream signalling cascades. Using a mouse model, Poltorak et al. (1998) confirmed that TLR4 mutation leads to impaired LPS signalling. Jiang and colleagues also provided more experimental evidence showing that LPS-induced activation of the TLR4-dependent signalling pathway in macrophages only occurs in the presence of CD14 (Jiang et al., 2005).

The role of CD14 in diabetic wound healing remains largely undefined, despite progress in understanding its functions as a co-receptor for TLRs and PRRs (Zanoni et al., 2009; Baumann et al., 2010; Zanoni et al., 2011). Considering its immunomodulatory functions in detecting pathogens and triggering inflammatory responses, studying the expression of both mCD14 and sCD14 in the context of hyperglycaemia and chronic inflammation may reveal mechanisms impacting healing outcomes in patients with DFUs. Dysregulated CD14 signalling may contribute to impaired pathogen clearance and prolonged inflammation in DFUs. Therefore, a better understanding of CD14 immunobiology in the diabetic wound microenvironment may uncover novel therapeutic approaches to combat immune dysfunction and infection susceptibility in DFUs.

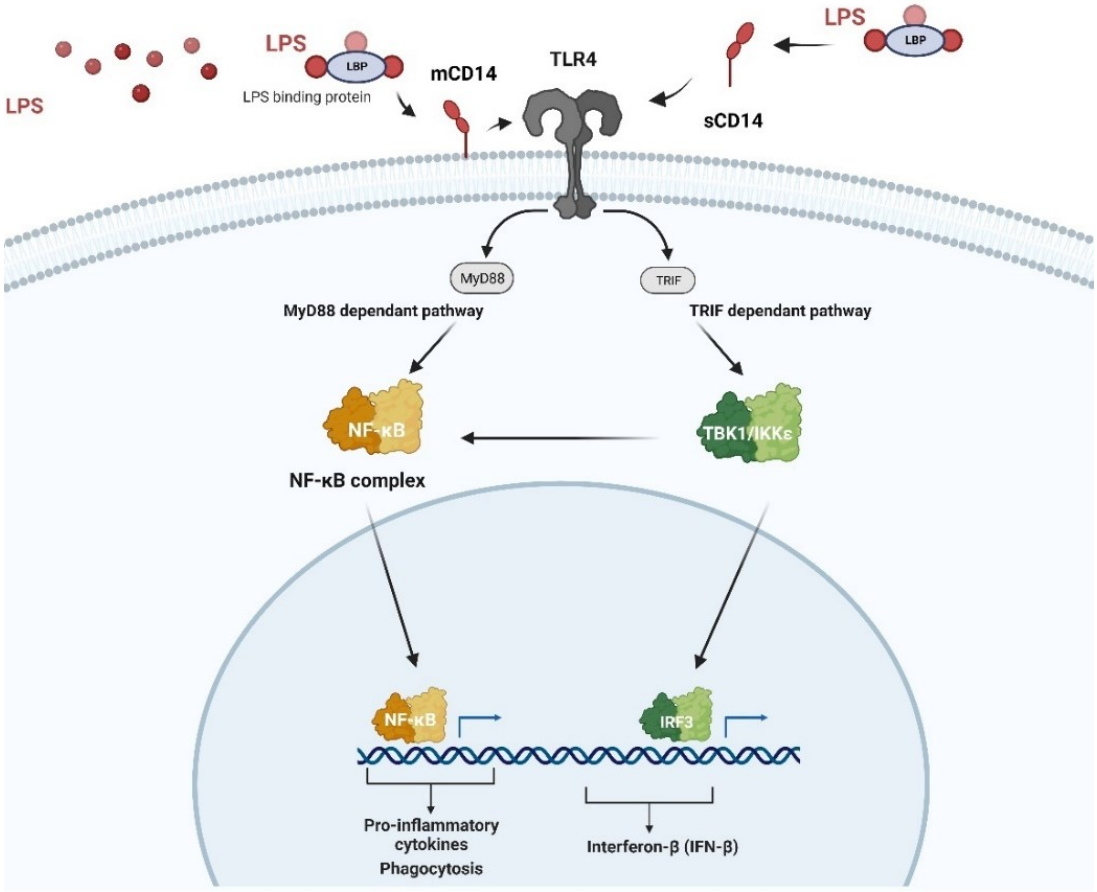

Figure 1.4 Schematic Presentation of the CD14 Signalling Pathway.
Upon detection of bacterial components (i.e, LPS), CD14 interacts with TLR4 and initiates downstream signalling events through a MyD88-dependent and TRIF-dependent pathway. This leads to activation of transcription factors like NF-κB, and subsequent induction of cytokine production, a key event in initiating innate immune responses. Figure created by the author based on information in Ouburg et al. (2005).

1.4.4 The Role of TREM2 in Phagocytosis and Inflammation

TREM2 protein is a novel immunoglobulin receptor that was first discovered in 2000 (Bouchon et al., 2000). It belongs to the TREM transmembrane glycoproteins family, which plays important roles in regulating myeloid cell functions (Colonna, 2003). TREM2 possesses two isoforms; a membrane-bound form mTREM2 and a soluble form sTREM2 (Cheng et al., 2018; Ferri et al., 2021). The mTREM2 receptor (Figure 1.5) is the most common isoform characterised by an extracellular immunoglobulin-like region, a transmembrane domain, and a short cytoplasmic tail. The sTREM2 isoform is characterised by the absence of both transmembrane and cytoplasmic

regions. This feature allows sTREM2 to be produced and secreted by the cells as a soluble protein (Yeh et al., 2017; Zhong et al., 2019).

TREM2 is primarily expressed on dendritic cells, monocyte-derived macrophages, and tissue-resident macrophages like osteoclasts, alveolar macrophages and Kupffer cells (Jay et al., 2017), but not on granulocytes or monocytes (Bouchon et al., 2000; Bouchon et al., 2001; Daws et al., 2001). The expression of TREM2 is highly regulated during inflammation. Research demonstrated that inflammatory molecules such as TNF-α, IL-1β and IFN-γ can reduce TREM2 protein levels in various cell types including tissue macrophages and microglia (Turnbull et al., 2006; Gao et al., 2013; Haure-Mirande et al., 2022; Wang et al., 2023). The downregulation of TREM2 expression in response to inflammatory signals suggests that it may serve as a negative feedback mechanism to modulate or dampen TREM2 signalling. By reducing TREM2 levels when inflammatory signals are present, this mechanism can prevent TREM2 signalling, which could then lead to dysregulated immune responses (Jay et al., 2015; Dabla et al., 2022).

Research suggests that TREM2 expression dampens inflammation and promotes bacterial phagocytosis and cell debris clearance (Chen et al., 2020; Cignarella et al., 2020). During inflammation, the up-regulation of TREM2 was shown to inhibit the production and secretion of inflammatory cytokine such as TNF-α, IL-1, IL-6 and nitric oxide (NO) by macrophages (Takahashi et al., 2005; Jones et al., 2014). TREM2 upregulation has also been reported to suppress TLR signalling cascades that trigger inflammation (Hamerman et al., 2006; Turnbull et al., 2006). Moreover, TREM2 triggers various anti-inflammatory pathways, like the phosphoinositide 3-kinase (PI3K)/Akt pathway (Peng et al., 2010), which may indirectly inhibit the pro-inflammatory NF-κB pathway (Peng et al., 2010; Sun et al., 2011). Additionally, TREM2 was shown to suppress the pro-inflammatory c-Jun N-terminal kinase (JNK) pathway, which can also suppress NF-κB signalling pathway (Zhong et al., 2017).

TREM2 expression in macrophages has been shown to promotes the phagocytosis of bacteria and apoptotic cells (Daws et al., 2003; Hsieh et al., 2009; Kawabori et al., 2015; Yeh et al., 2016). TREM2 can bind to a wide range of gram-positive and gram-

negative bacteria and bacterial-derived components, triggering signalling cascades that stimulate bacterial engulfment (Daws et al., 2003; N'Diaye et al., 2009; Kawabori et al., 2015). The mechanism by which TREM2 promotes phagocytosis is through recognition and binding to anionic lipids, LPS and lipoproteins including apolipoproteins (ApoE) (Yeh et al., 2016). However, it is essential to note that the exact mechanisms are still unclear, as receptor binding alone does not automatically trigger phagocytosis (Swanson and Baer, 1995; Henneke and Golenbock, 2004).

It is hypothesised that TREM2 couples with the adaptor protein DAP12 initiating a phagocytic signalling cascade through its immunoreceptor tyrosine-based activation motif (ITAM) (Peng et al., 2010; Zhong et al., 2017). Furthermore, research revealed that TREM2 activation induces cytoskeletal changes such as F-actin polarisation in macrophages, which suggests that TREM2 promotes the rearrangement of the actin cytoskeleton proteins necessary for phagocytosis (Phillips et al., 2018; Okuzono et al., 2021).

In summary, TREM2 plays a crucial role in the phagocytic clearance of bacteria during wound repair. The balance of its anti-inflammatory and pro-phagocytic functions is fundamental for the regulation of immune responses during infection. Further research is necessary to fully elucidate the mechanistic pathways resulting from TREM2 activation/blockade during wound infection. Nevertheless, current evidence highlights the potential of selectively targeting TREM2 signalling pathway as a novel approach to regulate phagocytic responses during DFIs (Cheng et al., 2018; Li et al., 2023).

1.4.5 Apolipoprotein E (ApoE), a key TREM2 Ligand

ApoE is an immunoregulatory lipoprotein that is essential for host immune defences (Zhang et al., 2011; Gong and Cun, 2019). Highly expressed in macrophage lineages, ApoE covers up to 10% of a macrophage's total secreted proteins (Kockx et al., 2008). It is also expressed in several cell types including liver cells, vascular smooth muscle cells, and neurons (Tedla et al., 2004). As part of the Apo family,

ApoE is involved in cholesterol transportation (Mahley and Rall Jr, 2000). Recently, multiple research studies described ApoE as a ligand with a high affinity to TREM2 receptors (Atagi et al., 2015; Bailey et al., 2015; Yeh et al., 2016). The three major isoforms of the ApoE; ApoE2, ApoE3, and ApoE4 have all been shown to bind to TREM2 receptors on innate immune cells (Mai et al., 2022).

The ApoE-TREM2 interaction has been shown to activate key physiological signalling pathways within innate immune cells (Shi and Holtzman, 2018). Binding of secreted ApoE molecules to TREM2 receptors has been shown to increase the phagocytic functions of macrophages and dendritic cells. However, the same studies reported that ApoE-TREM2 binding reduces TLR-induced inflammatory cytokine production in all cell subsets (Atagi et al., 2015; Yeh et al., 2016; Wolfe et al., 2018).

Although the ApoE-induced TREM2 signalling usually suppresses inflammation, several studies reported potential detrimental effects of this interaction, leading to the aggravation of brain inflammation particularly in Alzheimer's disease (Krasemann et al., 2017; Ulrich et al., 2017). These conflicting findings highlight the complexity of TREM2-ApoE axis. Hence, more research is needed to better understand the nature of the ApoE-TREM2 interaction and its influence on inflammation across various cell types and different disease states. Ongoing research continues to uncover the immunoregulatory role of the ApoE-TREM2 axis as a potential novel therapeutic option to overcome macrophages' phagocytic impairments during the inflammatory phase of wound repair, particularly in DFUs.

1.4.6 The Role of CD33 Receptor (Siglec-3) in Phagocytosis and Inflammation

CD33 is a member of the sialic acid-binding immunoglobulin-like lectins (Siglecs), an immunoglobulin superfamily (IgSF) receptor group involved in immune regulation (Crocker et al., 2007). Siglecs including CD33 are key modulators of innate immune responses during infection. In particular, CD33 modulates phagocytic responses in macrophages through recognition and binding to sialic acid ligands (Crocker et al., 2007; Jeswin et al., 2018; Islam et al., 2022).

CD33 is a transmembrane immunoglobulin receptor with intracellular immunoreceptor tyrosine-based inhibitory motifs (ITIMs). Its extracellular region has a V-set immunoglobulin domain that binds sialic acids (Crocker et al., 2007; Sereshki et al., 2023). This allows CD33 to recognise and bind to different Sialylated ligands and dampen immune responses against them. Upon sialic acid binding, the ITIMs are phosphorylated which leads to the recruitment of cytoplasmic phosphatases like SHP-1 and SHIP-1 (Figure 1.5). These phosphatases then block the activation pathways of macrophages triggered by other receptors like TLRs, resulting in the inhibition of their phagocytic activity which leads to reduced inflammatory responses (Taylor et al., 1999; Orr et al., 2007; Pillai et al., 2012; Griciuc et al., 2013).

The CD33-mediated immunoreceptor tyrosine-based inhibition motif (ITIM) signalling pathway functions as a counterbalance to activation mediated by immunoreceptor tyrosine-based activation motif (ITAM) receptors like TREM2 (Orr et al., 2007; Griciuc et al., 2019). TREM2 promotes the activation of myeloid cells, such as microglia and macrophages, through ITAM signalling cascades. These cascades involve downstream molecules that result in cytokine production, cell proliferation, and enhanced phagocytosis (Deczkowska et al., 2020). In contrast, CD33 can dampen these activation pathways through ITIM signalling (Paul et al., 2000; Chan et al., 2015). The opposing actions of TREM2 and CD33 enable a balanced immune cell response.

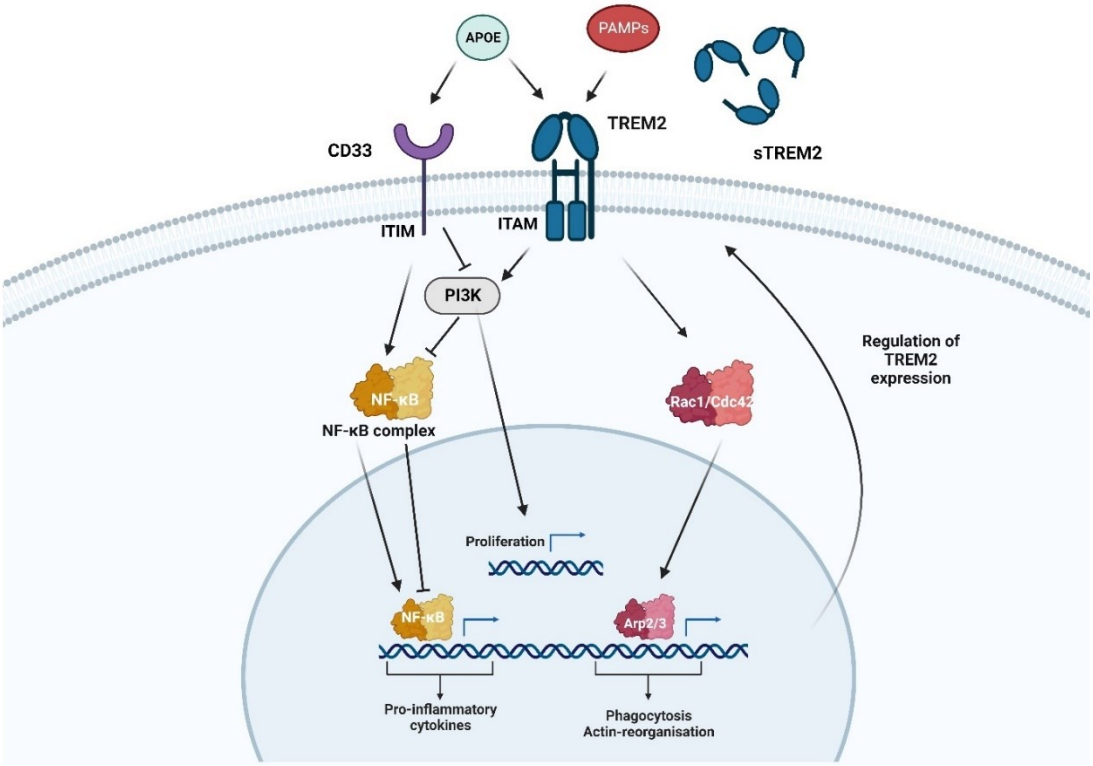

Figure 1.5 Schematic Presentation of TREM2/CD33 Signalling Pathway.
CD33 and TREM2 have opposing effect on macrophages activation during phagocytosis. The TREM2 receptor has multiple ligands, including PAMPs and ApoE, which upon binding stimulate intracellular signalling cascades leading to enhanced phagocytic activity and increased proinflammatory cytokine secretion in macrophages. TREM2-mediated signalling occurs via activation of the SYK and PI3K pathway. However, CD33 can also bind ligands such as ApoE which subsequently inhibits the TREM2-induced signals. Figure created by the author using Bio-render based on information in (Griciuc et al., 2019; Colonna, 2023)**.**

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In summary, CD33 is a key Siglec protein that modulates innate immune cell responses, particularly macrophage activation, through sialic acid binding and ITIM-mediated signalling (Ulyanova et al., 1999; Paul et al., 2000; Crocker et al., 2007). Further comprehensive understanding of its signalling mechanisms is essential to discover the full extent of its immunomodulatory impact.

1.4.7 NF-κB signalling.

NF-κB is an important transcription factor that plays a fundamental role in regulating various cellular processes including inflammation and phagocytosis, in immune cells like macrophages and neutrophils (Kracht et al., 2020; Cheng et al., 2023; Iacobazzi et al., 2023). In the absence of cellular stimulation, under non-inflammatory conditions, NF-κB is normally sequestered in the cytoplasm by inhibitory molecules (IκBs), blocking its nuclear translocation. However, in response

to pathogens, cytokines and other stimuli, NF-κB undergoes a dynamic activation process resulting in the activation of IκB kinase (IKK) complex. Then the IKK complex phosphorylates IκB proteins, resulting in ubiquitination and degradation of IκB. This process enables the nuclear translocation of NF-κB protein (Mulero et al., 2019), inducing the transcription of numerous target genes related to inflammation and immune responses (Nejatbakhsh Samimi et al., 2020). The most common genes that are targeted by NF-κB include pro-inflammatory cytokines such as TNF-α and IL-6, as well as chemokines and other mediators that regulate immune cells.

NF-κB activation increases the phagocytic capacity of macrophages to engulf and clear invading pathogens during infection. Dysregulation of NF-κB is linked to increased inflammation and impaired immune responses. Since DFUs are marked by exaggerated inflammatory responses and impaired immune functions, investigating NF-κB signalling is crucial in understanding the physiopathology of DFUs. Further research is needed to explore the intricate regulation of NF-κB pathways in the context of DFUs. Understanding the NF-κB signalling in inflammatory environments would potentially aid developing therapeutic approaches that lead to the downregulation of excessive inflammatory responses and restoration of normal innate immune functions in DFUs.

1.5 Estrogen and Wound Healing

Over the past decades, research investigating the role of estrogen on wound repair has significantly expanded (Gilliver et al., 2007; Guo and DiPietro, 2010). There is increasing evidence suggesting that systemic and peripheral levels of steroid hormones, mainly estrogen, have an impact on the inflammatory responses during wound healing (Ashcroft et al., 1999a; Horng et al., 2017; Mukai et al., 2022).

There are three main forms of estrogen found in human, estrone (E1), 17-β-estradiol (E2), and estriol (E3). 17-β-estradiol is the predominant systemic form of estrogen present in both males and females (Delgado and Lopez-Ojeda, 2019). Estrogen is often considered as a female sex steroid hormone. However, its role extends beyond the development of the female reproductive organs, such as

ovulation and menstrual cycle (Findlay et al., 2010). It has a crucial role in maintaining the reproductive health of males, particularly in spermatogenesis (O'donnell et al., 2001). Moreover, estrogen is fundamental to various functions in both genders, including its influence on bone metabolism, cardiovascular functions, and immune responses (Delgado and Lopez-Ojeda, 2019; Dama et al., 2021).

In females, the theca and granulosa cells within the ovaries are the primary sites of estrogen production (Moon et al., 1978; Mcnatty et al., 1979). Under the influence of luteinizing hormone (LH), the theca cells produce androgens, such as testosterone. Subsequently, the granulosa cells convert these androgens into estrogens, primarily estradiol (Imamichi et al., 2017). In males, smaller amounts of systemic estrogen are produced by the gonads and released into the blood circulation (Wierman, 2007; Cooke et al., 2017). Moreover, substantial quantities of inactive steroid precursors (e.g. Dehydroepiandrosterone (DHEA) and Dehydroepiandrosterone sulphate (DHEA-S)) are made by the adrenals and converted into active steroid hormones (e.g. estradiol) in peripheral tissues including adipose tissue and skin (Nelson and Bulun, 2001). This peripheric synthesis of estrogen exists in both genders and have a crucial impact of people's health, particularly when the systemic hormonal production is reduced during ageing. The production of steroid precursors (i.e. DHT and DHTS) also declines significantly during ageing, which results in a significant decline in the production of active estrogens in peripheral tissues (Labrie *et al*., 1998).

Research indicates that endogenous estrogen levels significantly decline with ageing in both male and female individuals, largely due to the decreased production of inactive adrenal-derived precursors (adrenally-derived DHEA) (Horstman et al., 2012). In postmenopausal women, this decline is also due to a reduced central estrogen production by the ovaries (Sherwin, 2003). Similarly, the production of estrogen in peripheral tissues decreases in aged males. Testosterone levels also decline with age, but to a lesser degree compared to estrogen (Ashcroft et al., 1999a).

Exogenous estrogen supplementation has been demonstrated to substantially accelerate wound healing in elderly subjects of both sexes through dampening inflammation and improving cellular proliferation (Ashcroft et al., 1997; Ashcroft et al., 1999a).

1.5.1 Estrogen Receptors

The expression of ERs; ER-α and ER-β in the nucleus and on the surface of inflammatory cells (e.g. neutrophils, monocytes and macrophages) suggests that topical and systemic estrogen supplementation influences inflammation during wound repair (Gulshan et al., 1990; Ashcroft et al., 1999a). In order to achieve its effect, estrogen binds to the ERs, which leads to the activation of signalling pathways (via surface receptors) or activation/repression of gene transcription (via nuclear receptors) (Murphy *et al.,* 2009). Although ER-α and ER-β serve as the primary receptors for estrogen, non-genomic signalling events mediated by transmembrane G protein coupled estrogen receptor (GPER) have also been identified as modulators of various cellular functions including inflammation and phagocytosis (Blasko et al., 2009; Rettew et al., 2010; Pelekanou et al., 2016).

ER distribution changes across different tissues and cell types. Their overlapping and distinct expression patterns indicate that ER-α and ER-β have separate interacting roles (Matthews and Gustafsson, 2003). Numerous studies confirmed the presence of both ER-α and ER-β across diverse physiological systems like the endocrine, cardiovascular, and central nervous systems (Weusten et al., 1986; Gulshan et al., 1990; Couse et al., 1997). The current understanding of ERs distribution in cutaneous tissue is still vague, despite several studies addressing this topic. ER-α has been identified in all skin-associated cell types, except in the sebaceous gland (Pelletier and El-Alfy, 2000). Conversely, ER-β is highly expressed in the epidermis, blood vessels, and dermal fibroblasts (Thornton et al., 2003). Furthermore, multiple studies confirmed the presence of both ER-α and Er-β in macrophages. However, the precise expression levels of ERs in macrophages remains unclear. Therefore, additional research is needed to clarify the specific impact of ER-α and ER-β on macrophage activity in cutaneous tissue.

Animal studies have demonstrated that the activation of both ER-α and ER-β improves wound repair. However, their precise role in this process is different. ER-α has been described to regulate inflammation and phagocytic responses. However, ER-β has been shown to enhance the later stages of wound healing, tissue proliferation and remodelling (Ashworth *et al.,* 2008; Campbell *et al.,* 2010).

In summary, estrogen is suggested to have a direct effect on monocytes and macrophages due to their possession of the ER proteins (Gulshan et al., 1990). Estrogen has been shown to decrease TLR4 signalling in macrophages and dendritic cells *in vivo* (Rettew et al., 2009; Guo et al., 2022). In addition, estrogen has been reported to produce anti-inflammatory effects through regulating the production of pro-inflammatory cytokines including IL-6, IL-1β, TNF-α, MCP-1, nitric oxide synthase (iNOS) and macrophage migration inhibitory factor (MIF) (Pfeilschifter et al., 2002; Hardman and Ashcroft, 2005).

1.5.2 Estrogen Signalling

Estrogen, along with its receptors (ERs), regulates diverse cellular processes through activation of three signalling pathways involving nuclear receptors, membrane receptors, and receptor-independent pathways (Fuentes and Silveyra, 2019) (Figure 1.6). In the classical nuclear pathway, estrogen binding to the ligand-binding domain (LBD) of ERs triggers receptor dimerization (Marino et al., 2006; Le Dily and Beato, 2018). This dimerized form of ERs then binds to specific DNA sequences called estrogen response elements (EREs). This process tiggers co-activators or co-repressors which consequently activate or suppress gene transcription (Stefkovich et al., 2018). Estrogen receptors can also indirectly regulate gene transcription through binding to other transcription factors such as NF-κB (Galien and Garcia, 1997; Kalaitzidis and Gilmore, 2005).

The second signalling pathway is the estrogen-dependent membrane pathway, also known as the non-genomic pathway, is initiated when estrogen binds to estrogen receptors located on the cell membrane. This binding triggers intracellular effects in

the cytoplasm that generate a physiological response, without directly regulating gene expression. One example of these cytoplasmic effects is changes in the levels of intracellular calcium. (Cui et al., 2013; Vrtačnik et al., 2014; Barton et al., 2018). Additionally, this membrane pathway can indirectly influence gene expression by activating cell membrane receptors such as tyrosine kinase receptors, which then initiate signalling cascades that ultimately modulate gene transcription (Lösel and Wehling, 2003).

In addition to the major ER-dependent pathways, estrogen can also act independently of its receptors to exert antioxidant effects. The antioxidant phenolic structure of estrogen molecule allows it to interact directly with different cytoplasmic enzymes and reduce oxidative stress by stopping the release of reactive oxygen species (ROS) (Richardson et al., 2012).

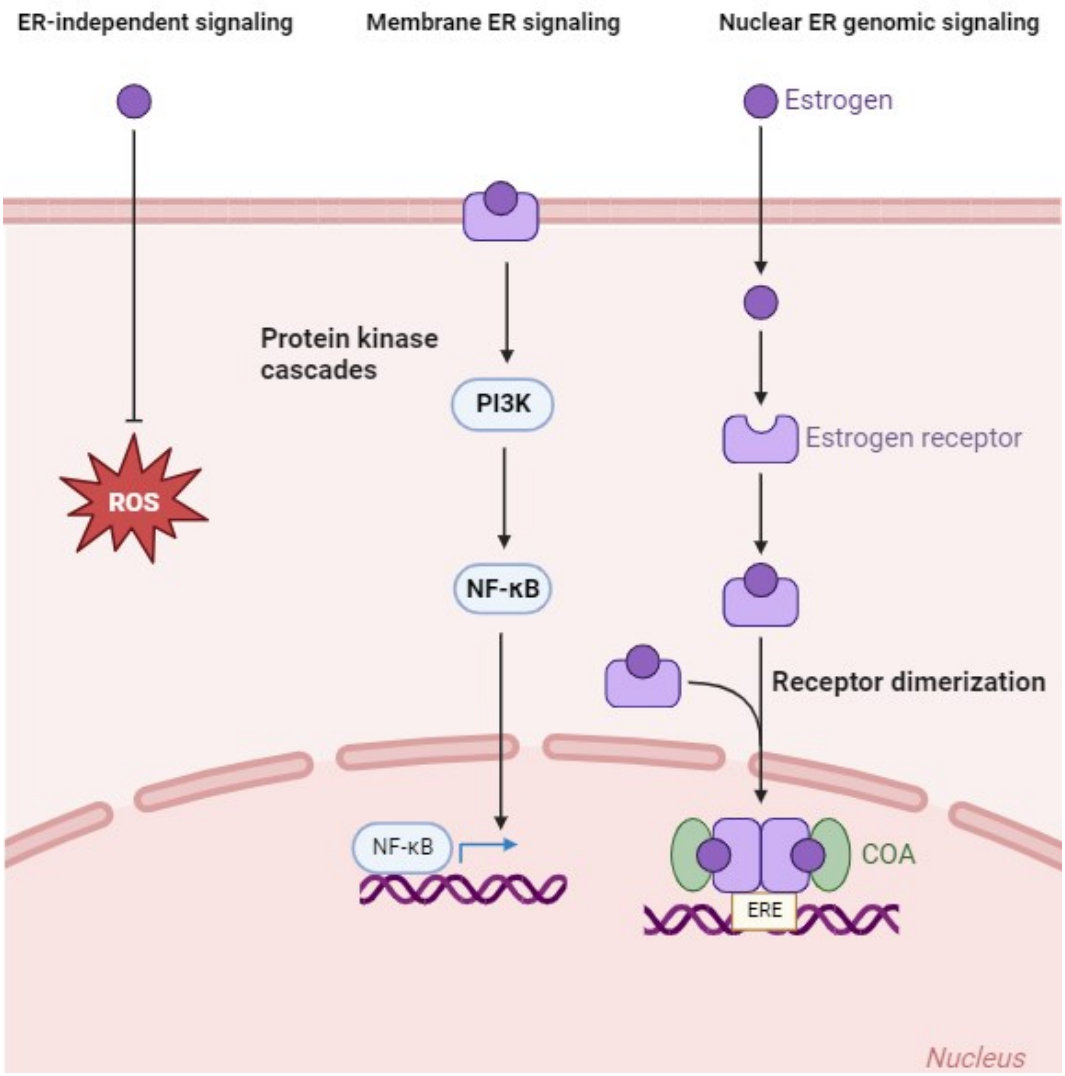

Figure 1.6 Schematic Representation of Estrogen Signalling Pathway.
Figure created with Bio-render based on information (Fuentes and Silveyra, 2019).

1.6 Estrogen and DFUs

Chronic wounds such as DFUs are characterised by a prolonged chronic inflammatory response, decreased re-epithelialisation, reduced angiogenesis and reduced ECM formation (Wetzler *et al.*, 2000; Leung, 2007). When DFUs are infected with bacteria, wound healing becomes severely impaired, leading to higher rates of mortality arising from associated morbidities, such as sepsis (Brem *et al.,* 2001).

Estrogen has been shown to accelerate re-epithelialisation, promote matrix deposition, stimulate wound contraction and dampen the inflammatory response by reducing the expression of pro-inflammatory cytokines such as TNF-α, IL-1, and

IL-6, which are key mediators of the activated macrophages (Hardman and Ashcroft, 2006; Kovats, 2015). Since the central and peripheral production of estrogen declines during ageing, estrogen deficiency in the elderly has been strongly associated with pathological chronic wounds. In addition, a clinical human study conducted in 2002 indicated that HRT-treated elderly patients were 30 to 40% less likely to develop a chronic ulcer compared to other non-treated age-matched patients (Margolis *et al.,* 2002).

Despite the well-documented anti-inflammatory effects of estrogen on the wound healing process, little is known about the effect of this hormone on innate immune responses during wound infection. Moreover, it remains unknown whether the decline in estrogen with age aggravates chronic wound infections, particularly those linked with DFUs. Specific and targeted enhancement of innate phagocytic function of immune cells using estrogen (and/or estrogen-like drugs that can act as ER agonists) could be an exciting approach to develop novel therapies to combat wound infections in older people with DFUs and warrants further investigation.

1.7 Aim and Objectives

DFUs are characterised by persistent inflammation and delayed healing processes marked by altered innate immune functions and deprived macrophage activity under hyperglycaemic conditions. Despite the advanced understanding of innate immune system regulations, the molecular mechanisms regulating pathogen recognition and phagocytosis by macrophages has not been completely understood, especially within the diabetic environment. Current evidence suggests the involvement of various pathways mediating host-pathogen interactions (figure 1.7). However, studies investigating the effects of hyperglycaemia on the expression of phagocytic receptors in pro-inflammatory macrophages are limited.

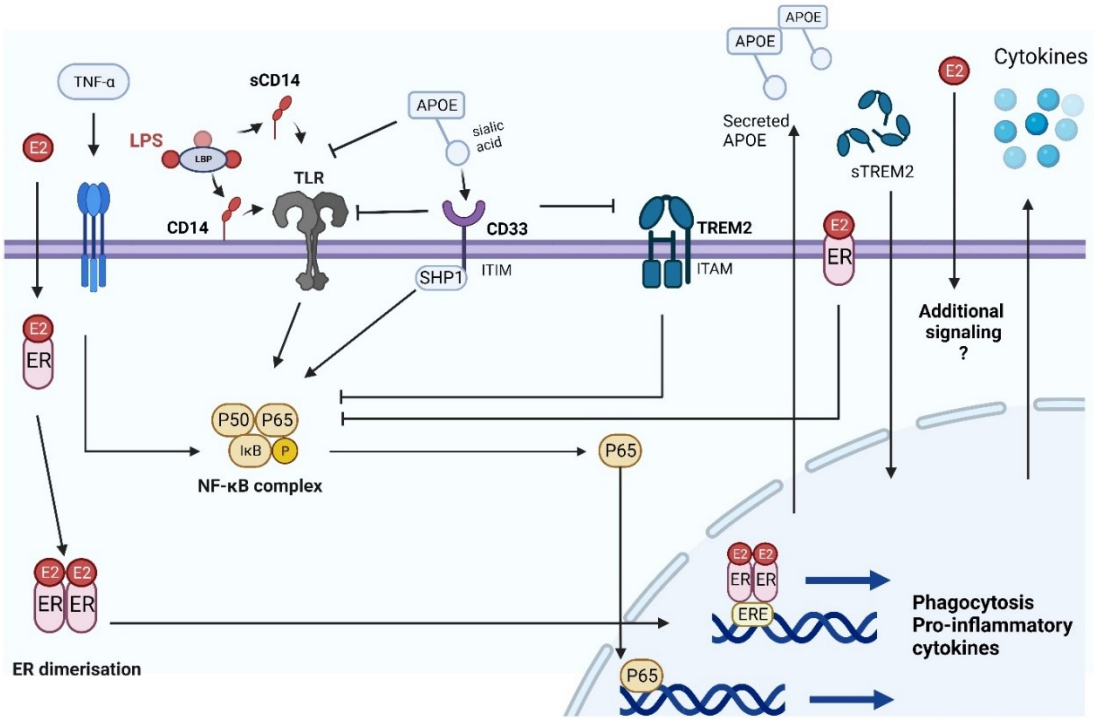

Figure 1.7 Schematic Representation of Pathways Representing Potential Targets for Estrogen-Mediated Immune Regulation.
Figure created by the author**.**

It is hypothesised that age-related estrogen deficiency contributes to macrophage dysregulation, leading to impaired phagocytosis and excessive inflammatory responses, similar to those seen in diabetic wound healing. Therefore, the aim of this PhD study was to determine the effects of age-related estrogen deprivation on the clearance of wound-associated bacteria by *in vitro* monocyte-derived macrophages under hyperglycaemic conditions.

The aim of the study was achieved through the following objectives:

- Establish and validate *in vitro* models of U937 monocytes derived macrophages and hyperglycaemic conditions mimicking diabetic inflammation.
- Investigate the impacts of hyperglycaemia on macrophage functions, including expression of pathogen recognition receptors, phagocytic capacity against planktonic and biofilm MRSA and *P. aeruginosa*, and inflammatory mediators like cytokine production and NO levels.

- Evaluate the effects of estrogen treatment on macrophage functions under hyperglycaemic conditions, such as bacterial phagocytosis and clearance, cytokine production, NO levels, and expression of key receptors (e.g. CD14, CD33 and TREM2) involved in pathogen recognition and signalling pathways.
- Investigate the roles of estrogen receptor isoforms ER-α and ER-β in mediating estrogenic modulation of macrophage responses to bacteria, including factors like bacterial clearance, cytokine production, and protein expression.
- Visualize host-pathogen interactions and phagocytic events using advanced microscopy techniques like confocal and scanning electron microscopy.

Chapter 2: General Methods

2.1 Media Preparation

Complete medium (CM): Roswell Park Memorial Institute RPMI-1640 Media containing 25 mM HEPES, L-Glutamine, 11 mM glucose, and supplemented with 10% heat-inactivated foetal bovine serum (FBS) and 2% penicillin-streptomycin.

Antibiotic-free (Ab-free) medium: RPMI-1640 medium containing 25 mM HEPES, L-Glutamine, 11 mM glucose, and supplemented with 10% FBS.

Glucose-supplemented medium: D-(+)-glucose was dissolved in a CM or Ab-free medium and used to prepare glucose-supplemented media with final glucose concentrations of 15 mM, 20 mM, and 30 mM by serial dilution in corresponding culture media.

Estrogen-supplemented medium (GS): Estrogen (17β-estradiol) was prepared to a final concentration of 1×10^{-7} M in antibiotic-free RPMI-1640 medium.
All supplemented media were sterile filtered (0.2 μm) prior to use in cell culture experiments.

2.2 Ethics

The project was screened for any ethical implications, and as no ethical issues were identified, a full ethics application was not required. Ethical approval was granted for the two applications submitted (RA_1875, RA_1876).

2.3 Cell Lines

The monocytic U937 cell line (Health Protection Agency Culture Collections, Salisbury) and CD33-knockout U937 cells (a kind gift from the University of Alberta) were used for this study.

2.4 Cell Culture

Euglycemic (11mM glucose)-treated cells

Routine monocytic or CD33-knockout U937 cells were cultured under aseptic conditions at 37 °C and 5% CO_2 using RPMI-1640 CM. The U937 cell suspension was maintained at 0.5 × 10^6 cells/mL by resuspension in the same medium every 48 hours.

Hyperglycaemic (15 mM, 20 mM and 30 mM glucose)-treated cells

Wildtype monocytic or CD33-knockout U937 cells cultured and maintained under aseptic conditions at 37 °C and 5% CO_2 at 0.5 × 10^6 cells/mL in glucose-supplemented (15, 20, 30 mM) medium, with media replacements every 48 hours. Glucose-treated cells were only used in host-pathogen experiments after a two-week period of culture in the appropriate glucose-supplemented media, representing an *in vitro* model of sustained hyperglycaemia. Glucose concentrations were chosen based on published methodologies to model *in vitro* type 2 diabetes (Paget et al., 1998; Padayatti et al., 2001; Zhang et al., 2010).

2.5 Cell Viability

Cell viability was assessed every other day using a TC10 automated cell counter (Bio-Rad, Hemel Hempstead, UK) using routine published methodologies (Strober, 1997). Equal volumes of cell suspension and trypan blue dye were mixed before loading into a dual-chamber slide (Bio-Rad, Hemel Hempstead, UK) and reading cell counts on the TC10 cell counter. Cell viability was maintained at a minimum of 90% for all experimental assays.

2.6 Differentiation of U937 Monocytes into M0 Macrophage-Like Cells

U937 cells cultured at a concentration of 1 × 10^6 viable cells/mL in RPMI-1640 CM or GS (15 mM, 20 mM, or 30 mM glucose) medium were differentiated into macrophage-like M0 cells using 50 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich, UK) for 24 hours at 37 °C and 5% CO_2. Cells were then washed twice in PMA-free medium prior to a further 48-hour incubation in PMA-free medium to

obtain resting M0 macrophages, following methods adapted and modified from (Tsuchiya et al., 1982; Takashiba et al., 1999).

2.7 Polarisation of M0 Macrophages into M1/M2 Macrophages

After differentiation of U937 monocytes into resting M0 macrophages, cells were polarized into M1 or M2 macrophages using methods adapted from Rios de la Rosa et al. (2017).

M1 Polarisation:

M0 resting macrophages were treated with 100 ng/mL LPS and 20 ng/mL IFN-γ in RPMI CM or GS (15 mM, 20 mM, or 30 mM glucose) medium for 24 hours at 37 °C and 5% CO_2. The adherent macrophages were washed twice in LPS/IFN-γ-free RPMI CM or GS (15 mM, 20 mM, or 30 mM). M1 macrophages were then obtained after 4 days of incubation in RPMI CM or GS medium at 37 °C and 5% CO_2, with medium changes every 48 hours.

M2 Polarisation:

M0 resting macrophages were incubated with 20 ng/mL IL-4 and 20 ng/mL IL-13 in RPMI CM or GS (15 mM, 20 mM or 30 mM glucose) medium for 24 hours. The adherent macrophages were washed twice with IL-4/IL-13-free RPMI CM or GS (15 mM, 20 mM or 30 mM) medium. M2 macrophages were then obtained after 4 days of incubation in RPMI CM or GS medium at 37 °C and 5% CO_2, with medium changes every 48 hours.

2.8 Estrogen Treatment

Estrogen (17β-estradiol) at a concentration of 1 x 10^{-7} M in antibiotic-free RPMI-1640 medium and 0 M in antibiotic-free RPMI-1640 CM was used. Concentrations of estrogen were chosen to model supraphysiological levels of estrogen (1 x 10^{-7} M) to represent estrogen supplementation (Viken, 1976), while estrogen deprivation

representing elderly diabetic patients with absence of estrogen (0 M) (Ashcroft and Ashworth, 2003b; Hardman et al., 2005; Sproston and Ashworth, 2018).

2.9 Flow Cytometry

2.9.1 CD11b Analysis for M0 Macrophage Differentiation

Following the differentiation of U937 monocytes into macrophage-like cells, flow cytometry was used to detect the macrophage-specific surface marker CD11b and validate the differentiation process. As described in 2.1.3, U937 cells were cultured in appropriate media, such as CM or GS medium and following incubation with PMA, cells were fixed with 4% paraformaldehyde in Dulbecco's phosphate-buffered saline (DPBS) for 10 minutes at room temperature. Subsequently, the cells were washed twice with DPBS and incubated for 1 hour at room temperature with FITC-conjugated anti-mouse-human CD11b antibody diluted 1:50 in 10% FBS in DPBS (Table 2.1). Afterwards, cells were washed twice with DPBS before a final suspension in 0.5 mL DPBS. The expression of CD11b was assessed on 10,000 events using a MACSQuant® Analyzer 16 flow cytometer (Miltenyi Biotec, Bisley, Surrey, UK). To identify the population of interest (monocytes) and exclude cellular debris, Forward Scatter (FSC), a measure of cell size, was plotted against Side Scatter (SSC), a measure of cell granularity. Once the monocyte population was identified, it was gated, and these cells were then taken forward to be gated to exclude doublets. For doublet exclusion, SSC-Area was plotted against SSC-Height, and cells that did not follow a linear distribution were excluded. Cells that did follow a linear distribution were gated as the single cell population of interest (Figure 2.1). Findings for CD11b-stained M0 macrophages were represented as average percentage CD11b+-labelled cells and median fluorescence intensity (MFI) relative to negative control U937 monocytes and unstained macrophages. Data analysis was performed using FlowJo software version 10.9 (Ashland, OR, USA).

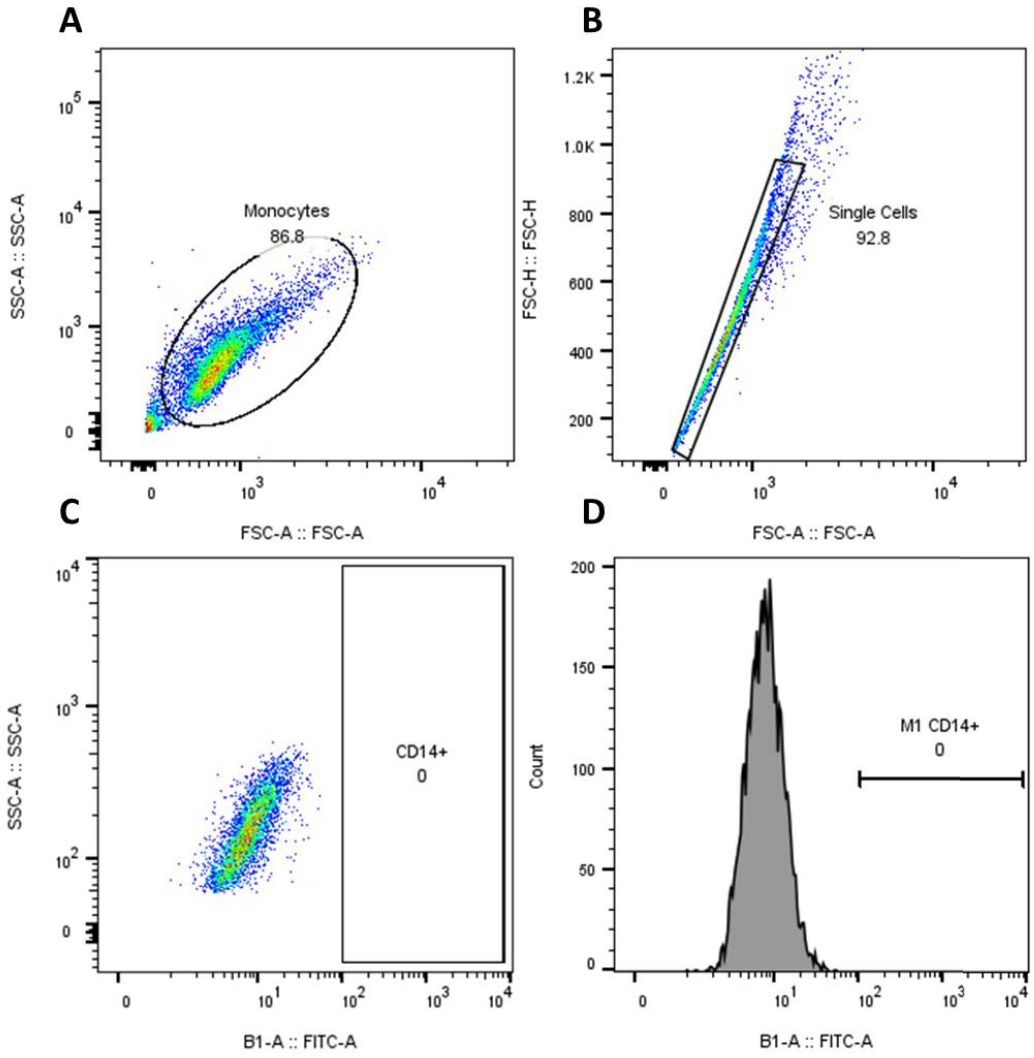

Figure 2.1 Flow Cytometry Gating Strategy.
A) Initial gating of the cell population by plotting forward scatter (FSC) against side scatter (SSC). B) The single cell population were gated by plotting FSC-H against FSC-A. C. The cell population is again displayed by plotting SSC against each protein parameter. D. Histogram was used to further identify the expressing of each protein marker of interest.

2.9.2 Analysis of CD197 to Confirm M1 Polarisation.

Flow cytometry was used to confirm the polarization of U937 M0 macrophages into M1 macrophages by assessing the surface protein marker CD197 (CCR7) (Mueller and Schultze-Mosgau, 2011). M0 and M1 macrophages were generated as described in 2.5 and 2.6. Macrophages were detached by incubation with trypsin for 5 minutes at 37 °C and 5% CO_2 and then adding an equal volume of CM or GS medium to neutralise the trypsin. The macrophages were then fixed using 4% paraformaldehyde for 10 minutes at room temperature. For staining, monocytes, M0 and M1 cells were stained with APC-conjugated anti-human CD197 antibody

diluted 1 in 50 for 1 hour at room temperature. The remaining unstained monocytes, M0 and M1 cells were incubated with 10% FBS in DPBS without antibody to serve as negative controls for gating purposes. After staining, all samples were washed twice with DPBS and resuspended in 500 µL DPBS. Expression levels of CD197 were assessed on 10,000 events using a MACSQuant® Analyzer 16 flow cytometer (Miltenyi Biotec). Data analysis was performed using FlowJo software. To identify the populations of interest and exclude debris, a gating strategy similar to section 2.8.1 was employed, using FSC vs SSC to gate on cells, followed by FSC-A vs FSC-H to separate singlets. Results for polarized M1 macrophages were recorded as the average percentage of CD197+ cells and MFI relative to the M0 controls. This allowed confirmation of successful M1 polarization by comparing the upregulated CD197 levels against the unpolarized monocyte and M0 populations.

2.9.3 Analysis of CD14, CD33 and TREM2 Protein Expression

M1 U937 macrophages were generated in RPMI-1640 CM or high glucose (30mM)-supplemented medium as described in 2.3. Cells were detached, fixed, and resuspended in PBS as described previously in 2.8.3. The cell-surface markers CD14, CD33 and TREM2 were respectively labelled with Alexa fluor 647 anti-human CD14 antibody, APC anti-human CD33 antibody and anti-TREM-2 antibody clone 78 diluted in 1:10 FBS in DPBS (Table 2.1) for 1 hour at room temperature. Control samples of M1 macrophages were left unstained and incubated in 1:10 FBS in DPBS in the absence of antibody. Following incubation, cells were washed twice and resuspended in 500 µl PBS. The cell surface membrane expression of CD14, CD33 and TREM2 was assessed on 10,000 events using The MACSQuant® Analyzer 16 flow cytometer. The macrophage population was gated according to the forward and side scatter parameters, with dead cells and cell debris gated out by determining a region around the population of interest. Data were analysed using FlowJo v10.9 software (Ashland, OR, USA) and the amount protein expression estimated based on the MFI.

2.9.4 Analysis of ApoE Protein Expression

U937-derived M1 macrophages were cultured in 24 well plates as described in 2.3. Cells were gently detached by trypsinisation and then fixed using 4% paraformaldehyde in DPBS for 10 minutes at room temperature. Samples were washed with DPBS before adding triton X-100 in DPBS (0.1%) to the cell pellet for 10 minutes to permeabilise cell membrane and facilitate the access of specific antibodies to intracellular protein targets such as ApoE. Following the permeabilization step, cells were blocked with 3% BSA in DPBS and then incubated with recombinant Alexa fluor 488 anti-apolipoprotein E antibody diluted in 1:10 FBS in DPBS (Table 2.1) for 24 hours in the dark at 4^{o}C. ApoE protein levels were then quantified using a MACSQuant® Analyzer 16 flow cytometer. A gating strategy similar to previous flow experiments was employed, using FSC vs SSC to identify the cell population, followed by FSC-A vs FSC-H to exclude singlets. The gated single cell population was then analysed for Alexa 488 fluorescence, with MFI values reported for quantification of intracellular ApoE levels. Unstained M1 cells processed in parallel were used as controls. Data was analysed using FlowJo software.

Table 2-1 Antibodies Used for Flow Cytometry Experiments

Target	Host Species	Concentration	Fluorophore	Manufacturer
Anti-Human CD11b	Rat	1 µg/mL	FITC	BioLegend, London, UK
Anti-Human CD197	Mouse	1:50	FITC	BioLegend, London, UK
Anti-Human CD206	Mouse	1:500	Alexa Fluor 647	BioLegend, London, UK
Anti-Human CD14	Mouse	1:50	Alexa Fluor 647	BioLegend, London, UK
Anti-Human CD33	Mouse	1:500	APC	BioLegend, London, UK
Anti-Human TREM2	Rat	1:500	Alexa Fluor 488	Merck Life Science Gillingham, UK
Anti-Human ApoE	Rabbit	1µg/mL	Alexa Fluor 488	Abcam, Cambridge, UK

2.10 Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) was used for the detection and quantification of several pro-inflammatory cytokines and soluble proteins in culture supernatants. U937 and CD33 knockout U937 cells were seeded at 1×10^6 cells/mL in RPMI-1640 CM or high glucose (30mM)-supplemented medium in 96-well plates, and then differentiated into M0 macrophages and subsequently stimulated with 100 ng/mL LPS and 20 ng/mL to obtain M1 macrophages as described in 2.6. M1 macrophages were treated with or without 1×10^{-7} M estradiol for 24 hours. Culture supernatants were collected and levels of TNFα (R&D Systems), IFN-β (R&D

Systems), Soluble CD14 (R&D Systems), Soluble CD33 (Abcam), soluble TREM2 (Abcam) and soluble ApoE (Thermofisher) proteins secreted by M1 macrophages were measured by ELISA according to the manufacturer's protocol. Cytokine and protein concentrations were estimated based on interpolation from standard curves generated in the ELISA assays.

2.11 Nitric Oxide Assay

U937 derived M1 macrophages ($1x10^5$) were seeded in CM or GS media in a 96-well plate following the protocols described in sections 2.3, 2.5 and 2.6. These M1 macrophages were then treated with or without estrogen (n=6) for 24 hours. After the 24-hour treatment, 100 µL of the supernatant from each well was transferred to a new 96-well plate and mixed with an equal volume of modified Griess reagent. This mixture was incubated at 37 °C and 5% CO_2 for 15 minutes. The nitric oxide (NO) produced by the macrophages was then quantified by measuring the absorbance at 540 nm using GloMax Plate Reader (Prometheus) (Promega GloMax Explorer. A standard curve was generated using sodium nitrite standards ranging from 1 to 100 µM to calculate the NO concentrations.

2.12 Microbiology Techniques

Standard sterile techniques were used during the culturing and manipulation of bacteria.

2.12.1 Bacterial Culture Preparation

A single colony of MRSA strain 11 (Hospital Isolates, Manchester, UK) or *P. aeruginosa* strain PAO1 (Culture Collections, Salisbury, UK) from the bacterial culture plate was transferred to 20mL nutrient broth (Oxoid, Basingstoke) and incubated at 37 °C on an orbital shaker overnight. Ten-fold serial dilutions of each broth were performed in sterile saline (Sigma-Aldrich, UK) using a 1 mL starting aliquot of neat broth to enumerate the number of colonies in the neat broth using the Miles and Misra technique (Brugger *et al.,* 2012). Briefly, while broths were refrigerated overnight at 4°C to inhibit further bacterial growth, serial dilutions

were inoculated onto duplicate agar plates (Oxoid, Basingstoke), incubated at 37 °C for 24 hours, followed by counting of bacteria to calculate the density of bacteria (CFU/mL) in the original broth by taking the dilution factor into consideration. Bacterial cultures for subsequent assays were prepared then prepared by centrifugation of the broth at 3500 RPM for 10 minutes and washing the cell pellet twice in saline before re-suspension at a density of $1x10^5$ (CFU/mL) in Ab-free (glucose-supplemented) culture medium.

2.12.2 Biofilm Generation

MRSA and *P. aeruginosa* were cultured at an initial density of $1x10^5$ (CFU/mL) in Ab-free GS medium on polycarbonate membranes (12µm pore size) by incubation at 37 °C for 24 hours. After 24 hours the supernatant was removed, and the membranes were washed with Ab-free (glucose-supplemented) medium to remove any loose bacteria.

2.12.3 *In vitro* Host-Pathogen Interaction Assay

2.12.3.1 Planktonic Host-Pathogen Interaction Assay

U937 M1 macrophages were treated with estrogen for 24 hours as described in 2.7. After the estrogen treatment, the M1 cells were inoculated with $1x10^5$ CFU/mL bacteria in antibiotic-free GS medium, and subsequent incubation at 37 °C and 5% CO_2 for 3 hours of host-pathogen interaction. Following the 3-hour incubation period, M1 macrophage viability and counts were measured in replicate wells (n=12) by detachment of cells with trypsin EDTA followed by cell counting on using a TC10 cell counter.

In other replicate wells (n=12) both the supernatant, together with adherent macrophages released by trypsin EDTA were collected and diluted 1:10 with antibiotic-free GS medium. Duplicate nutrient agar plates were inoculated with one tenth of the sample and incubated for 24 hours at 37°C. The recovery of MRSA and *P. aeruginosa* colonies (CFU/mL) per million viable macrophages was then calculated using the bacterial counts from replicate agar plates, taking into account the sample dilution factor and viable M1 macrophage counts.

2.12.3.2 Host-Biofilm Interaction Assay

The membranes of retained MRSA biofilms (prepared as described in 2.11.2) were clamped against the membranes of adhered macrophages prepared as described in 2.3 treated with or without 1 x 10^{-7} M estradiol for 24 hours.

Additional control treatments were added in this experiment:

- Membranes lacking M1 macrophages,
- Membranes of adhered M1 macrophages attached to membranes lacking MRSA/ *P. aeruginosa* biofilms,
- Membranes lacking both M1 cells and MRSA/ *P. aeruginosa* biofilms.

The membranes were incubated for 1 hour at 37 °C in 500µl Ab-free (glucose-supplemented) cell culture medium. After incubation, supernatants together with adherent cells released by trypsinisation were collected in 1mL Ab-free (glucose-supplemented) cell culture medium and diluted 1:1000 in Ab-free (glucose-supplemented) medium. Duplicate nutrient agar plates were inoculated with one tenth of the sample and incubated for 24 hours at 37°C. The bacterial recovery was then calculated using the bacterial counts from replicate agar plates, taking into account the sample dilution factor.

2.12.4 Biofilm Quantification

The ability of MRSA and *P. aeruginosa* to form biofilms was measured using the crystal violet biofilm assay. The quantification of biofilm was performed following the protocol outlined in Merritt et al. (2011) with some minor changes. Briefly, *P. aeruginosa* and MRSA strains were grown on polycarbonate membranes in 24 well plates at 37 °C as described in 2.11.2. After 24 hours of incubation, the supernation was gently removed and the biofilms were washed two times with DPBS to eliminate free planktonic bacteria. The membranes were then dried for 30min before adding 200µl of crystal violet solution (0.2%) to the adherent biofilm and incubated at room temperature for 15 minutes. Following the incubation time, the stained membranes were carefully washed twice and dried. Afterward, 30 % glacial acetic acid was added to each well to release all the crystal violet from the membranes. Finally, relative biofilm formation was assessed by comparison of the

absorbance of each well contents measured at 600 nm using a SPECTROstar nano (BMG SPECTROstar nano, Ortenberg, Germany) plate reader.

2.13 Cell Staining and Confocal Microscopy

2.13.1 Visualising Bacterial Internalisation

To confirm bacterial internalisation and visualise the phagocytosis of both planktonic and biofilm bacteria by M1 macrophages cultured in euglycemic and high (30mM) glucose conditions, confocal analysis was performed using a Leica STELLARIS 5 confocal microscope (Leica STELLARIS 5, Milton Keynes,UK). U937-derived M1 macrophages were seeded onto polycarbonate membranes inside 24 well plates containing CM or high glucose (30mM)-supplemented medium as previously described in 2.3. Cells were treated with or without 1 x 10^{-7} M etradiol for 24 hours prior to carrying out the host-pathogen assay using GFP *S. aureus* or mCherry *P. aeruginosa* bacteria using methods described in 2.11.3.2. After 1 hour of incubation, samples were washed in DPBS and fixed with 4% paraformaldehyde for 10-15 minutes at room temperature. Following fixation, samples were washed twice with DPBS and incubated with 3% of BSA in DPBS (blocking buffer) for 1 hour at room temperature to block non-specific binding of antibody. Samples were washed twice in DPBS and incubated in the dark for 1 hour at room temperature with either Alexa fluor 647 phalloidin (diluted 1:500 in DPBS) to stain M1-associated F-actin, thereby providing a contrasting colour to help visualise the internalisation of GFP-*S. aureus*, or with FITC CD197 (diluted 1:50 in DPBS) to provide a contrasting stain to help visualise the internalisation of mCherry *P. aeruginosa.* Following incubation, DAPI stain was added to the membranes at a concentration of 1 ug/mL for 5 minutes at room temperature in the dark to stain M1 nuclei. The membranes were then transferred onto a new microscope slide and observed on a STELLARIS 5 confocal microscope. Z-stack image analysis was performed on each sample to confirm the internalisation of bacteria by M1 macrophages. In addition, three-dimensional (3D) video representations were created from the Z-stack images to reveal detailed 3D host-pathogen interaction.

2.13.2 Visualising NF-kB/p65 Nuclear Translocation.

Confocal microscopy was performed to study the localisation and the translocation of NF-κB in M1 macrophages under euglycemic and high glucose (30mM) conditions, with or without estrogen treatment. U937 M1 cells were seeded onto glass coverslips in a CM or GS medium as described in 2.3 and 2.5. Cells were then treated with or without estrogen for 24 hours, fixed with 4% paraformaldehyde for 10 minutes at room temperature and then permeabilised using 0.1% Triton X-100 in PBS for 5 minutes at room temperature to allow the NF-κB/p65 antibody to enter the M1 cells. M1 cells were then washed twice in PBS and incubated for 1 hour at room temperature with blocking buffer (5 % goat serum in DPBS). M1 cells were then stained immediately with alexa fluor 647 anti-NF-κB p65 antibody (Abcam, USA) diluted at 1:100 in DPBS and incubated for 1 hour in the dark at room temperature. Finally, M1 cells were counterstained with DAPI for 5 minutes to label nuclei (Garrido-Gomez et al. 2017), washed twice with DPBS and mounted using VECTASHIELD antifade mounting medium to a microscope slide. All Images were captured using a 100× objective on a STELLARIS 5 confocal microscope and converted into two new separate image files for DAPI and rel A staining (blue and red channels respectively) using ImageJ software (1.54f, National Institute of Health, USA). ImageJ software was used to quantify nuclear NF-κB p65 translocation following each treatment (compared to untreated control macrophages) based on twelve image fields captured across the full coverslip range. To establish NF-κB/p65 translocation, fluorescence intensity of regions of interest (ROI) were measured by ImageJ software such that the fluorescence intensity of the cytoplasm was obtained after subtracting measurements for the nucleus, thereby allowing separation of nuclear and cytoplasmic staining within each selected region and determination of the nuclear to cytoplasmic fluorescence intensity ratio. The corrected total cell fluorescence (CTCF) was also measured for each sample by measuring the integrated density across all image pixels within a ROI. To measure the CTCF the following calculation was used as described by McCloy et al. (2014).

CTCF = integrated density – (area of interest × mean fluorescence of background measurements)

2.13.3 Receptor Localisation

Confocal microscopy was used to study CD33/TREM2 receptor localisation on M1 macrophages under euglycemic and high (30mM) glucose conditions, with/without 1 x 10^{-7} M etradiol treatment for 24 hours. M1 macrophages were grown on glass coverslips as described in 2.3 and 2.5. The cells were fixed with 4% paraformaldehyde for 10 minutes at RT followed by 1 hour of incubation at room temperature with APC anti-human CD33 antibody (diluted at 1:800) and Alexa fluor 448 anti TREM2 antibody (1:500). The cells were then stained with DAPI for 5 minutes, washed twice in PBS and mounted onto a microscope slide. After mounting onto a microscope slide, images were captured using a Stellaris 5 confocal microscopy system at a magnification of 100x. Z-stack images were acquired to capture multiple section and to create 3D videos, ensuring comprehensive visualisation of the cellular structures.

2.14 Scanning Electron Microscopy

Scanning electron microscopy (Zeiss Supra 40VP, Germany) was used to provide detailed visualisation of the initial interactions of bacteria with M1 macrophages and any gross phenotypic effects of hyperglycaemia on the cellular appearance or spatial arrangements of macrophages and/or bacteria. U937-derived M1 macrophages were seeded onto polycarbonate membranes exposed to CM or glucose-supplemented (30mM) medium as previously described in 2.3, 2.5 and 2.6. M1 cells were then treated with or without 1 x 10^{-7} M estradiol for 24 hours prior to the membrane being clamped against another membrane containing either retained MRSA or *P. aeruginosa* biofilms and incubated for 1 hour at 37 °C and 5% CO_2 as described in 2.11.3.2. Following the 1-hour period of host-pathogen interaction, both membranes were collected and fixed overnight in 2.5% glutaraldehyde in DPBS at 4°C. Membranes were washed twice in DPBS, then dehydrated with 20% ethanol for 30 minutes, 40% ethanol for 30 minutes, 60%

ethanol for 30 minutes, 80% ethanol for 30 minutes, and finally 100% ethanol for 30 minutes. Subsequently, samples were dried and sputter-coated with gold/palladium before being stored in a vacuum-assisted desiccator (Sigma-Aldrich, UK).

2.15 Data Analysis

Statistical analysis was conducted using IBM SPSS (Version 24) and GraphPad (version 9). Differences between treatment groups were evaluated through either two-way ANOVA or one-way analysis of variance (ANOVA), followed by Tukey post-hoc comparison tests or Student's t-tests. For data not conforming to normal distribution, non-parametric tests such as the Kruskal-Wallis test for ANOVA equivalence were employed. A significance level of $P<0.05$ was considered statistically significant and the Bonferroni correction was applied for multiple comparisons.

Chapter 3: High Glucose Impairs the Innate Immune Functions of Macrophages *In Vitro*

3.1 Introduction

The chronic nature of DFUs is characterised by impaired healing and a prolonged inflammatory phase. Unlike normal acute wounds, DFUs exhibit dysregulated inflammation with increased influx of immune cells, particularly macrophages and neutrophils (Mi et al., 2007; Martin and Nunan, 2015; Strang et al., 2020; Louiselle et al., 2021; Mu et al., 2022). This leads to excessive production of pro-inflammatory cytokines and matrix metalloproteinases (MMPs), damaging the extracellular matrix and impeding the development of healthy granulation tissue (Eming et al., 2014; MacLeod and Mansbridge, 2016; Chen et al., 2021; Dasari et al., 2021; Horikawa et al., 2021).

Hyperglycaemia plays a crucial role in promoting bacterial growth in DFUs. Elevated glucose levels provide energy for bacterial metabolism, enhance virulence, and facilitate biofilm formation (Gottschalk and Gottschalk, 1986; Wang et al., 2021; Pari et al., 2023). The altered tissue microenvironment in DFUs, including changes in oxygenation and pH levels, further supports bacterial survival and proliferation (Chávez-Reyes et al., 2021; Nagendra et al., 2022).

Macrophage dysfunction is a key factor in the impaired healing of DFUs (Goren (Goren et al., 2007; Mirza et al., 2013). Sustained hyperglycaemia promotes the persistence of pro-inflammatory M1 macrophages, resulting in reduced phagocytic capacity and enhanced secretion of inflammatory markers (Khanna et al., 2010; Sindrilaru et al., 2011). This leads to an overproduction of pro-inflammatory cytokines and reactive oxygen species (ROS), contributing to oxidative stress and exacerbating the inflammatory phase of wound healing (Arango Duque and Descoteaux, 2014; Zgheib et al., 2017; Bolajoko et al., 2020; Suzuki et al., 2021).

Hyperglycaemia also alters the expression of cell surface receptors, including PRRs, disrupting intracellular signalling pathways crucial for immune function (Al-Rashed (Al-Rashed et al., 2020; Matuschik et al., 2022). This dysregulation compromises the ability of macrophages to effectively recognise and respond to bacterial stimuli, affecting processes such as phagocytosis, cytokine production, and antimicrobial activity (Ayala et al., 2019).

Studies have shown that macrophages grown in high glucose environments exhibit significantly lower phagocytosis of S. aureus bioparticles compared to those in normal glucose conditions (Pavlou et al., 2018; Sousa et al., 2023). Additionally, aberrant macrophage responses to bacterial components like LPS have been observed in hyperglycaemic environments, potentially due to dysregulated Toll-like Receptor (TLR) signalling (Dasu et al., 2008; Dasu et al., 2010b; Jafar et al., 2016).

In summary, sustained and uncontrolled high blood glucose levels in DFUs significantly alter macrophage function, leading to excessive inflammation, defective bacterial clearance, and delayed wound healing. Further research is needed to elucidate the precise cellular and molecular mechanisms regulating macrophage responses in diabetic wounds. Identifying specific pathways that control pro-inflammatory M1 processes under hyperglycaemic conditions may lead to the development of new therapeutic targets for reducing inflammation, promoting bacterial clearance, and stimulating tissue repair mechanisms in DFU patients (Bajpai et al., 2019; Li et al., 2019; Holzer-Geissler et al., 2022; Loder et al., 2022).

3.2 Aim and Objectives

3.2.1 Aim

The aim of this chapter was to investigate the effect of diabetes on the key functions of macrophage in DFUs. In particular, this chapter examined how the high glucose that is associated with uncontrolled diabetes affects the ability of macrophages to eliminate wound-associated bacteria (MRSA and *P. aeruginosa*) via phagocytosis *in vitro*. Determining the effect of high glucose on the phagocytic functions of macrophages may highlight disrupted signalling processes that promote the development and progression of wound infections in diabetic patients.

3.2.2 Objectives

The aim of this chapter will be achieved through the following objectives:

- Assess the effect of hyperglycaemia on M1 polarization of macrophages *in vitro.*
- Assess the effect of hyperglycaemia on the growth of planktonic and biofilm arrangements of MRSA and *P. aeruginosa* to model typical diabetic wound infections.
- Assess the phagocytic activity of monocyte-derived macrophages under hyperglycaemic conditions *in vitro*, using biofilm arrangements of MRSA and *P. aeruginosa* as models of typical diabetic wound infections.
- Assess the effect of hyperglycaemia on the downstream production of inflammatory mediators and secretion of pro-inflammatory/anti-inflammatory cytokines by macrophages *in vitro* as a way to identify cellular and molecular pathways that may contribute to defects in bacterial clearance.
- Determine the impact of hyperglycaemic wound environments on macrophage cell surface receptors and receptor ligands involved in pathogen recognition or signalling pathways that mediate bacterial clearance.

3.3 Methods

3.3.1 Cell Culture

The monocytic cell line U937 was cultured under aseptic conditions at 37 °C and 5% CO_2 using RPMI-1640 CM. An *in vitro* model of diabetes was generated by growing the monocytes in CM with stepwise increasing glucose concentrations (15, 20 and 30 mM) as described in 2.3. Negative control (NC) cells were also grown in standard CM containing 11mM glucose to model maximal euglycemic levels observed in random glucose tests.

3.3.2 Cell Viability

After growing the monocytes in glucose-supplemented (GS) media for two weeks, the cells were differentiated into M0 and M1 macrophages. The viability of the

differentiated macrophages was then quantified using a TC10 automated cell counter using equal volumes of cell suspension and trypan blue loaded into dual cell counting chamber slides, as described previously in 2.4.

3.3.3 Differentiation of U937 Monocytes into M0 and M1-like Macrophages

U937 monocytes ($1x10^6$ cells/mL) were differentiated into macrophage-like cells by culturing in CM or GS medium (15 mM, 20 mM or 30 mM glucose), stimulating with 50 ng/mL PMA for 24 hours, and then resting for 48-hour resting in PMA-free medium (Rios de la Rosa et al., 2017; El Mohtadi, 2019)

For M1 polarization, resting M0 macrophages were stimulated with 100 ng/mL LPS and 20 ng/mL IFN-γ in CM or GS medium for 24 hours. Cells were washed and incubated in LPS/ IFN-γ free medium for 4 days with media changes every 48 hours to generate classically-activated M1 macrophages as described in 2.5 and 2.6.

3.3.4 Flow Cytometry

Flow cytometry was used to assess successful differentiation and polarisation states by quantitative cell surface protein detection. CD11b expression was assessed to validate the differentiation of U937 monocytes into M0 macrophages. Undifferentiated NC monocytes and PMA-differentiated macrophages were stained with FITC-anti-CD11b antibody and analysed on a MACSQuant Analyser to quantify the percentage (%) CD11b+ cells and CD11b-associated MFI as described in 2.8.1. Following treatment of M0 macrophages with LPS/IFN-γ, cells were stained with APC-anti-CD197 antibody to confirm successful polarization of M0 macrophages to an M1-like phenotype. The M1-specific cell surface marker CD197 was analysed on a MACSQuant Analyser by quantifying the percentage (%) CD197+ cells and CD197-associated MFI relative to non-polarised NC M0 macrophages as described previously in 2.8.2.

Finally, cell surface expression of mCD14, mCD33, TREM2 and ApoE in low (11mM) and high (30mM) glucose-exposed M1 macrophages was quantified through labelling of cells with fluorochrome-conjugated antibodies (Table 2.1; FITC anti-mCD14, AlexaFluor 647 anti-TREM2, FITC anti-mCD33, or FITC anti-ApoE) and

determining protein levels by flow cytometry. Cell surface marker expression was analysed on a MACSQuant Analyser using an acquisition of 10,000 events to determine % positive cells and marker-associated MFI. Flow data were analysed using FlowJo software to determine surface marker expression as described in 2.8.3.

3.3.5 Effect of High Glucose on Bacterial Growth and Biofilms Formation

MRSA11 and *P. aeruginosa* (PAO1) strains were prepared at a concentration of 1 x 10^5 CFU/mL as described previously in 2.11.1. Following preparation, bacterial suspensions were cultured in CM (11mM glucose) or GS-medium (15 mM, 20 mM, 30 mM glucose) at 37 °C for 3 hours. After incubation, 100 µL culture suspension was inoculated onto duplicate NA plates and incubated overnight at 37 °C. The number of colony-forming units per millilitre (CFU/mL) on each plate were counted to determine bacterial growth.

In order to quantify biofilm formation, the crystal violet assay was performed as described in 2.11.2 and 2.11.4. Biofilms grown for 24 hours in CM or GS -medium were dried, stained with crystal violet, and detached with acetic acid prior to measuring the absorbance at 600nm on SPECTROstar nano plate reader. Relative biofilm formation was determined by quantitative comparison of mean absorbance data between glycaemic growth conditions, with higher absorbance values being indicative of greater biofilm growth.

3.3.6 *In Vitro* Host-Pathogen Interactions

M0 and M1-like monocyte-derived macrophages were generated in different glucose concentrations (11 mM, 15 mM, 20 mM and 30 mM) as described in 2.3, 2.5 and 2.6. Macrophages were incubated with MRSA or *P. aeruginosa* (1 x 10^4 CFU) for 3 hours at 37 °C and 5% CO_2 to allow host-pathogen interactions to occur. Bacterial recovery (CFU/mL) was then determined as previously detailed in 2.11.3.1.

3.3.7 Enzyme-Linked Immunosorbent Assay (ELISA)

Secreted inflammatory cytokines TNF-α, IFN-β and soluble proteins sCD14, sTREM2, sCD33 and ApoE were quantified from cell culture supernatants of GS classically-activated M1 macrophages by ELISA as described in 2.9.

3.3.8 NO Assay

U937 monocytes were seeded at 1 x 10^6 cells/mL in increasing glucose concentrations (11 mM, 15 mM, 20 mM or 30 mM) and differentiated into M1 cells as described in 2.6. 100 µL supernatant from each well was added to modified Griess reagent and incubated at 37 °C, 5% CO_2 for 15 minutes, in accordance with the manufacturer's instructions. Absorbance was measured at 540 nm and NO concentrations calculated against a standard curve of sodium nitrite standards, correcting for background absorbance.

3.3.9 Confocal Microscopy

Inverted confocal microscopy (STELLARIS 5, Leica, UK) was used to measure the intracellular localisation and nuclear translocation of NF-kB/p65 in M1 macrophages under euglycemic and hyperglycaemic conditions as described in 2.12.2. Furthermore, confocal microscopy was performed to visualise filamentous actin (F-actin) in GS-cultured M1 macrophages. To achieve this, M1 Macrophages were cultured on coverslips, fixed with 4% formaldehyde, and labelled with fluorescent Alexa Fluor 546-conjugated phalloidin at 4 °C overnight. Subsequently, the coverslips were mounted on glass slides and phalloidin-stained macrophages visualised by confocal microscopy using a 100X oil objective to obtain high-resolution fluorescence images.

3.4 Results

3.4.1 Differentiation of U937 Monocytes into M0 macrophages under Hyperglycaemic Conditions

The differentiation of U937 monocytes into M0 macrophage-like cells was assessed by flow cytometry through detection of the macrophage-specific cell surface marker CD11b (Sproston and Ashworth, 2018). Figure 3.1 consists of four panels, each representing the conversion of U937 monocytes cultured at different glucose concentrations (11 mM, 15 mM, 20 mM or 30 mM) into M0 macrophages. The findings indicate the expression of CD11b was almost exclusive in PMA-treated cells compared to NC monocytes at all glucose concentrations (Figure 3.1). Within each panel (glucose concentration), (A) shows flow cytometry histograms where PMA-treated cells showed significantly ($P<0.05$) higher expression of CD11b compared to undifferentiated U937 monocytes [and unstained control cells].

Figure 3.1, Panel A) higher proportion of CD11b+ cells cultured at 11mM (98.4%±2.3), 15mM (86.26%±4.1), 20mM (81.6%±3.4) and 30mM (78.2%±2.6) compared to unpolarized U937 monocytes cultured at 11mM (30.94%±2.1), 15mM (24.01%±3.4), 20mM (30.71%±3.1) and 30mM (34.54%±2.5). (B) provides an overlay of the histograms, highlighting a shift in CD11b staining intensity between U937 monocytes and PMA-differentiated macrophage populations [and unstained control cells]. (C) quantifies the MFI, with PMA-differentiated CD11b+ macrophages demonstrating significantly ($P<0.05$) higher CD11b levels compared to CD11b+ monocytes. The results consistently demonstrate that PMA treatment effectively induced monocyte differentiation into M0 macrophages across all glucose concentrations, evident from the upregulated CD11b surface marker expression compared to undifferentiated controls.

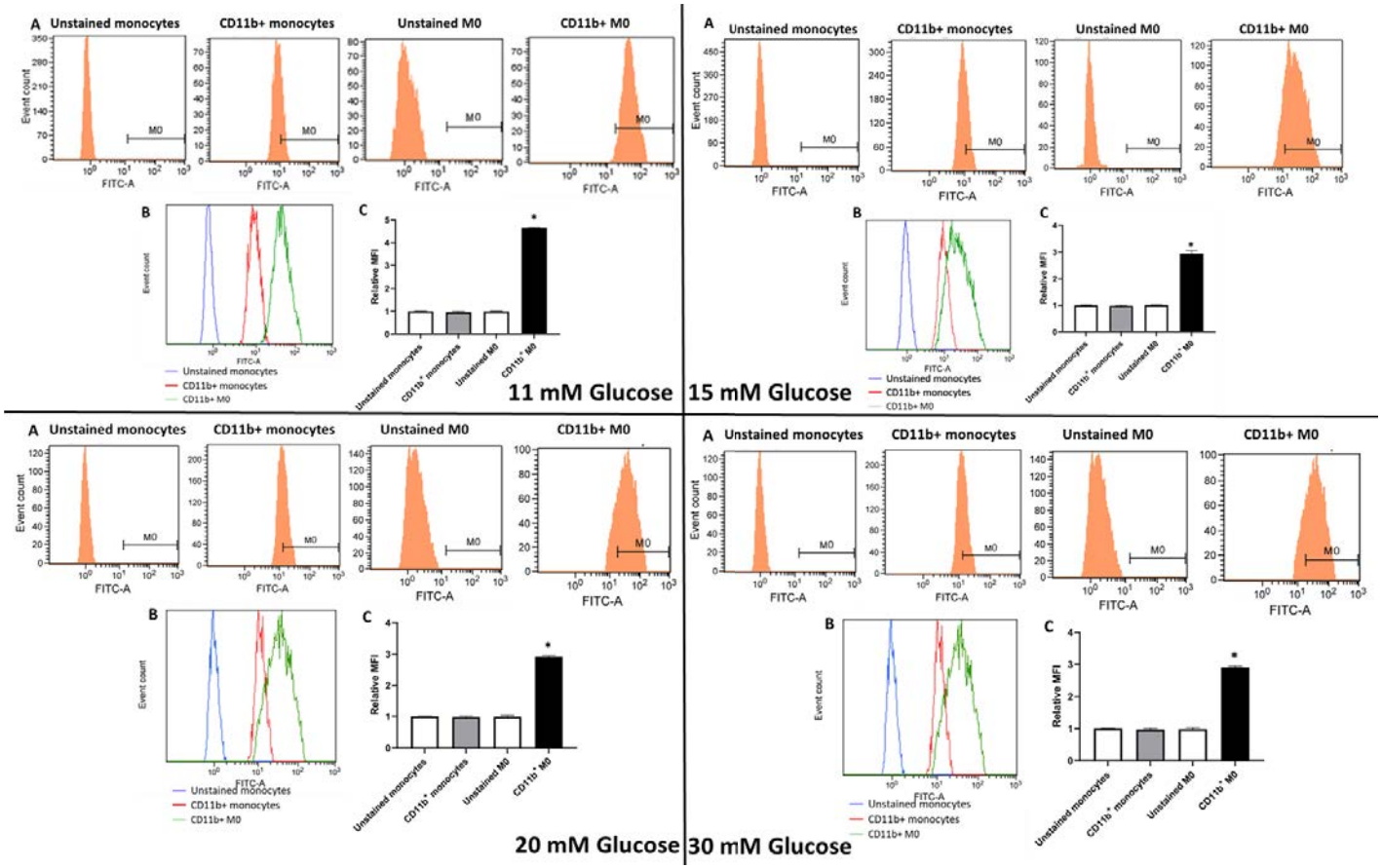

Figure 3.1 PMA Induced the Differentiation of U937 Monocytes into M0 Macrophages.

PMA induced differentiation of U937 monocytes into M0 macrophages cultured at 11 mM, 15 mM, 20 mM or 30 mM glucose. A) Flow cytometry histograms demonstrate PMA-treated cells have significantly higher CD11b expression compared to undifferentiated monocytes and unstained cells. B) Histogram overlays highlight the shift in CD11b levels between untreated U937 monocytes and PMA-differentiated macrophages. C) Quantified MFI data shows significantly (*:P< 0.05) elevated CD11b levels in PMA-differentiated CD11b+ macrophages compared to CD11b+ monocytes. Data are mean ± StEM from n=6 experiments.

3.4.2 Polarisation of M0 Macrophages into M1-Like Macrophages

The generation of M1 macrophages was confirmed by assessing the expression of the M1-associated CD197 surface marker on U937-derived macrophages by flow cytometry (Mantovani et al., 2004). U937-derived M0 macrophages were treated with/without LPS and IFN-γ across a range of glucose concentrations (11, 15 mM, 20 mM and 30 mM). Macrophages polarized with LPS/IFN-γ displayed a significantly ($P<0.05$; Figure 3.2, Panel A) higher proportion of CD197+ cells cultured at 11mM (67.4%), 15mM (67.5%), 20mM (78.6%) and 30mM (80.2%) compared to unpolarised U937 macrophages cultured at 11mM (5.94%), 15mM (3.91%), 20mM (4.39%) and 30mM (4.74%). The MFI for M1 surface marker CD197 was significantly ($P<0.001$) higher in U937 macrophages treated with LPS/IFN-γ compared to undifferentiated U937 monocytes or M0 macrophages treated with PMA across all glucose concentrations, validating the successful polarization of M0 macrophages into the pro-inflammatory M1-like phenotype.

(B) provides an overlay of the histograms, highlighting the shift in CD197 staining intensity between unpolarised M0 macrophages [and U937 monocyte populations} and polarised M1 macrophages. (C) indicates the MFI, with LPS/IFN-γ polarized $CD197^{+}$ macrophages demonstrating significantly ($P<0.001$) higher CD197 levels compared to unpolarised M0 macrophages [and U937 monocytes].

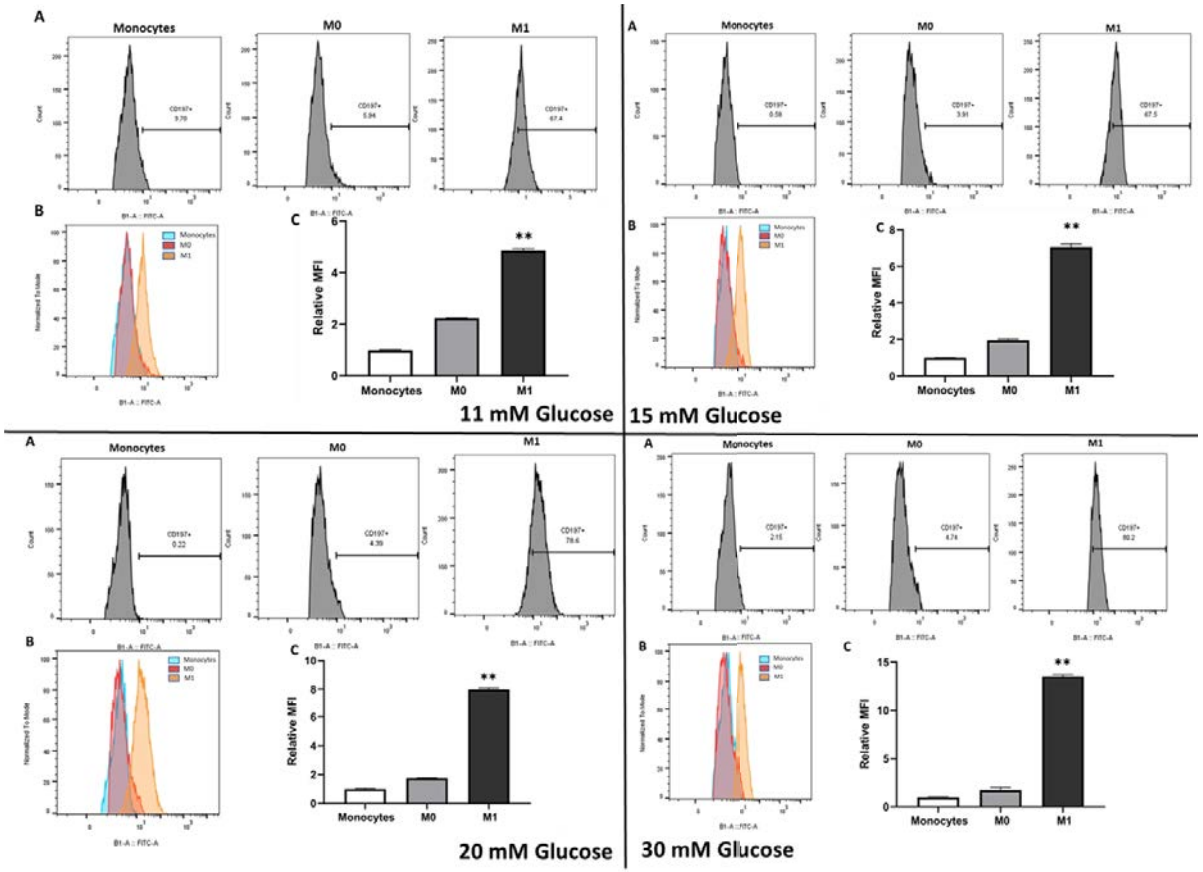

Figure 3.2 Flow Cytometry Assessing U937-M0 Macrophages Polarisation into M1 Macrophages.
LPS/IFN-γ stimulation induced M1 polarization of U937-M0 macrophages cultured under increasing glucose concentration (11, 15 mM, 20 mM and 30 mM). (A) Flow cytometry histograms demonstrate LPS/IFN-γ-treated cells had significantly ($P<0.05$) higher expression of the M1 marker CD197 compared to undifferentiated U937 monocytes and PMA-differentiated M0 macrophages. (B) Histogram overlays highlight the shift in CD197 staining between M0 macrophages [and U937 monocytes] and polarised M1 macrophages. (C) Quantified MFI data shows significantly (**$P<0.001$) elevated CD197 levels in LPS/IFN-γ-polarized CD197+ M1 macrophages compared to both CD197+ monocytes and M0 macrophages. Data are mean ± StEM from n=6 experiments.

3.4.3 The Link Between Diabetes and Susceptibility to Bacterial Infection

3.4.3.1 Effect of High Glucose on The Growth of Planktonic Bacteria

The effect of elevated glucose concentration (15 mM, 20 mM and 30 mM) on the growth of both MRSA and *P. aeruginosa* was investigated compared to NC bacteria cultured under euglycaemic conditions (11mM). Elevated glucose significantly increased ($P<0.05$) the growth of MRSA compared to NC bacteria in a concentration-dependent manner (Figure 3.3.A). Similarly, hyperglycaemia caused a significant ($P<0.05$) concentration-dependent rise in PA01 colonies in comparison with NC bacteria cultured under 11mM glucose conditions (Figure 3.3.B).

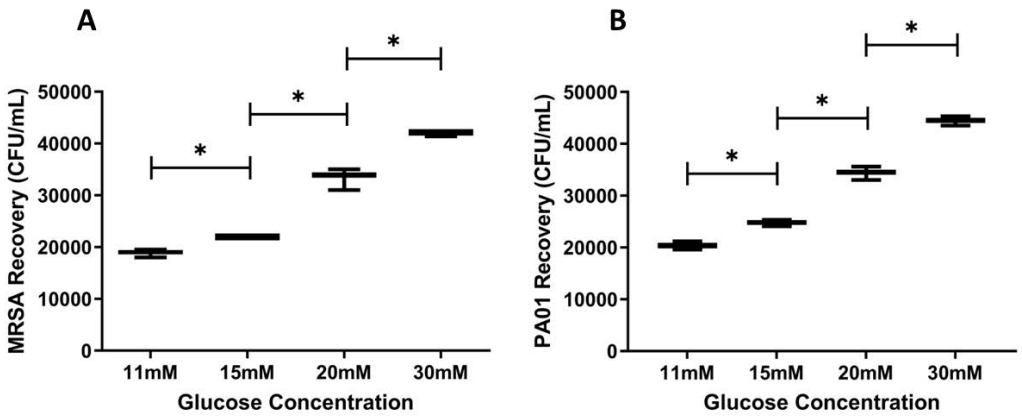

Figure 3.3 Effect of High Glucose on the Growth of MRSA and *P. aeruginosa*
Elevated glucose levels (15, 20, 30mM) significantly (*$P<0.05$; n=24) increased the growth of both MRSA (A) or PAO1 (B) compared to bacteria cultured under 11 mM glucose conditions. Data indicate mean bacterial recovery (CFU/mL) ± standard error of the mean (StEM). * Designates significant differences between glucose treatments (One-way ANOVA tests).

3.4.3.2 Effect of High Glucose on Biofilm Formation

The crystal violet assay was used to quantify the biofilm mass of MRSA and *P. aeruginosa* isolates following culture at different glucose levels (11 mM, 15 mM, 20 mM, 30 mM). Absorbance was measured at 600 nm using a plate reader. Compared to the NC with lowest glucose concentration (11 mM), biofilm mass was significantly higher with increasing glucose concentrations for both MRSA and PAO1 strains ($P<0.05$). This suggests that hyperglycaemia promotes biofilm production.

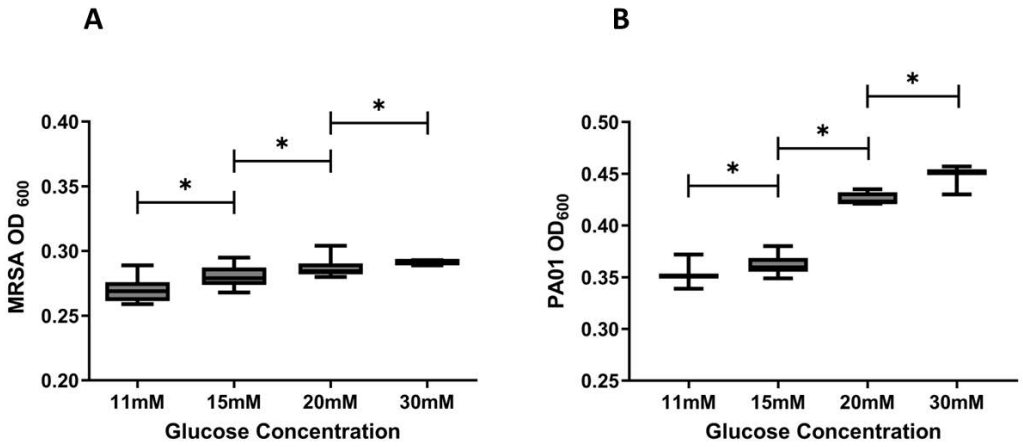

Figure 3.4 High Glucose Stimulates Biofilm Formation in *Vitro*.
Elevated glucose levels (15, 20, 30mM) significantly (*P<0.05; n=24) increased biofilm formation of both MRSA (A) and *P. aeruginosa* PAO1 (B) compared to bacteria cultured under 11 mM glucose conditions. * Indicates significant differences between glucose treatments (One-way ANOVA tests). Error bars represent the standard error of the mean (StEM).

3.4.4 The Effect of Diabetes on Macrophages

3.4.4.1 The Effect of High Glucose on Macrophages Viability

U937 monocytes were cultured under elevating glucose concentrations (11, 15, 20, 30 mM) for two weeks to model chronic hyperglycaemia *in vitro*. Monocytes were then differentiated into M0 and M1 macrophages cultured under corresponding glucose conditions. The viability of the macrophages was then quantified using a TC10 automated cell counter using methods described in 2.4. A significant (P<0.05) concentration-dependant decline in M0 and M1 macrophage viability was observed with increasing glucose concentration (Figure 3.5). These results suggest that the prolonged hyperglycaemic microenvironment associated with diabetes negatively affects both M0 and M1 macrophage populations through the promotion of cell death.

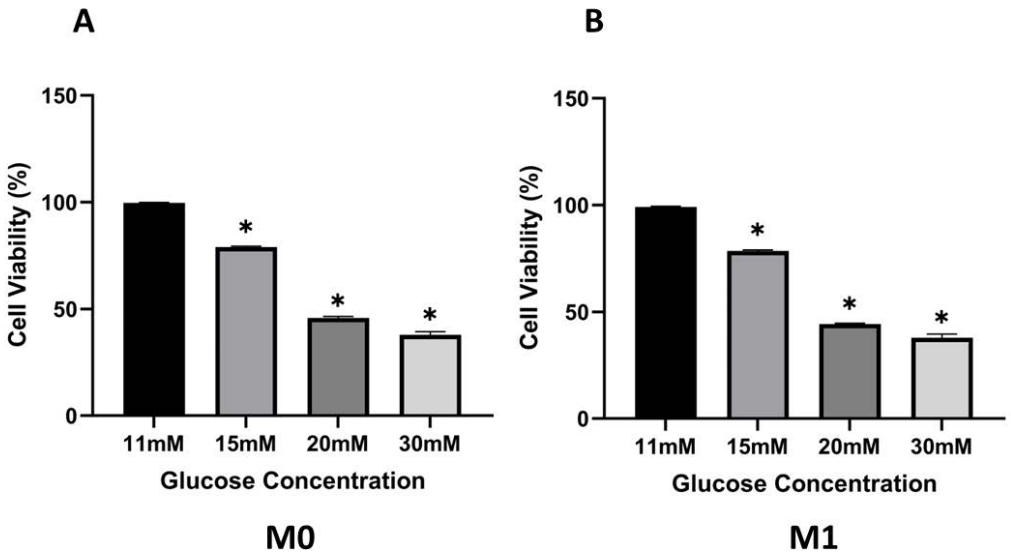

Figure 3.5 Glucose Reduces the Viability of Both M0 and M1 Macrophages *in Vitro*.
Elevated glucose levels (15, 20, 30mM) significantly (*P<0.05) decreased the viability of both M0 (A) and M1 (B) macrophages compared to cells treated with euglycemic glucose levels (11 mM). Data are shown as average of n = 24 experiments. * Represents significant differences between elevated glucose concentrations and the 11mM euglycemic control (One-way ANOVA tests). Error bars represent the standard error of the mean (StEM)

3.4.4.2 The Effect of High Glucose on the Classical-Activation of Macrophages

To investigate the influence of glucose on the phenotypic polarization of macrophages *in vitro*, monocyte-derived macrophages were cultured under control (11 mM) or GS (15, 20 and 30 mM) conditions prior to examining the expression of CD11b and CD197 via flow cytometry (Figure 3.6). Panel A presents flow cytometry histograms showing the expression of CD11b and CD197 in both resting M0 macrophages (top rows) and classically activated M1 macrophages (bottom rows) cultured under 11 mM and 30 mM glucose conditions. For M0 macrophages, a left shift in the CD11b histogram was observed for cells grown in 30 mM glucose media compared to 11 mM, indicating significantly lower surface expression of CD11b. This suggests that high glucose levels impair the differentiation of monocytes into resting M0 macrophages compared to euglycemic exposure levels. In contrast, the histograms reveal a right shift in CD197 expression in M1 macrophages cultured under 30 mM glucose compared to 11 mM control. This rightward shift, representing increased CD197 levels, demonstrates that glucose enhanced the classical activation of M0 macrophages into the M1 phenotype in a concentration-dependent manner. Panel B presents the relative MFI, confirming the findings

observed in the histograms of panel A. Hyperglycaemia (15, 20 and 30 mM) resulted in a concentration-dependent significant ($P<0.05$) decrease in the MFI of CD11b on M0 macrophages compared to 11 mM control levels, indicating impaired differentiation into the resting state. Hyperglycaemia (15, 20 and 30 mM) also resulted in a concentration-dependent significant ($P<0.05$) increase in the MFI of CD197 on M1 macrophages compared to 11 mM control levels, indicative of enhanced M1 polarization under elevated glucose conditions. These novel findings suggest that hyperglycaemia promotes the pro-inflammatory polarization of tissue macrophages into an M1 phenotype.

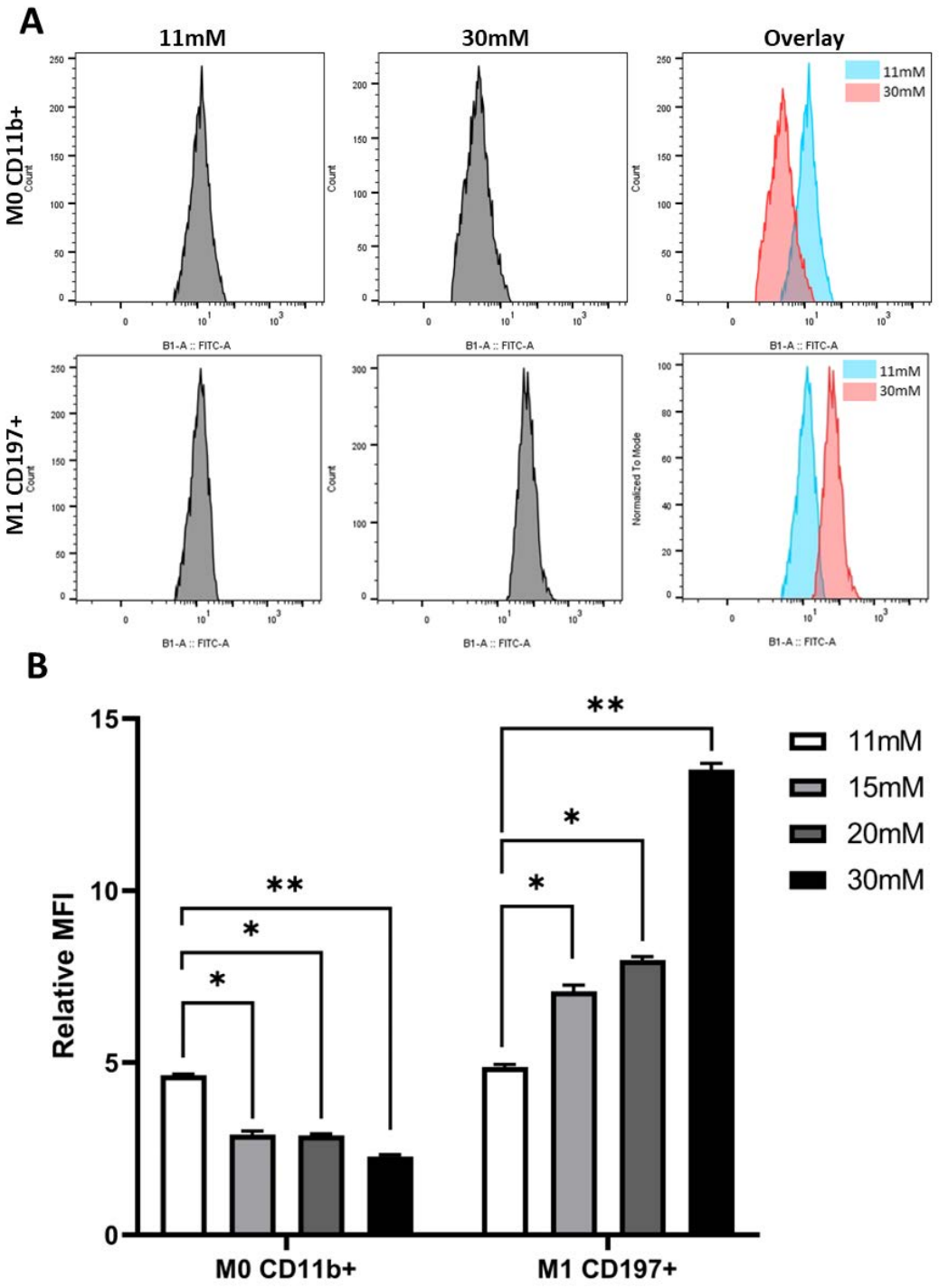

Figure 3.6 High Glucose Impairs the Differentiation of Monocytes into M0 Macrophages but Stimulates Subsequent Polarisation toward a M1 Phenotype.
Compared to the 11mM glucose control, elevated glucose (15 mM, 20 mM and 30 mM) impaired the differentiation of monocytes into resting M0 CD11b+ macrophages but enhanced polarization towards a pro-inflammatory M1 CD197+ phenotype. (A) Histograms showing CD11b (M0) and CD197 (M1) expression in macrophages cultured at 11mM or 30mM glucose, with left shift in CD11b and right shift in CD197 observed at 30mM glucose. (B) Quantification of MFI showing levels for CD11b in M0 significantly decreased while levels for CD197 in M1 significantly increased (*P<0.05, **P<0.001) in a glucose concentration-dependent manner compared to the 11mM control. Data represent the mean MFI ± StEM, n=6.

3.4.4.3 The Effect of High Glucose on the Phagocytosis of MRSA and *P. aeruginosa* Planktonic Bacteria

Monocyte-derived M0 macrophages were cultured in increasing glucose concentrations (11, 15, 20, 30mM) prior to incubation with MRSA11 and *P. aeruginosa* (PA01) strains for 3 hours to allow host-pathogen interactions to occur. The MRSA recovery (CFU/mL) significantly ($P< 0.05$) increased with increasing glucose concentration (Figure 3.7.A). Similarly, the PAO1 recovery (CFU/mL) significantly ($P< 0.05$) increased as the glucose concentration increased (Figure 3. 7.B). These findings indicated that elevated glucose impaired the clearance of both MRSA and PA01 by M0 macrophages in a dose-dependent manner, particularly at 30mM (Figure.3.7).

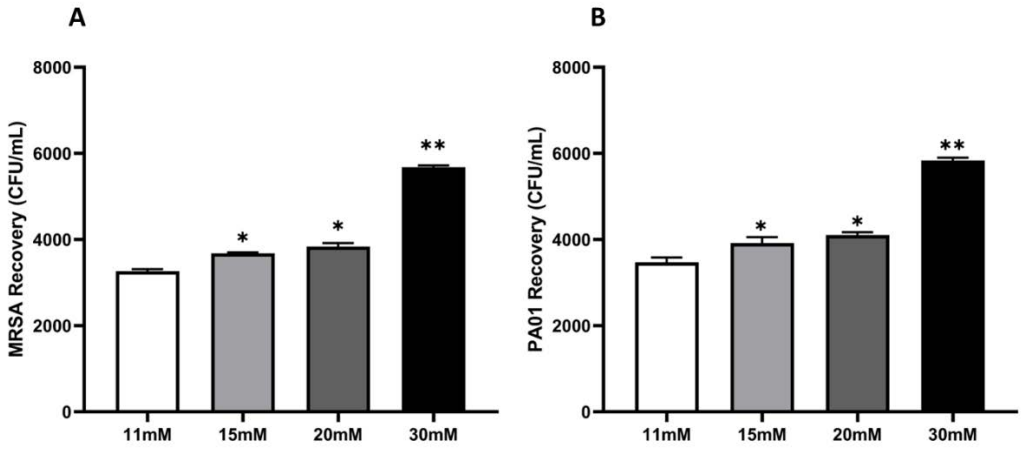

Figure 3.7 Glucose Reduces the Phagocytosis of MRSA and PAO1 by M0 Macrophages. The mean MRSA and PAO1 recovery (CFU/mL) per million viable macrophages was determined after 3 hours of host-pathogen interaction. The clearance of MRSA (A) and PAO1 (B) by M0 macrophages significantly (*$P<0.05$, **$P<0.001$; n=12) reduced in a glucose concentration-dependent manner. Data represent bacterial recovery (CFU/mL) ± StEM.

Similarly, elevated glucose inhibited the phagocytosis of MRSA and *P. aeruginosa* by M1 macrophages (Figure 3.8). The number of recovered MRSA (A) and PAO1 (B) colonies significantly increased in a glucose concentration-dependent manner ($P<0.05$), suggesting glucose has a dose-dependent detrimental effect on M1-mediated bacterial clearance.

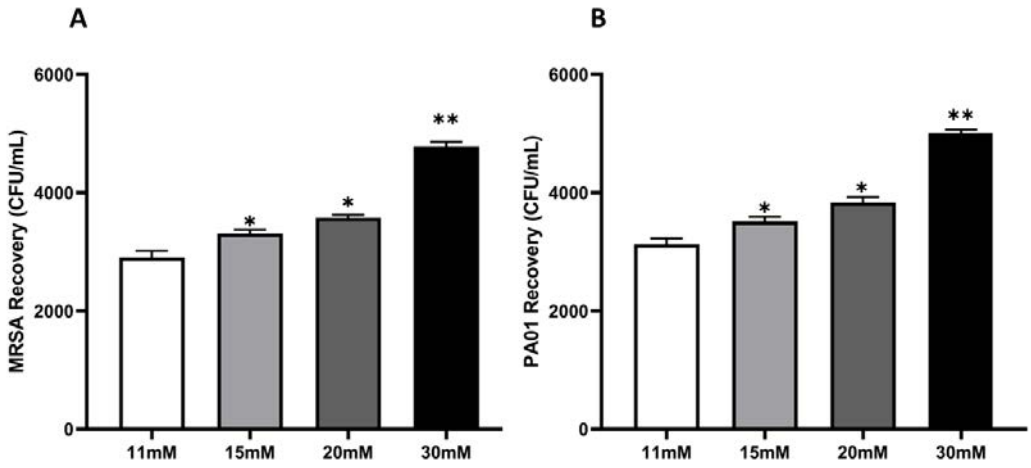

Figure 3.8 Glucose Dampens the Phagocytosis of MRSA and PAO1 by M1 Macrophages. The mean MRSA and PAO1 recovery (CFU/mL) per million viable macrophages was determined after 3 hours of host-pathogen interaction. The clearance of MRSA (A) and PAO1 (B) by M1 macrophages significantly (*$P<0.05$, **$P<0.001$; n=12) reduced in a glucose concentration-dependent manner. Data represent bacterial recovery (CFU/mL) ± StEM.

3.4.5 The Effect of High Glucose on Cytokine Production

3.4.5.1 Effect of High Glucose on the Secretion of TNF-α

TNF-α is a pro-inflammatory cytokine that plays a fundamental role in the pathogenesis of DFUs (Goren et al., 2007). To investigate the effect of high glucose levels on inflammation, TNF-α protein secretion was measured in glucose supplemented (GS) M1-like macrophages via ELISA. Elevated glucose levels (15 mM, 20 mM and 30 mM) significantly increased the production of TNF-α by M1 macrophages compared to the 11mM GS macrophages in a concentration-dependent manner ($P<0.05$). Since TNF-α is a key marker for inflammation (Ashcroft et al., 2012; Zelová and Hošek, 2013), our results indicate that

macrophage exposure to elevated glucose could potentially exacerbate the prolonged and excessive inflammatory responses observed in DFUs (Figure 3.9).

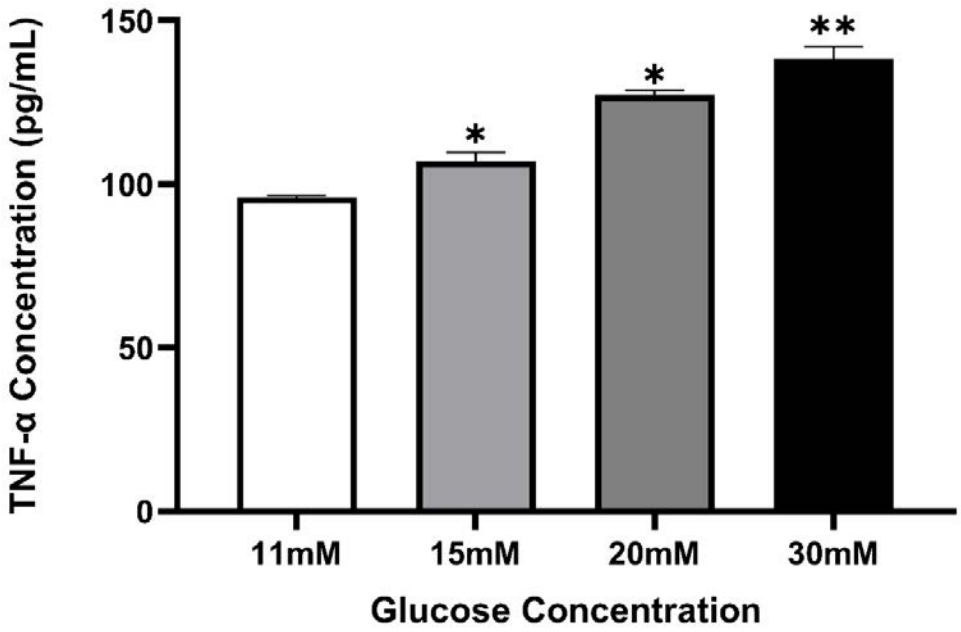

Figure 3.9 Glucose Induces TNF-α Secretion by M1 Macrophages *in Vitro*.
Compared to 11mM glucose control macrophages, the secretion of TNF-α by M1 macrophages significantly (*P< 0.05, **P< 0.001; n=6) increased with elevated glucose concentrations (15 mM, 20 mM and 30 mM) in concentration-dependent manner. Data represent mean TNF-α concentration (pg/mL) ± standard error of the mean (StEM).

3.4.5.2 Effect of High Glucose on the Secretion of Interferon beta (IFN-β)

IFN-β is an anti-inflammatory cytokine that plays a fundamental role in wound repair in DFUs (Raziyeva et al., 2021; Song et al., 2022). To investigate the effect of elevated glucose levels on the later stages of inflammation, IFN-β protein secretion by glucose-supplemented M1 macrophages was measured via ELISA. Increasing the glucose concentration resulted in a significant concentration-dependent reduction (P< 0.05) in the levels of IFN-β compared to control macrophages cultured under 11mM GS. This finding indicates that glucose inhibited the secretion of IFN-β from M1 macrophages, suggesting glucose may prolong the inflammatory phase in DFUs by dampening the anti-inflammatory effects of IFN-β (Figure 3.10).

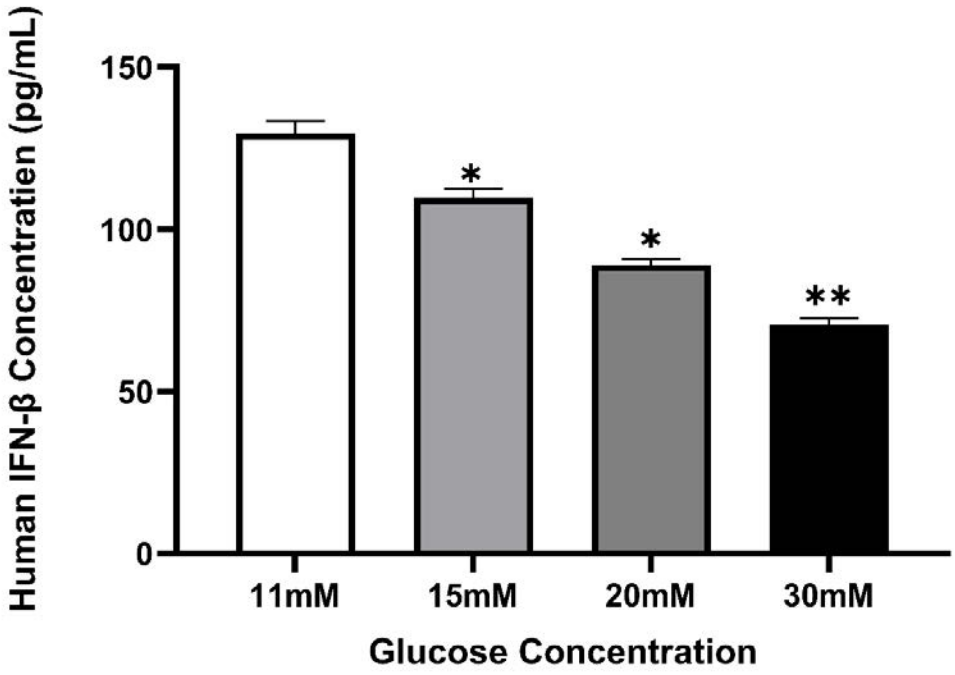

Figure 3.10 Glucose Decreases the Levels of IFN-β Released by M1 Macrophages *in Vitro*. Compared to control macrophages cultured under 11mM glucose, the secretion of IFN-β by M1 macrophages significantly (*P<0.05, **P<0.001; n=6) decreased with elevated glucose concentration (15 mM, 20 mM and 30 mM) in concentration-dependent manner. Data represent mean IFN-β concentration (pg/mL) ± StEM.

3.4.6 Effect of High Glucose on Nitric Oxide Production by M1 Macrophages

Over time, it has been established that nitric oxide (NO) plays significant physiological functions by facilitating vasodilation, boosting immune defences against infections, and promoting the regeneration of tissues (Ignarro, 1996; Lundberg and Weitzberg, 2022; Andrabi et al., 2023). Therefore, understanding the impact of hyperglycaemia on NO production by macrophages is important, since both excessive and deficient NO levels have been implicated in the pathogenesis of diabetic foot ulcers (Y. Yang et al., 2016; Deng et al., 2023). There was a significant (P<0.05) decrease in NO production by M1 macrophages cultured at all glucose concentrations (15, 20 and 30 mM) compared to 11mM GS control macrophages (Figure 3.11), suggesting hyperglycaemia inhibits NO production from M1 macrophages.

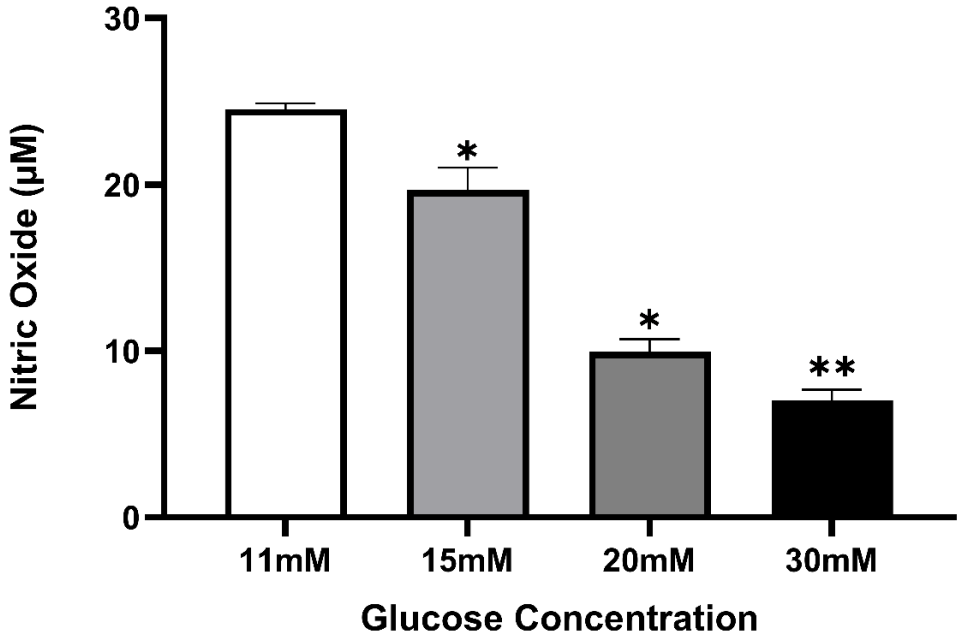

Figure 3.11 Glucose Reduced the Production of NO by M1 Macrophages.
Compared to control macrophages cultured at 11mM glucose, the production of NO by M1 macrophages significantly (*P<0.05, **P<0.001; n=6) decreased with elevated glucose concentration (15 mM, 20 mM and 30 mM) in concentration-dependent manner. Data represent mean IFN-β concentration per million viable M1 macrophages (pg/mL) ± StEM.

3.4.7 Effect of High Glucose on the NF-κB Inflammatory Pathway in M1-like Macrophages

To gain insight into the impact of high glucose levels on the NF-κB inflammatory pathway, confocal microscopy was used to visualise NF-κB nuclear translocation in glucose-treated M1 macrophages. M1 macrophages were cultured in either 11mM or 30mM glucose representing euglycaemic and hyperglycaemic conditions respectively. Cells were fixed with 4% paraformaldehyde and stained with a fluorescent NF-κB subunit p65 antibody as described in 2.12.2. Z-stack imaging on a STELLARIS 5 confocal microscope and subsequent analysis on ImageJ software allowed the visualisation and quantification of nuclear and cytoplasmic NF-κB localisation (Figure 3.12).

Compared to euglycemic (11mM glucose) control conditions, elevated glucose levels significantly induced an increase in both total NF-κB expression (Figure 3.12C, *P<0.05) and its nuclear translocation (Figure 3.12A, B, **P<0.001) in M1 macrophages under high glucose (30mM) conditions. Quantitatively,

hyperglycaemia augmented the nuclear/cytoplasmic ratio of the RelA P65 subunit from 0.23 at 11mM glucose to 0.48 at 30mM glucose (**P<0.001). These findings reveal a direct relationship between hyperglycaemia and activation of the pro-inflammatory NF-κB pathway in M1 macrophages, highlighting their sensitivity to high glucose levels. Further studies investigating the downstream inflammatory effects mediated by NF-κB signalling under hyperglycaemic conditions could provide additional mechanistic insight.

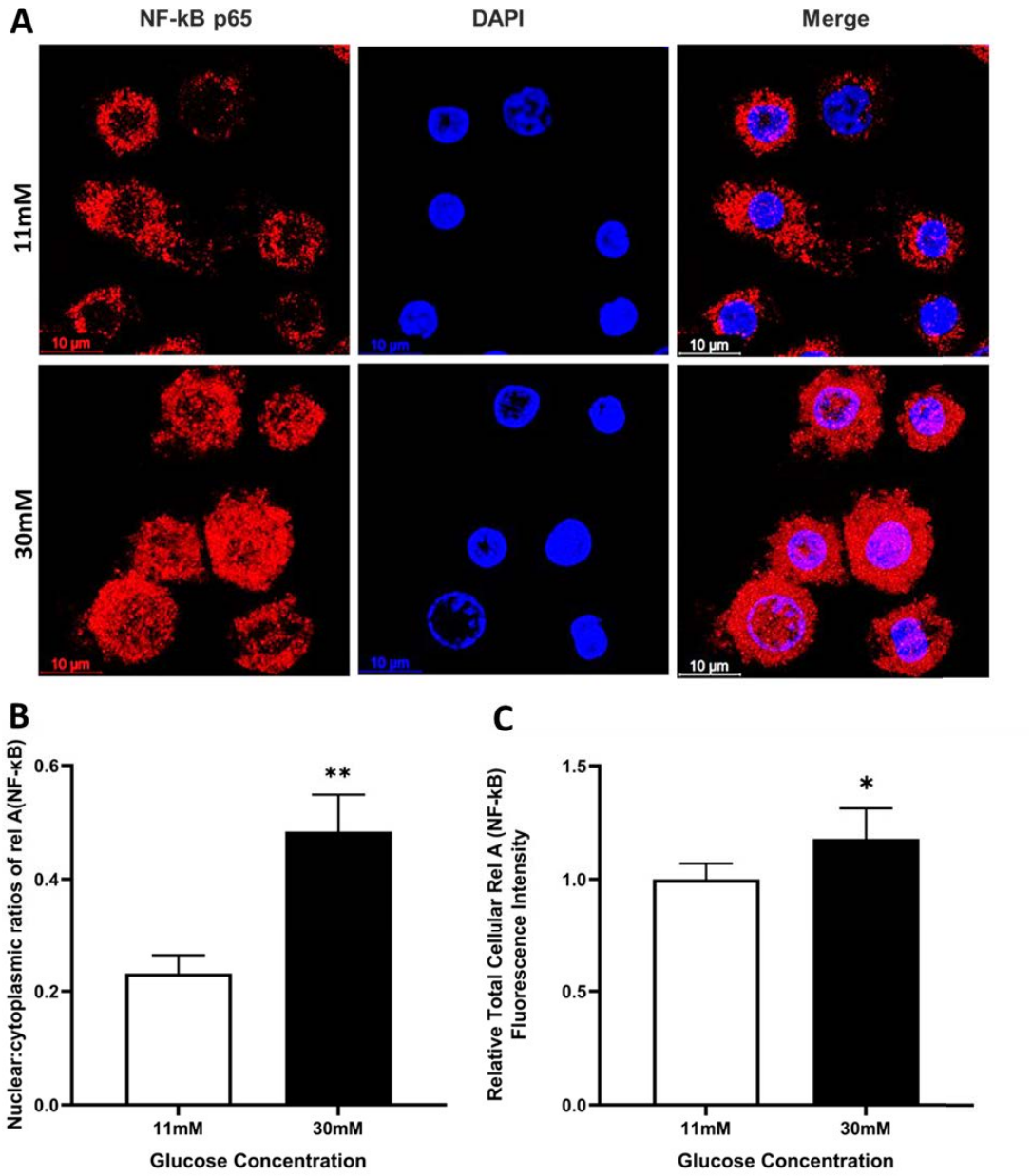

Figure 3.12 High Glucose Induces the Translocation of NF-κB p65 from the Cytoplasm to Nuclei.

Compared to control macrophages cultured under 11mM glucose, the nuclear translocation of the NF-κB p65 subunit was increased under high (30mM) glucose conditions. A: Representative confocal images of the p65 subunit of NF-κB (red) in M1 macrophages and cell nuclei (blue: DAPI) showed nuclear translocation (purple: merged blue & red) when M1 were cultured under 30mM glucose conditions. Images were captured with x100 objective magnification, scale bars: 10 µm. B: Nuclear/cytoplasmic ratios quantified using ImageJ software showing elevated Rel A (NF-κB) subunit p65 expression in M1 cultured under 30mM hyperglycaemic conditions (**P≤ 0.001) compared to the 11mM control. C: Relative total cellular NF-κB fluorescent intensity quantified using ImageJ (*P≤ 0.05 compared to 11mM glucose control). Results represent an average of n = 12 experiments. Error bars indicate the standard error of the mean (StEM).

3.4.8 The Effect of High Glucose on Protein Expression of Macrophage-Associated Receptors and Ligands

3.4.8.1 The Effect of High Glucose on M1 Macrophage-Associated CD14 Expression

The pattern recognition receptor CD14 has a major effect on the control of innate immune responses and pathogen identification (Pugin et al., 1994). The effect of hyperglycaemia on M1 expression of mCD14 and secretion sCD14 protein levels were investigated by flow cytometry and ELISA assays respectively. Monocyte-derived M1 macrophages were grown in CM containing euglycemic glucose (11 mM) levels or GS-media containing elevated glucose concentrations (15 mM, 20 mM and 30 mM) to model the hyperglycaemic associated with uncontrolled diabetes. Flow cytometry analysis (Figures 3.12) revealed that elevated glucose (30mM) significantly increased the expression of mCD14 in M1 macrophages compared to 11mM control levels ($P<0.05$; n=6). The proportion of mCD14+ M1 macrophages (Figures 3.12.A) increased from 22.4% (11mM) to 44.7% (30mM). The MFI for mCD14 was significantly ($P<0.05$) higher in M1 macrophages cultured in high glucose level 30mM compared to M1 macrophages cultured at 11mM glucose (Figure 3.12.B). Following a similar pattern, sCD14 levels significantly increased in GS (15, 20, 30mM) M1 macrophages compared to control macrophages cultured at 11mM glucose ($P<0.05$; n=6). This concurrent glucose-induced expression of both mCD14 and sCD14 by M1 macrophages potentially implicates both forms of CD14 in the dysregulated immune signalling responses observed in DFU patients.

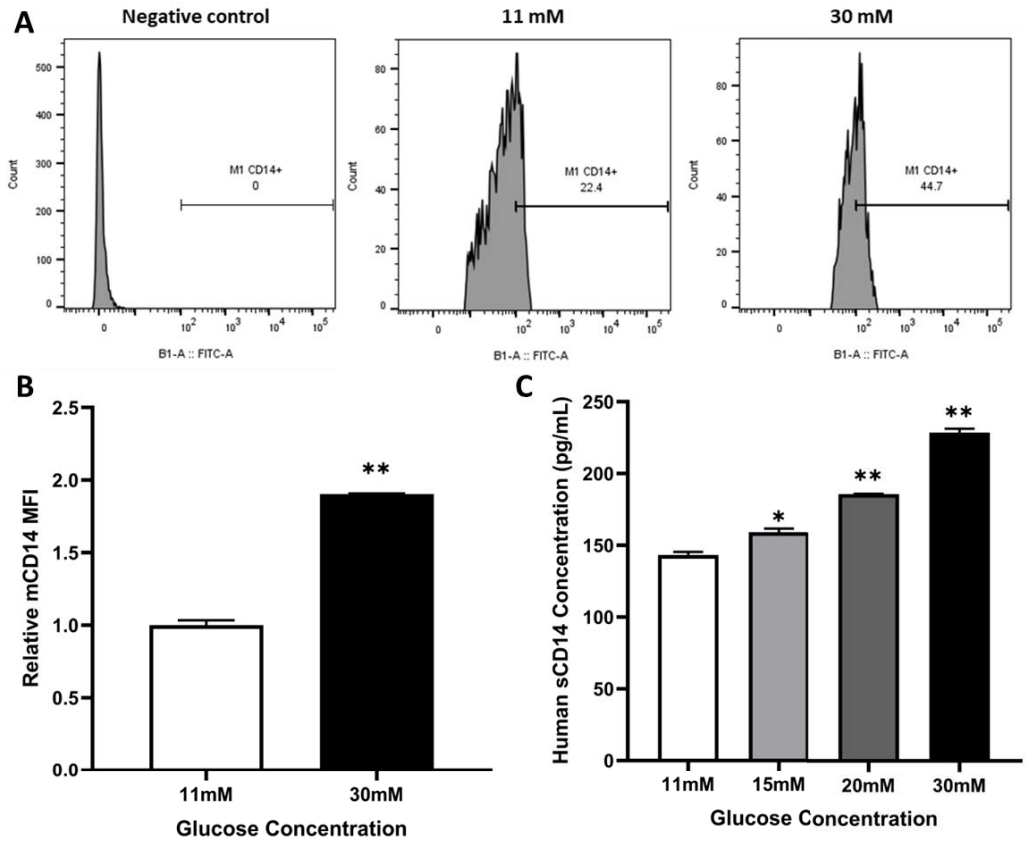

Figure 3.13 High Glucose Induces the Expression of M1 Macrophage-Associated CD14 Expression

M1 macrophages were cultured in the presence of control (11mM) or elevated (15, 20, or 30 mM) glucose concentrations. High glucose (30mM) increased the percentage of mCD14-positive M1 macrophages (44.7%) compared to M1 macrophages cultured at low (11mM) glucose (22.4%), with unstained negative control (NC) macrophages showing no cellular fluorescence (A). This correlated with significantly (**P<0.001, n=6) higher MFI at 30mM glucose compared to 11mM control levels (B). The secretion of sCD14 (pg/mL) by M1 macrophages significantly (*P<0.05, **P<0.001; n=6) increased with elevated glucose concentration (C). Data in B and C represent mean CD14 levels ± StEM.

3.4.8.2 The Effect of High Glucose on M1 Macrophage-Associated CD33 Expression

The cell surface receptor CD33 is essential for pathogen identification and phagocytosis (Paul et al., 2000; Butler et al., 2021). Thus, the effect of hyperglycaemia on mCD33 expression and secretion of sCD33 by M1 macrophages was assessed by flow cytometry and ELISA respectively. Monocyte-derived M1 macrophages were grown in CM containing euglycemic glucose (11 mM) levels or GS-media containing elevated glucose concentrations (15 mM, 20 mM and 30 mM) to model the hyperglycaemic levels associated with uncontrolled diabetes. Flow cytometry analysis revealed a substantial decrease in mCD33 expression in

macrophages supplemented with elevated glucose (15mM - 22.15%, 20mM - 15.45%, 30mM - 12.01%) compared with those cultured in control levels (11mM - 24.29%) of glucose. Similarly, ELISA quantification showed that elevated glucose (15, 20, 30mM) significantly reduced sCD33 secretion in M1 macrophages compared with macrophages cultured in control (11mM) levels of glucose (Figure 3.11C, $P < 0.05$; n=6). These findings collectively suggest that the diabetic milieu may cause downregulation of CD33 expression in pro-inflammatory M1 macrophages.

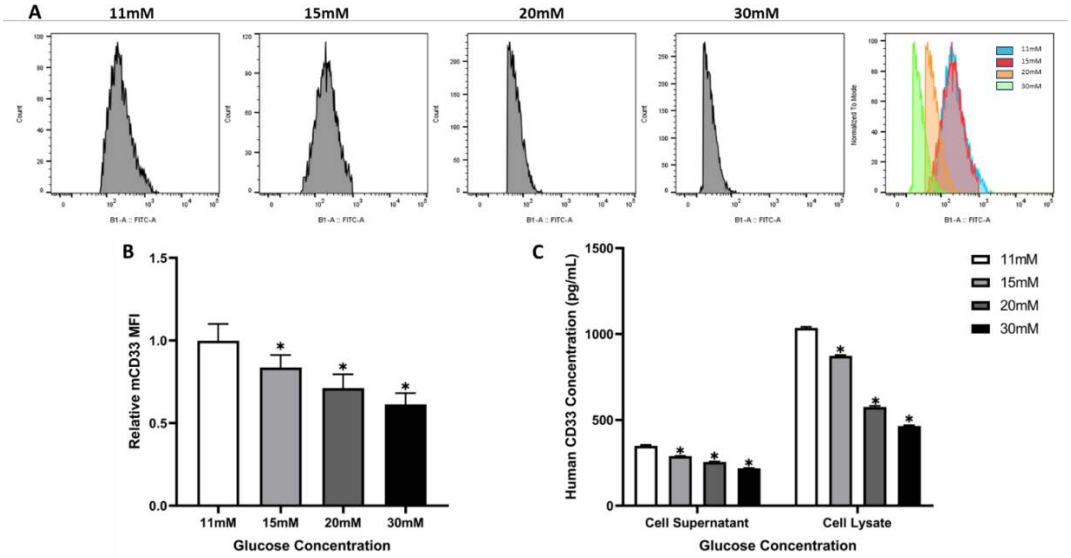

Figure 3.14 . High Glucose Reduces CD33 Expression in M1 Macrophages *in Vitro*.
M1 macrophages were cultured in the presence of control (11mM) or elevated (15, 20, or 30mM) glucose concentrations. (A) Representative flow cytometry histograms showing mCD33 protein expression on M1 macrophages cultured at each glucose concentration. A left shift in the cell population was observed with increasing glucose concentration, indicating reduced CD33 expression compared to the 11 mM control. (B) Quantitative analysis indicating a significant decrease in CD33 MFI in macrophages treated with elevated glucose versus normal 11mM glucose conditions (*P<0.05). (C) ELISA analysis confirmed the glucose-mediated effect on sCD33 expression by M1 macrophages *in vitro*. Elevated glucose levels significantly reduced sCD33 protein levels compared to 11mM normal glucose (*P<0.001). Data in B and C represent mean CD33 levels ± StEM from n=6 independent experiments.

3.4.8.3 The Effect of High Glucose on M1 Macrophage-Associated TREM2 and ApoE Expression

TREM2 is an immunomodulatory receptor that regulates phagocytosis as well as inflammation (Deczkowska et al., 2020; Dabla et al., 2022). Given the role of TREM2 signalling in innate immunity, the effect of hyperglycaemia on M1 macrophage-associated mTREM2 and sTREM2 protein expression was measured in GS macrophages via flow cytometry and ELISA respectively. Flow cytometry histograms (Figure 3.14A) revealed a left shift in the fluorescence intensity curve for mTREM2 in M1 macrophages exposed to elevated glucose (red histogram) compared to the euglycemic control (blue histogram), indicating decreased mTREM2 expression under hyperglycaemic conditions. Quantitative analysis (Figure 3.14B) confirmed that elevated glucose levels significantly (*P<0.001, n=6) reduced the MFI of mTREM2 in M1 macrophages compared to untreated controls.

ELISA analysis (Figure 3.14C) further demonstrated that elevated glucose concentrations (15, 20, and 30 mM) significantly decreased the levels of sTREM2 secreted by M1 macrophages compared to the 11 mM control (*P<0.05).

In summary, these findings indicate that high glucose exposure inhibits the expression of both mTREM2 and sTREM2 proteins in M1 macrophages *in vitro*, potentially impacting immunomodulatory function under hyperglycaemic conditions.

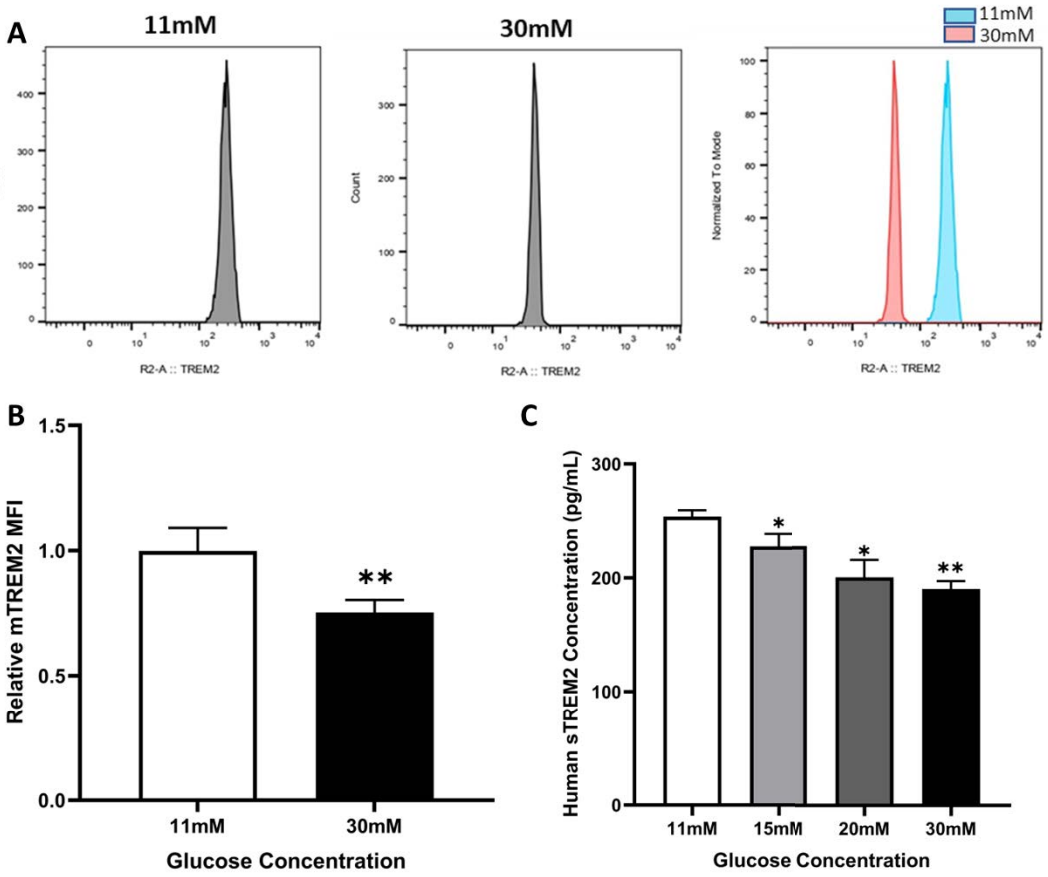

Figure 3.15 High Glucose Reduces TREM2 Expression in M1-Like Macrophages.
M1 macrophages were cultured in the presence of control (11mM) or high (15, 20, or 30mM) glucose concentrations. (A) Representative overlaid flow cytometry histograms showing mTREM2 protein expression in M1 macrophages cultured at different glucose concentrations. A left shift in the cell population is observed with increasing glucose levels, indicating reduced mTREM2 expression compared to 11mM control conditions. (B) Quantitative analysis indicating a significant decrease in mTREM2 MFI in macrophages treated with high glucose 30mM versus normal 11mM glucose conditions (*P<0.05). (C) ELISA analysis confirmed the glucose-mediated effect on sTREM2 expression by M1 macrophages *in vitro*. Elevated glucose levels significantly reduced sTREM2 levels compared to 11mM normal glucose (*P<0.05, **P<0.001). Data in B and C represent the mean ± StEM from n=6 independent experiments.

To further investigate the TREM2 inflammatory pathway, the expression of the TREM2 ligand ApoE was assessed via flow cytometry and ELISA to determine intracellular and secreted levels respectively. M1 macrophages were cultured in euglycemic (11mM) or high glucose concentration (30mM) prior to quantifying the intracellular levels of ApoE by flow cytometry. ELISA was used to measure the amount of secreted ApoE released from macrophages cultured across a range of glucose concentrations (11 mM, 15 mM, 20 mM and 30 mM). Intracellular ApoE was significantly reduced (P<0.05; n=6) in M1 macrophages cultured in high glucose

(30mM) compared to control macrophages cultured in 11mM glucose. Similarly, macrophages cultured in elevated glucose (15 mM, 20 mM and 30 mM) significantly ($P<0.05$; n=6) reduced secreted levels of ApoE compared to macrophages cultured in 11mM glucose. In summary, these data indicate elevated glucose levels downregulate protein expression of ApoE and its secretion from M1 macrophages.

Collectively, the TREM2 and ApoE findings suggest elevated glucose levels in M1 macrophages may lead to inhibition of the TREM2 signalling pathway through synergistic downregulation of both ApoE and its TREM2 receptor.

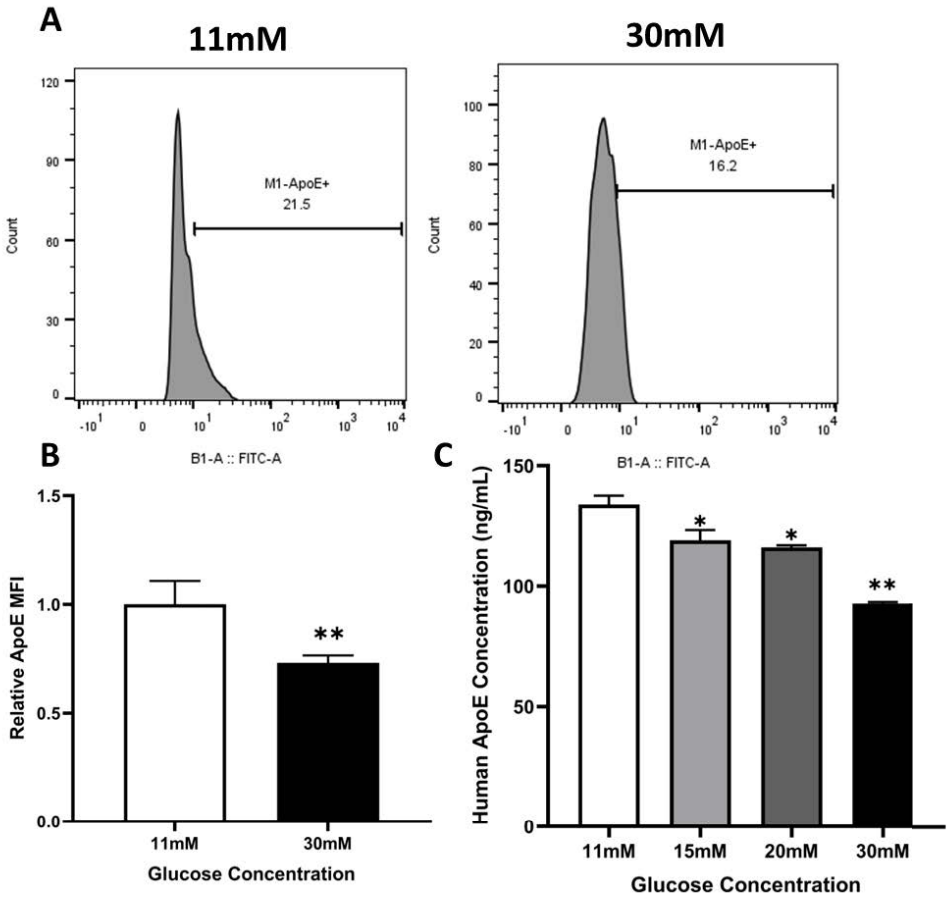

Figure 3.16 High Glucose Reduces ApoE Expression in Monocyte-Derived M1 Macrophages.
M1 macrophages were cultured in the presence of control (11mM) or elevated (15, 20, or 30mM) glucose concentrations. (A) Representative flow cytometry histograms showing intracellular ApoE protein expression in M1 macrophages cultured at 11 and 30mM glucose concentrations. A reduction in the ApoE+ cells is observed with increasing glucose levels, indicating reduced ApoE expression compared to 11mM control conditions. (B) Quantitative analysis indicating a significant decrease in ApoE MFI in macrophages cultured under high (30mM) glucose compared the 11mM control (*P<0.01. (C) ELISA analysis showed elevated glucose significantly reduced ApoE secretion from M1 macrophages compared to the 11mM control in a dose-dependent manner (*P<0.01; **P<0.001). Data in B and C represent the mean ± StEM from n=6 independent experiments

3.4.9 The Effect of High Glucose on the Actin Cytoskeleton Organisation in M1 Macrophages

The dynamic movement of actin cytoskeleton proteins is required for effective immune responses, particularly actin cytoskeleton polymerisation during the phagocytosis of pathogens by macrophages (Rougerie et al., 2013). The effect of hyperglycaemia (30mM glucose) on filamentous actin (F-actin) was investigated by confocal microscopy following phalloidin staining of M1 macrophages. Structural

alterations in the actin cytoskeleton were observed between M1 macrophages cultured under hyperglycaemic conditions and control macrophages grown in euglycemic (11mM) levels of glucose (Figure 3.16) with decreased number and length of F-actin filament extensions from M1 macrophages cultured under hyperglycaemic conditions, together with the development of prominent, rounded and punctate F-actin aggregates that were not observed in control macrophages cultured under euglycemic conditions. The quantitative measure of F-actin filament extension length (Figure 3.16.C) showed a significant ($P<0.001$) decrease in M1 macrophages cultured under hyperglycaemic conditions. Additionally, the number of extensions per M1 macrophage (Figure 3.16.E) also significantly decreased ($P<0.001$) under hyperglycaemic conditions compared to control.

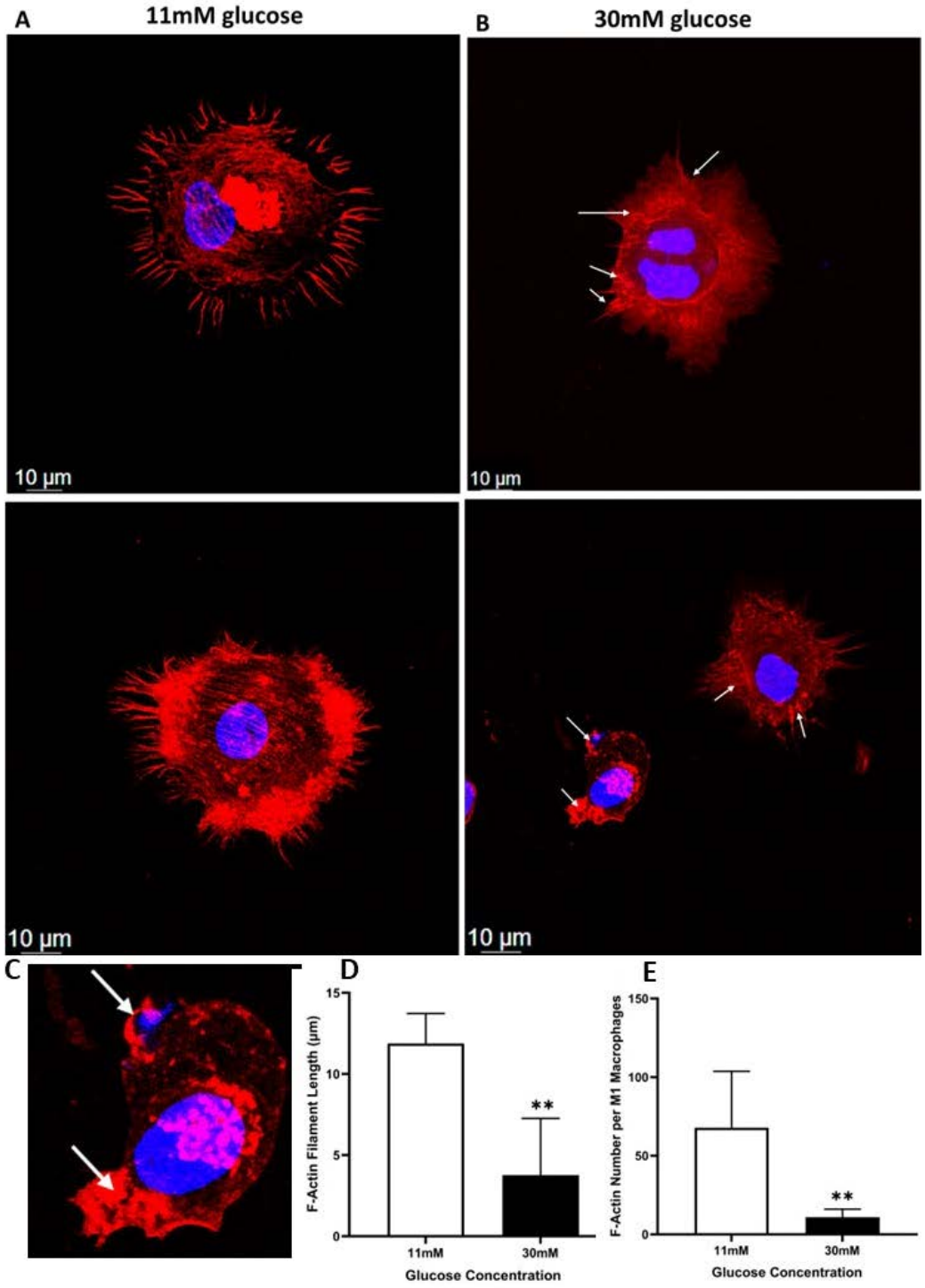

Figure 3.17 High Glucose Induces Alteration in Actin Organisation in M1 Macrophages.
The effect of elevated (30mM) glucose on cytoskeleton organisation was observed in M1 macrophages by confocal microscopy via labelling of F actin filaments with phalloidin (red). Compared to control macrophages cultured under euglycemic (11mM glucose) conditions (A), hyperglycaemia (30mM glucose) significantly (**P<0.001; n=12) decreased F-actin filament extension length and number in M1 macrophages (D,E), and promoted the formation of distinct, rounded and punctate F-actin aggregates (white arrows; B and C). Images were captured using a x100 oil objective, with scale bars: 10 µm. Data in panel D and E represent mean filament extension length and number from three independent captured images with > 5 cells per captured field.

3.5 Discussion

DFUs are a serious complication of diabetes that are characterised by chronic, non-healing lesions that are often colonised by bacteria such as *S. aureus* and *P. aeruginosa* (Macdonald et al., 2021). Sustained hyperglycaemia underlies the impaired wound healing in DFUs and results in peripheral systemic effects that compromise the function of various cells typically engaged in wound repair processes (Burgess et al., 2021; Swoboda and Held, 2022). In normal conditions, the process of wound healing is a well-coordinated event that starts with the inflammatory response involving a variety of immune cells, including tissue macrophages (Ellis et al., 2018). Macrophages play a crucial role in wound healing because of their host defence functions to eradicate microbes and promotion of cell proliferation in the tissue regenerative phase of wound repair through the release of cytokines and growth factors (Koh and DiPietro, 2011; Krzyszczyk et al., 2018).

The aim of this chapter was to establish evidence for the range of negative consequences of prolonged hyperglycaemia on the function of M1 macrophages in order to form the basis for subsequent chapters investigating whether estrogen supplementation can successfully reverse one or more observed detrimental effects of diabetes that is attributed to elevated glucose. Four major effects of elevated glucose were investigated: (1) diminished phagocytic capacity, (2) impaired bacterial clearance due to either enhanced bacterial growth and/or biofilm formation, (3) dysregulated inflammatory responses such a M1 polarization, and (4) altered expression of receptors, receptor ligands or downstream mediators involved in pathogen recognition or signalling pathways that control bacterial clearance.

Before conducting investigations of the effects of hyperglycaemia on M1 macrophages, it was essential to establish an appropriate *in vitro* model of chronic hyperglycaemia to mimic the development of diabetes *in vitro*. According to established guidelines, an individual is diagnosed with diabetes when their random plasma glucose level reaches 11.1 mmol/L or higher (International Expert

Committee, 2009; The American Diabetes Association, 2014). In the present study which utilised *in vitro* cultured cells that do not undergo daily periods of fasting, a model was developed based on the upper limit of the random glucose test. Therefore, 11mM glucose was used as the 'maximal' euglycemic control in all experiments, and in order to simulate moderate to severe hyperglycaemia, further glucose concentrations of 15, 20 and 30 mM were used.

U937 monocytes were then differentiated into M0-like macrophages, confirmed via the detection of the cell surface marker CD11b (Geissmann et al., 2003; Arndt et al., 2007; Lumeng et al., 2007), and subsequent polarization of M0 macrophages into the proinflammatory M1-like phenotype was confirmed via detection of the cell surface marker CD197 (Martinez et al., 2008). M0 and M1-like macrophages were also maintained at the same glucose conditions as the corresponding U937 monocytes they were derived from throughout investigations to provide a model that closely mirrors sustained hyperglycaemia observed in wound tissue fluids of DFU patients with chronic, uncontrolled diabetes.

The effect of hyperglycaemia on both planktonic bacterial growth and biofilm formation were assessed using MRSA and *P. aeruginosa*. In comparison to the euglycemic glucose (11mM) controls, the planktonic growth of both bacterial strains was significantly ($P<0.05$) promoted by elevated glucose in a concentration-dependent manner. Similarly, the biofilm mass of both MRSA and *P. aeruginosa* increased proportionately with rising glucose levels in a concentration-dependent manner. These findings highlight the stimulatory effect of hyperglycaemia on the proliferation of bacteria and the development of mature bacterial biofilms. Other researchers have also found that elevated glucose levels provide an ideal environment for bacterial growth and biofilm formation (Chávez-Reyes et al., 2021; Fernández-Grajera et al., 2022). Although there is evidence linking hyperglycaemia to increased bacterial growth, it is important to appreciate these effects may differ between experimental conditions and bacterial strains. Moreover complex *in vivo* wound environments are typically colonised by multiple bacteria, and often fungal organisms, whose relative abundance and virulence with depend on multiple, interdependent factors (Byrd et al., 2018; Maheswary et al., 2021; Short et al.,

2023). Hyperglycaemia can promote bacterial growth by providing an abundant glucose source for bacterial nutrition (Garnett et al., 2013; S. K. Gill et al., 2016), impairing immune functions (Chávez-Reyes et al., 2021), increasing protein glycosylation that facilitates bacterial adhesion (Ozer et al., 2015), altering wound environment pH and osmolarity, and inducing oxidative stress that compromises host defences (Chávez-Reyes et al., 2021). However, the specific mechanisms and extent of increased bacterial growth may vary across bacterial species, strains, and the complex interplay of factors in *in vivo* wound environments colonised by diverse microbial communities.

Prolonged hyperglycaemia caused a significant ($P<0.05$) glucose concentration-dependent reduction in M0 and M1 viability. The findings suggested that M0/M1 macrophage populations may be negatively impacted by diabetes-related chronic exposure to high glucose. These findings are in accordance with research showing immune cells, including macrophages, can experience apoptosis through compromised cell viability and cell function when subjected to hyperglycaemia (Smart and Li, 2007; Dasu et al., 2010a; Morey et al., 2019). Indeed, evidence suggests hyperglycaemia may induce cellular damage through various mechanisms, such as oxidative stress, advanced glycation end-product (AGE) formation, and altered intracellular signalling pathways (Rattan et al., 1997; Vlassara and Striker, 2011; Xiu et al., 2016). Hyperglycaemia has been shown to increase reactive oxygen species (ROS) production (Dludla et al., 2017; Kaludercic and Di Lisa, 2020; González et al., 2023), leading to oxidative damage to cellular components like DNA and cell death (Newsholme et al., 2007; Newsholme et al., 2016; Kaludercic and Di Lisa, 2020). Additionally, the formation of AGEs and their interaction with receptors (RAGE) can trigger inflammatory responses and apoptotic pathways in macrophages (Ramasamy et al., 2011; Asadipooya and Uy, 2019). Furthermore, hyperglycaemia has been demonstrated to impair intracellular signalling cascades, such as the PI3K/Akt, MAPK/ERK, and NF-κB pathway, which are crucial for cell survival and function (Singh et al., 2014; Asadipooya and Uy, 2019; Peng et al., 2022).

Regulation of inflammatory responses requires a coordinated transition between macrophage states and an imbalance in macrophage polarization, particularly delayed M1 activation, can lead to the development of chronic inflammation (Wang et al., 2014; Parisi et al., 2018; Xia et al., 2023). To investigate the influence of glucose on the polarization of macrophages, monocyte-derived macrophages were cultured under control (11 mM) or elevated (15, 20 and 30 mM) glucose concentrations prior to examining the expression of CD11b and CD197 via flow cytometry. Resting M0 macrophages cultured in 30mM glucose had significantly ($P<0.05$) reduced CD11b expression, indicating hyperglycaemia has a detrimental impact on M0 macrophage differentiation. M1-directed macrophages cultured under hyperglycaemic conditions, on the other hand, revealed a more than 2-fold increase in CD197 expression, indicating increased polarization toward the M1 phenotype when exposed to elevated glucose. The observed decrease in CD11b expression in M0 macrophages and the increase in CD197 expression in M1 macrophages under hyperglycaemic circumstances are consistent with evidence showing metabolic factors, particularly blood sugar levels, can influence immune cell activity (MacLeod and Mansbridge, 2016; Daryabor et al., 2020). Macrophages are known to be microenvironment sensitive, and variations in glucose concentrations may affect their activation states and activities (Grosick et al., 2018; Ayala et al., 2019; Sousa et al., 2023). According to Zhao et al. (2023), when exposed to high glucose levels, macrophages convert to a pro-inflammatory M1 phenotype, which causes chronic inflammation and fibrosis in diabetic kidney disease (DKD). Other evidence also indicates high glucose levels promote inflammation through the activation of murine macrophages towards the M1 phenotype (Oh et al., 2019).

Findings of the study showed glucose significantly impaired the clearance of MRSA and *P. aeruginosa* ($P<0.05$) by U937-derived macrophages in a concentration-dependent manner (Figure 3.6 and Figure 3.7). These findings are supported by several other *in vitro* research studies where hyperglycaemia impaired the phagocytosis of bacteria, including MRSA and *P. aeruginosa*, by macrophages and

neutrophils in a dose-dependent manner (Marhoffer *et al.,* 1992; Peleg *et al.*, 2007; Yano *et al.,* 2012).

The impaired phagocytosis of bacteria by macrophages under hyperglycaemic conditions *in vitro* provides a framework model for elucidating immune dysfunction in diabetes-associated infections (Fu and Harrison, 2021). Impairments in macrophage function may have cascading effects on wider immune responses given the integral role macrophages play in orchestrating immunity via cytokine signalling (Stow et al., 2009; Kumaran Satyanarayanan et al., 2019). Key opposing cytokine mediators of inflammation include TNF-α and IFN-β (Cantaert et al., 2010). When exposed to elevated glucose, M1 macrophages exhibited significantly ($P<0.05$) increased TNF-α protein secretion. Identified as a driver of excessive inflammation in DFUs, pronounced TNF-α secretion establishes and sustains a pro-inflammatory state (Acosta et al., 2008; Xu et al., 2013). In contrast, elevated glucose exposure substantially reduced secretion of the anti-inflammatory cytokine IFN-β by M1 cells. IFN-β plays an essential role in wound healing by assisting in the reduction of inflammation and promoting tissue repair (Graves and Dasu, 2011; Wolf et al., 2022). Bolívar et al. (2018) found that IFN-β triggers a significant anti-inflammatory effect in fibroblasts by activating STAT2 and/or STAT3. This anti-inflammatory effect is characterized by the inhibition of pro-inflammatory cytokines such as IL-6, TNF-α, and MCP-1 secretion, as well as a decrease in neutrophil recruitment (Kumaran Satyanarayanan et al., 2019). Furthermore, Kimball et al. (2019) highlighted the importance of IFN-β in facilitating the switch of macrophages from a pro-inflammatory to an anti-inflammatory phenotype during wound healing. This transition is crucial for resolving inflammation and promoting tissue repair. In a study by Mizutani et al. (2020), enhanced IFN-β expression resulted in accelerated wound healing in mice, underscoring its beneficial role in this process. Lubina and colleagues found that IFN-β treatments inhibited the production of pro-inflammatory cytokines (IL-6, IL-1β, TNF-α) in a rat model. They observed that IFN-β reduced the neurological inflammation by different mechanisms, including a reduction in inducible nitric oxide synthase (iNOS) expression and the enhancement of the anti-inflammatory cytokine IL-10 (Lubina-Dąbrowska et al., 2017). These

findings collectively highlight the importance of IFN-β in regulating inflammation and promoting wound healing. By inhibiting pro-inflammatory cytokines, modulating macrophage phenotypes, and influencing key signalling pathways, IFN-β exerts anti-inflammatory effects and facilitates tissue repair processes.

Having observed the modulation of cytokine secretion in response to elevated glucose, M1-associated generation of the free radical nitric oxide (NO), known to be a key signalling molecule in immune functions (Bogdan, 2001), was subsequently investigated. Elevated glucose exposure resulted in a significant ($P<0.05$) reduction in NO generation by M1 macrophages in a concentration-dependent manner. NO is well-known for playing an important role in various immune responses, including enhancing phagocytosis and direct antibacterial actions (Fang, 1997; Bogdan, 2015). Reduced NO generation by M1 macrophages exposed to hyperglycaemic conditions is consistent with research linking diabetes with suppression of antimicrobial immune activity (Ayala et al., 2019; Sousa et al., 2023).

To better understand the mechanism through which hyperglycaemia might lead to pronounced inflammatory responses such as M1-associated TNF-α secretion, the NF-κB transcription factor was investigated. Hyperglycaemia was shown to significantly increase total cellular NF-κB protein expression ($P<0.05$; $n=12$) and, more importantly, enhance NF-κB nuclear translocation ($P<0.05$; $n=12$). The increased nuclear translocation observed in macrophages exposed to high (30mM) glucose is indicative of enhanced NF-κB activation, which in turn stimulates the inflammatory response via gene transcription of inflammatory mediators, particularly TNF-α (López-Bojórquez et al., 2004; Iacobazzi et al., 2023). These findings highlight NF-κB translocation as an important molecular process through which hyperglycaemia might impact bacterial clearance and the inflammatory milieu in DFUs. Other researchers have confirmed both the pro-inflammatory and immune-suppressive effects of NF-κB activation in macrophages exposed to prolonged hyperglycaemia (Lawrence, 2014) (Suzuki et al., 2021).

The final investigations in this chapter investigated the effect of hyperglycaemia on key receptor protein/ligand expression in M1 macrophages. CD14 is a PRR essential

for innate immunity (Anas et al., 2010b). Flow cytometry analysis showed M1-like macrophages cultured in elevated glucose had significantly ($P<0.05$; n=6) higher mCD14 protein expression and significantly ($P<0.05$; n=6) increased sCD14 secretion than control macrophages cultured in 11mM glucose. This rise in CD14 expression is consistent with previous research that found high glucose exposure increased CD14 expression in U937 cells, possibly through increased activity of transcription factors that target CD14 gene transcription (Nareika et al., 2008). Furthermore, obesity-induced hyperglycaemia has been demonstrated to increase CD14 mRNA levels in human adipocytes, linking glucose-induced CD14 expression to pronounced inflammation and insulin resistance (Fernández-Real et al., 2011a). While these findings collectively suggest that hyperglycaemia upregulates CD14 expression in macrophages and related cell types, it is important to note that opposing effects have been observed in monocytes, which are precursor cells to macrophages. Blanks et al. (2022) reported that hyperglycaemia decreased mCD14 protein and gene expression levels in blood monocytes. These results highlight the potential for differential effects of hyperglycaemia on CD14 expression between monocytes and differentiated macrophage population, likely reflecting the phenotypic and functional changes that occur during the monocyte-to-macrophage differentiation process.

sCD14 released from cells expressing CD14 can act as an inflammatory mediator linked to numerous inflammatory diseases (Ogawa et al., 2013; Leveque et al., 2017; Mabrey et al., 2021). The elevated levels of sCD14 observed in hyperglycaemic conditions are consistent with the broader literature indicating that sCD14 levels are elevated in various disease states, including HIV infection (Kelesidis et al., 2012), cardiovascular disease (Anker et al., 1997; Kelesidis et al., 2012), inflammatory lung diseases (Marcos et al., 2010; Elias-Oliveira et al., 2022), viral infections (Sandler et al., 2011; Schlatzer et al., 2013; Teixeira et al., 2021), and rheumatoid arthritis (Bas et al., 2004). Furthermore, Increased sCD14 likely contributes to the propagation of inflammation through its ability to facilitate innate immune activation by LPS (Anas et al., 2010b). By binding and transferring LPS to the TLR4/MD-2 receptor complex on responsive cells, sCD14 can amplify pro-

inflammatory signalling cascades and drive the secretion of cytokine mediators (Kitchens and Thompson, 2005; Anas et al., 2010b). Indeed, elevated sCD14 levels correlate with increased circulating inflammatory markers in various pathological conditions (Leveque et al., 2017). Moreover, sCD14 has emerged as an independent risk factor for diabetic macrovascular complications, such as accelerated atherosclerosis and cardiovascular disease, likely due to its ability to potentiate cytokine release, including TNF-α, IL-1B, and IL-8, and upregulate TLR4 expression in macrophages, thereby exacerbating the inflammatory response characteristic of atherosclerosis in diabetes (Sanjurjo et al., 2023). Thus, the hyperglycaemia-induced upregulation of sCD14 observed in this PhD study might contribute to exaggerated inflammatory responses and impaired bacterial clearance in diabetic conditions.

A significant decrease in both mCD33 ($P<0.05$; n=6) and sCD33 ($P<0.05$; n=6) protein expression was observed in M1 macrophages cultured under hyperglycaemic conditions compared to control macrophages exposed to 11mM glucose. The role of CD33 in immune responses associated with hyperglycaemia is not fully known but decreased CD33 expression in monocytes under hyperglycaemic conditions has been linked to an increase in inflammatory cytokines, increased reactive oxygen species (ROS) generation, and increased suppressor of cytokine signalling protein-3 (SOCS-3) expression (Gonzalez et al., 2012). Notably, Moreno-Indias et al. (2016) demonstrated that CD33 levels in adipose tissue macrophages contribute to sustained inflammation and insulin resistance in obesity-induced diabetes. Furthermore, Zhang et al. (2013) and Crocker et al. (2012) reported that downregulation of CD33 in monocytes and macrophages exacerbated inflammation and promoted the development of obesity and associated metabolic disorders, such as insulin resistance and fatty liver disease. This heightened inflammatory state and metabolic dysregulation could potentially impair the ability of macrophages to effectively clear bacterial infections. Chronic inflammation and insulin resistance are known to compromise various aspects of the immune response, including macrophage function (Shoelson et al., 2006; Ndisang et al., 2014). Additionally, obesity has been linked to impaired

bacterial clearance and increased susceptibility to infections (Milner and Beck, 2012; Huttunen and Syrjänen, 2013). Therefore, the decreased CD33 expression observed in M1 macrophages under hyperglycaemic conditions may contribute to a compromised immune response, potentially hindering bacterial clearance in DFUs. Furthermore, the role of CD33 in modulating inflammatory responses and phagocytic activity of macrophages (Zhang et al., 2013; Bhattacherjee et al., 2021) suggests that its downregulation could directly impact the ability of these cells to effectively recognise, engulf, and eliminate bacterial pathogens present in DFUs.

Hyperglycaemia decreased the expression of both mTREM2 and sTREM2 by M1-like macrophages. Similarly, both intracellular and secreted levels of ApoE, the natural ligand for TREM2, were also significantly inhibited in macrophages by sustained high glucose. Taken together, these findings indicate notable disruption of the TREM2/ApoE immunomodulatory axis by chronic hyperglycaemic environments. Inhibition of the TREM2/ApoE signalling pathway can induce inflammation (Jay et al., 2015; Chen et al., 2020; Li et al., 2021) and impair phagocytosis (Kawabori et al., 2015; Yeh et al., 2016; Krasemann et al., 2017; Dabla et al., 2022). Specifically, TREM2 deficiency has been linked to reduced phagocytic activity and increased production of pro-inflammatory cytokines, such as TNF-α and IL-6, in macrophages (Painter et al., 2015; Zhong et al., 2017; Zhao et al., 2018) . Hyperglycaemia has been shown to reduce ApoE gene expression in adipose tissue, thereby altering lipid metabolism and promoting inflammation (Al-Malki et al., 2013; Ghiraldini and Mello, 2013). Moreover, these studies indicate that this decrease in ApoE is mediated through oxidative stress and activation of inflammatory pathways such as NF-κB in response to high glucose levels. Decreased ApoE production in adipocytes under hyperglycaemic conditions has been associated with impaired cholesterol efflux and exacerbated inflammation (Martins et al., 2006; Espiritu et al., 2010; Huang et al., 2015). Thus, in summary, the inhibitory effects of hyperglycaemia on TREM2 and ApoE expression may lead to dysregulation of the TREM2/ApoE axis with downstream consequences on inflammatory responses and phagocytic activity.

Using high-resolution confocal imaging, this chapter showed structural changes in the actin cytoskeleton of M1 macrophages in response to increased glucose concentration. Notably, there was a significant reduction ($P<0.05$; $n=12$) in F-actin filament extensions and number and the formation of F-actin aggregates in M1 macrophages under hyperglycaemic conditions, suggesting actin depolymerization was taking place. These modifications are likely to impair cellular motility and phagocytosis (Castellano et al., 2001). Hyperglycaemia-mediated actin disruption may be caused by increased oxidative damage, the generation of advanced glycation end products (AGEs), and/or the induction of AGE receptors (RAGEs), all of which have been shown to influence cytoskeletal dynamics (Egaña-Gorroño et al., 2020). Structure alterations in hyperglycaemic conditions can also indicate changes in small Rho GTPases (e.g., CDC42, Rac1) that act as important regulators of macrophage actin polymerization (Möller et al., 2002; Q.-Y. Huang et al., 2019). In addition to affecting motility and phagocytosis, cytoskeletal modifications can affect multiple cellular functions including intracellular transport and cellular respiration that also depend on stable and functional actin scaffolding (Möller et al., 2002; Plekhova et al., 2017).

The findings of this chapter are consistent with previous findings that show hyperglycaemia leads to multiple changes in macrophages that alter innate immune function, giving rise to increased inflammation but impaired infection clearance (Dasu et al., 2008; Gonzalez et al., 2012; Hsu et al., 2015; Ayala et al., 2019; S.-M. Huang et al., 2019). Additionally, it is important to consider whether the detrimental effects of hyperglycaemia on macrophage function may be exacerbated by age-related changes in endogenous hormone levels, particularly estrogen. Estrogen has been shown to modulate macrophage function and immune responses (Toniolo et al., 2015; Villa et al., 2015), and its decline with age could potentially amplify the impact of hyperglycaemia on innate immune cells. Therefore, estrogen supplementation may be a potential option to explore for reversing or mitigating such age-related effects in conjunction with hyperglycaemia. The subsequent chapters of this thesis aim to investigate this potential link

between age-related hormonal changes and the impact of hyperglycaemia on macrophage function and bacterial clearance in diabetic foot ulcers.

Chapter 4: Estrogen Reverses the Detrimental Effect of Elevated Glucose on Bacterial Clearance by Monocyte-Derived Macrophages

4.1 Introduction

4.1.1 Diabetic Foot Infections

DFIs represent a prevalent and serious complication among individuals with DM, often leading to hospitalisation, amputation, and even death (Bader and Brooks, 2012; Turzańska et al., 2023; Senneville et al., 2024). DFUs act as entry points for bacterial infections, which can rapidly progress if left untreated (Driver et al., 2010; Noor et al., 2017). The progression from sterile wounds to subsequent microbial contamination and colonisation is influenced by various factors within the DFU microenvironment, including hyperglycaemia and ischemic tissue along with immune system dysfunctions that impair inflammatory cell functions (Brinkmann et al., 2004; Chastain et al., 2019; Akash et al., 2020).

Early detection of DFIs is challenging due to the absence of clear symptoms, often masked by underlying ischemia and neuropathy (Armstrong et al., 1996; Gardner et al., 2009). Approximately half of DFUs cases involve infected wounds with a polymicrobial consortium, including *Pseudomonas, Staphylococcus, Enterococcus, Corynebacterium*, and *Enterobacteriaceae* species (Ishwarya and Neelusree, 2019; Ogba et al., 2019; Ruke and Savai, 2019; Rodríguez-Rodríguez et al., 2022).

Antibiotic therapy is typically the first line of treatment for DFIs (Gardner et al., 2013). However, antibiotic resistance is a growing concern, complicating treatment (Kandemir et al., 2007). Several studies have highlighted the increasing rates of antibiotic resistance in bacteria isolated from DFUs. For example, a study conducted on 225 patients with DFIs at a hospital in Italy found significantly higher resistance to vancomycin and linezolid in 2020 compared to 2019 (Caruso et al., 2021). Another systematic review and meta-analysis with a total of 1174 diabetic patients and 1701 isolated bacteria from DFUs in Sub-Saharan Africa found substantial antibiotic resistance rates for antibiotics, these bacteria displayed high resistance rates towards key antibiotics such as Gentamycin and Ciprofloxacin among *S. aureus* isolates, with resistance rates reaching 57.96% and 52.45% respectively. Additionally, gram-negative bacteria including *E. coli* and *K. pneumoniae* showed significant resistance to Amoxicillin, with resistance rates of 72.42% and 62.67%

respectively. Moreover, resistance to other important antibiotics such as Ampicillin and Ceftriaxone was also observed. Highlighting an urgent need for context-specific successful therapy procedures (Wada et al., 2023).

The formation of biofilms by virulent pathogens further complicates treatment, conferring even greater resistance to multiple classes of antibiotics (Wolcott and Cox, 2013; Noor et al., 2017). Biofilms require exceptionally high concentrations of antibiotics and antiseptics, making standard treatment protocols less effective (Clinton and Carter, 2015; Lavery et al., 2019).

The rapidly escalating challenge of antibiotic resistance urgently requires a rigorous effort to develop novel and potent antimicrobial agents. Further research into novel antimicrobial treatments and strategies to overcome bacteria and biofilm resistance will be crucial for improving outcomes in complex infections such as DFIs.

4.1.2 The Role of Estrogen in Wound Repair and DFIs

Estrogen has been shown to accelerate wound repair in humans and animals (Ashcroft et al., 1999a; Ashcroft et al., 1999b; Asilian et al., 2001; Thornton, 2013). The positive influence of estrogen on the wound healing processes originated from clinical evidence demonstrating that premenopausal women exhibit accelerated healing rates compared to postmenopausal women (Thornton, 2013; Horng et al., 2017). The decline in steroid hormone precursors like DHEA-S with age correlates with delayed healing in both elderly males and females (Ashcroft et al., 2002; Ashcroft and Ashworth, 2003b).

Initially, researchers hypothesized that estrogen significantly impacts the complex pathways involved in wound healing. Upon investigating the intricate molecular and cellular aspects of wound healing, they revealed the multifaceted positive effects that estrogen exerts throughout the sequential stages of the healing process. Estrogen treatment was able to dampen inflammation, restore collagen deposition and promote matrix remodelling in a model of delayed wound healing (Ashcroft et al., 2003; Zomer and Cooke, 2023). Moreover, estrogen promotes cutaneous wound healing by accelerating keratinocyte proliferation and migration, which are

crucial for re-epithelialization. This is mediated through the AKT and ERK signalling pathways (Zhou et al., 2016). Additionally, estrogen was found to downregulate inflammation via ER-mediated inhibition of Macrophage Migration Inhibitory Factor (MIF) (Ashcroft et al., 2003). However, the potentially impacts of estrogen on bacterial clearance in wounds, specifically in the context of DFIs, remain largely unclear and represent a critical gap in current knowledge.

Diabetic patients often suffer from chronic, non-healing wounds due to vascular and neuropathic complications, and bacterial colonisation (Chauhan et al., 2023). The high glucose environment and systemic immune deficits promote microbial growth, often leading to infected wounds with polymicrobial biofilm communities (Noor et al., 2017; Bondi et al., 2021).

Estrogen modulates early innate immune responses to tissue damage and microbial invasion (Giannoni et al., 2011). Estrogen has also been shown to regulate the production of pro-inflammatory cytokines and chemokines responsible for recruiting monocytes and macrophages to the wound which helps maintaining a balanced non-exaggerated inflammatory state in the tissues surrounding the wound site (Giannoni et al., 2011; Pelekanou et al., 2016; Dragin et al., 2017). This controlled local inflammatory environment is critically required to initiate the subsequent coordinated stages of tissue regeneration, while preventing excessive inflammatory responses that can impede wound closure (Barman and Koh, 2020). Estrogen also stimulates fibroblast migration and proliferation (Brufani et al., 2017), induces angiogenesis (Huang et al., 2016). and promotes collagen production (Vodegel et al., 2022).

In addition to its influences on cellular behaviours, estrogen has been shown to broadly augment skin suppleness and hydration which aids the healing process and prevents excessive scar formation (Brincat et al., 2005; Thornton, 2013). Through these mechanisms, estrogen creates an optimal local environment to support each phase of the intricate wound healing process.

In recognition of the multifaceted roles estrogen plays in wound healing, therapeutic approaches such as systemic and local hormone replacement have

been explored as strategies to correct deficiencies and/or imbalances in estrogen signalling, particularly those that develop after menopause (Margolis et al., 2002; Zomer and Cooke, 2023). Estrogen supplementation has been demonstrated to successfully restore age-related delays and aberrations in wound healing in both animal models and human patients (Asilian et al., 2001; Mukai et al., 2016; Jiang et al., 2022; Mukai et al., 2022).

While systemic estrogen therapy remains controversial due to risks of unintended hormonal side effects in non-target organ systems, topical application of estrogen for diabetic wounds is being explored while avoiding widespread systemic effects (Jiang et al., 2022; Mukai et al., 2022). Early studies show promising improvements in wound closure rates with topical estrogen treatments, although definitive large-scale clinical evidence to support efficacy is still lacking at this stage (Ashcroft et al., 1999a; Mukai et al., 2016; Mukai et al., 2022). Identifying the optimal dosage, timing, and delivery system for topical estrogen therapy in diabetic wounds will be essential to maximize therapeutic benefits while mitigating potential risks before this approach can be widely adopted.

Despite its beneficial impact on wound healing, the specific mechanisms by which estrogen influences bacterial clearance in diabetic wounds remains unclear. Elucidating these mechanisms will provide invaluable knowledge to guide targeted therapeutic interventions that could potentially treat DFIs.

Previous preliminary studies conducted by the Ashworth research group showed that estrogen supplementation can potently modulate host immune responses to bacterial infections by enhancing the phagocytic clearance of bacteria *in vitro* (El Mohtadi, 2019). These findings underscore the profound influence sex steroid hormones exert on regulating immune defences against microbial invaders.

To further investigate the specific effects and mechanisms of estrogen signalling during wound infections, the current study utilised *in vitro* models of DFIs using both planktonic bacterial cultures and surface-attached biofilm cultures of MRSA and *P. aeruginosa*. By evaluating estrogen's effects on both cultured suspension bacteria and *in vitro* wound biofilm models, this study provides the necessary

reductionist approach needed to systematically explore the immunomodulatory role of estrogen in combating DFIs prior to translation to more complex clinical settings.

4.2 Aim and Objectives

4.2.1 Aim

To investigate the impact of estrogen on the phagocytic function of distinct monocyte-derived macrophage phenotypes (M0, M1, and M2) using an *in vitro* model of DFUs infected with planktonic and biofilms of MRSA or *P. aeruginosa*.

4.2.2 Objectives

- Investigate the effect of estrogen on the clearance of planktonic form of MRSA and *P. aeruginosa* by M0, M1, and M2 macrophages using an *in vitro* model of an infected DFU.
- Compare the potential variations in phagocytic responses among different macrophage phenotypes (M0, M1, and M2) under hyperglycaemia conditions.
- Investigate the specific impact of estrogen on the phagocytosis of MRSA and *P. aeruginosa* biofilms by pro-inflammatory M1 macrophages *in vitro*.
- Visualise the estrogen-mediated internalisation of Gram positive (MRSA) and Gram negative (*P. aeruginosa*) biofilms by monocyte-derived M1 macrophages.

4.3 Methods

4.3.1 The Effect of Estrogen on Bacterial Growth

To determine the effect of estrogen on the phagocytosis of MRSA and *P. aeruginosa* by macrophages, it was first necessary to assess its direct impact on bacterial growth. MRSA and *P. aeruginosa* were grown as described in 2.11.1 and resuspended at 1 x 10^5 CFU/mL in increasing glucose concentrations (11, 15, 20,

30mM) with or without estrogen treatment (1 x 10^{-7} M). The suspensions were incubated at 37 °C for three hours. A volume of 100 µL of each bacterial sample was inoculated into duplicate nutrient agar plates and incubated overnight at 37°C. The number of bacterial CFUs grown on the agar plates were counted to determine the growth rate of both MRSA and *P. aeruginosa* under each glucose condition.

4.3.2 *In vitro* Host-Pathogen Interaction Experiments

4.3.2.1 Planktonic Bacteria

U937 monocyte at a concentration 1 x 10^6 cells/mL were differentiated into M0 macrophages and polarized into M1-like and M2-like macrophages under increasing glucose levels (11, 15, 20, 30mM) as described in 2.3, 2.5 and 2.6. Macrophages were then treated with 1 x 10^{-7} M estrogen for 24 hours as explained in 2.7. and subsequently incubated with 1 x 10^4 CFU planktonic MRSA or *P. aeruginosa* for 3 hours to allow host-pathogen interactions to take place (Figure 4.1). Bacterial recovery was then determined as outlined in 2.11.3.1.

4.3.2.2 Biofilms of MRSA and *P. aeruginosa*

MRSA and *P. aeruginosa* biofilms were generated on polycarbonate membranes as described in 2.10.2. The bacterial biofilm membranes were clamped to membranes containing M1-like macrophages previously cultured under increasing glucose concentrations (11, 15, 20, 30mM), with or without estrogen treatment (1 x 10^{-7} M) for 24 hours. In addition, appropriate controls lacking biofilms and/or macrophages were prepared. After the 1-hour host-pathogen interaction at 37 °C, the supernatants and any adherent cells released by trypsinization were collected, serially diluted, and plated onto duplicate nutrient agar plates to determine the bacterial recovery by CFUs as detailed previously in section 2.11.3.2 (Figure 4.1).

4.3.3 Biofilm Quantification Assay

MRSA and *P. aeruginosa* biofilms were grown on polycarbonate membranes for 24 hours as described in 2.11.2. The bacterial biofilm membranes were clamped to membranes containing M1-like macrophages previously cultured under increasing

glucose concentrations (11, 15, 20, 30mM), with or without estrogen treatment (1 x 10^{-7} M) for 24 hours. Following a 1-hour period of host-pathogen interaction, bacterial biofilms were washed and stained with crystal violet as described in 2.11.4. The crystal violet dye was released using glacial acetic acid prior to measuring the absorbance at 570nm to quantify biofilm formation. The crystal violet biofilm assay allowed quantification of the effects of estrogen-treated macrophages on established MRSA and *P. aeruginosa* biofilm formation under different glucose conditions.

4.3.4 Visualising Bacterial Interaction and Internalisation by Macrophages

The interaction and internalisation of GFP-*S. aureus* or mCherry-*P. aeruginosa* biofilms by M1 macrophages previously cultured under increasing glucose concentrations (11, 15, 20, 30mM), with or without estrogen treatment (1 x 10^{-7} M) for 24 hours, was visualised by SEM (2.12.2) and confocal microscopy (2.12.1) respectively.

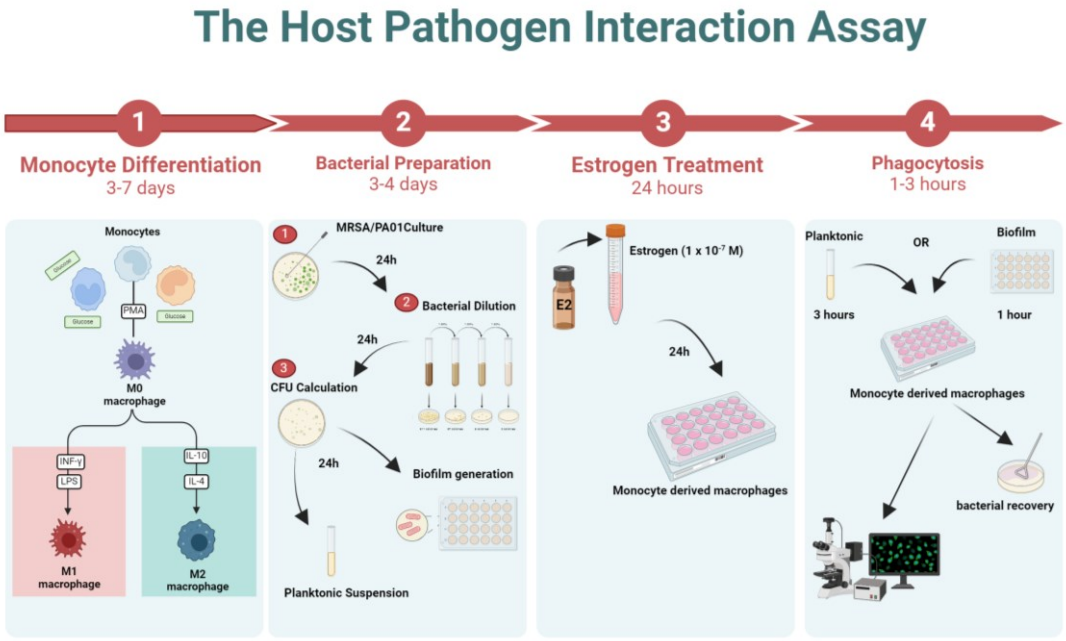

Figure 4.1 A Detailed Workflow of the Host-Pathogen Interaction Experiments.
1: Generation of *in vitro* monocytes-derived macrophages under increasing glucose concentrations. 2: Bacterial biofilm generation. 3: Estrogen stimulation of macrophages under different glucose concentrations. 4: Phagocytosis assay: co-culturing macrophages and bacteria to allow phagocytosis followed by microscopy to visualise host-pathogen interactions.

4.4 Results

4.4.1 Effect of Estrogen on The Growth of MRSA and *P. aeruginosa*

Prior to conducting host-pathogen interaction assays, it was important to assess the effect of estrogen (1 x 10^{-7} M) on the growth of MRSA and *P. aeruginosa* at all glucose concentrations (11, 15, 20, 30mM). Following 3 hours of incubation, there was no significant effect of estrogen (P>0.05; n=12) on MRSA (Figure 4.2.A) or *P. aeruginosa* (Figure 4.2.B) recovery at all glucose concentrations compared to untreated bacterial controls. These results showed that estrogen had no direct effect on the growth of either MRSA or *P. aeruginosa*, indicating that any potential effect of the hormone on bacterial clearance would be due to its influence on macrophages rather than bacteria (Figure 4.2).

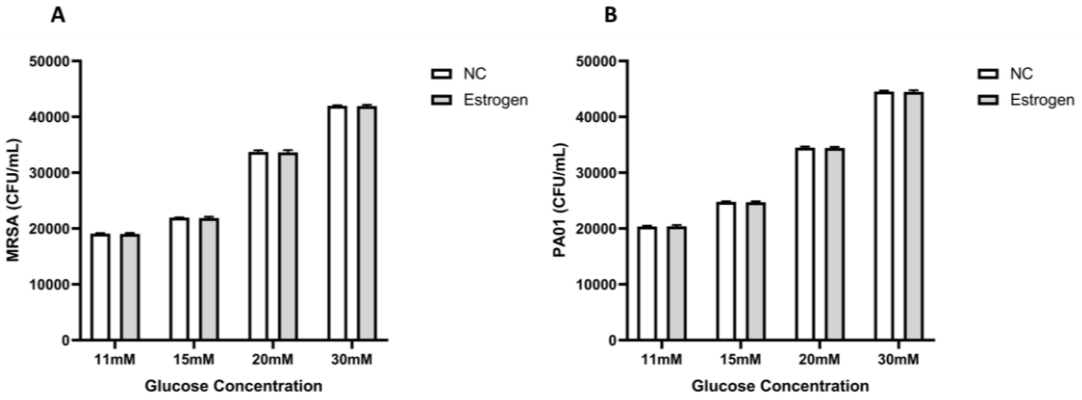

Figure 4.2 The Effect of Estrogen of the Growth of MRSA and *P. aeruginosa*.
Estrogen (1 x 10^{-7} M) had no significant direct influence (P>0.05) on the growth of MRSA (A) or *P. aeruginosa* (B) at all glucose concentrations (11, 15, 20, 30mM) in cmparison to the corresponding negative control (untreated) bacteria (NC). Data represent an average of n = 12 independent experiments with error bars indicating the standard error of the mean.

4.4.2 Effect of Estrogen on the *In Vitro* Phagocytosis of Planktonic MRSA and *P. aeruginosa* by M0 Monocyte-Derived Macrophages

The effect of estrogen on the phagocytosis of planktonic MRSA and *P. aeruginosa* by M0 macrophages was investigated under increasing glucose concentrations (11 mM, 15 mM, 20 mM and 30 mM) (Figure 4.3). Estrogen (1 x 10^{-7} M) significantly (P < 0.05; n = 12) reduced the recovery of MRSA by M0 macrophages compared to untreated negative control (NC) macrophages, regardless of the glucose concentration. Similarly, the recovery of *P. aeruginosa* was significantly (P < 0.05; n

= 12) reduced following treatment of M0 macrophages with estrogen (1 x 10^{-7} M) in comparison with untreated negative control (NC) macrophages, regardless of glucose concentration (Figure 4.3.B). Collectively, these findings indicate that estrogen stimulated the phagocytosis of planktonic MRSA and *P. aeruginosa* by M0 macrophages.

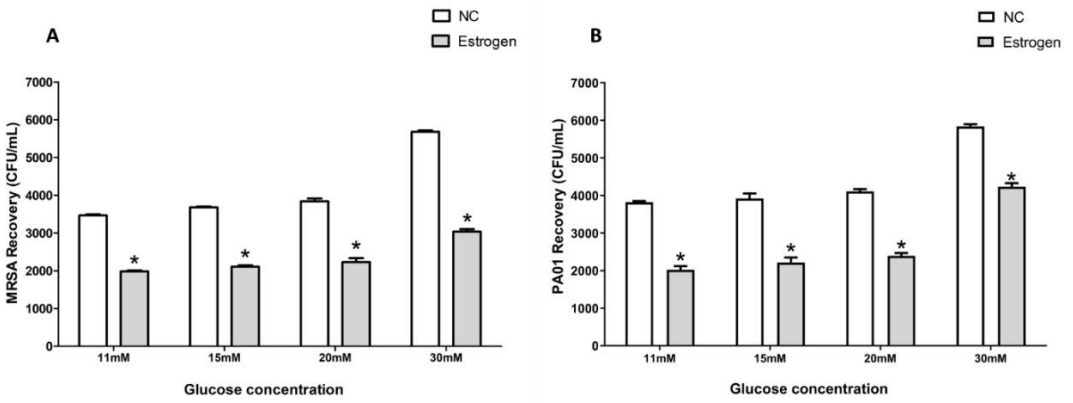

Figure 4.3 Estrogen Enhances the *In Vitro* Clearance of Planktonic MRSA (A) and PA01 (B) by M0 Macrophages.

* Indicates significant reductions (*P<0.05) in MRSA (A) and PA01 (B) recovery following exposure of M0 macrophages to estrogen treatment (1 x 10^{-7} M) in comparison to the corresponding untreated negative control (NC) M0 macrophages cultured at different glucose concentrations (11 mM, 15 mM, 20 mM and 30 mM). Data represent an average of 12 independent experiments, with error bars indicating the standard error of the mean.

4.4.3 Effect of Estrogen on the *In Vitro* Phagocytosis of Planktonic MRSA and *P. aeruginosa* by M1-Derived Macrophages

The impact of estrogen (1 x 10^{-7} M) on the phagocytosis of planktonic MRSA and *P. aeruginosa* by M1 macrophages was investigated under varying glucose concentrations (11 mM, 15 mM, 20 mM and 30 mM). Estrogen supplementation (1 x 10^{-7} M) significantly reduced M1-mediated MRSA recovery compared to untreated M1 macrophages (P<0.05, n = 12) (Figure 4.4.A), regardless of glucose levels. Likewise, the number of recovered *P. aeruginosa* colonies significantly (P < 0.05; n = 12) decreased following interaction with M1 macrophages treated with estrogen (1 x 10^{-7} M) compared to corresponding untreated M1 macrophages (Figure 4.4.B), irrespective of the glucose concentration. Collectively, these findings indicate that estrogen stimulated the phagocytosis of planktonic MRSA and *P. aeruginosa* by M1 macrophages.

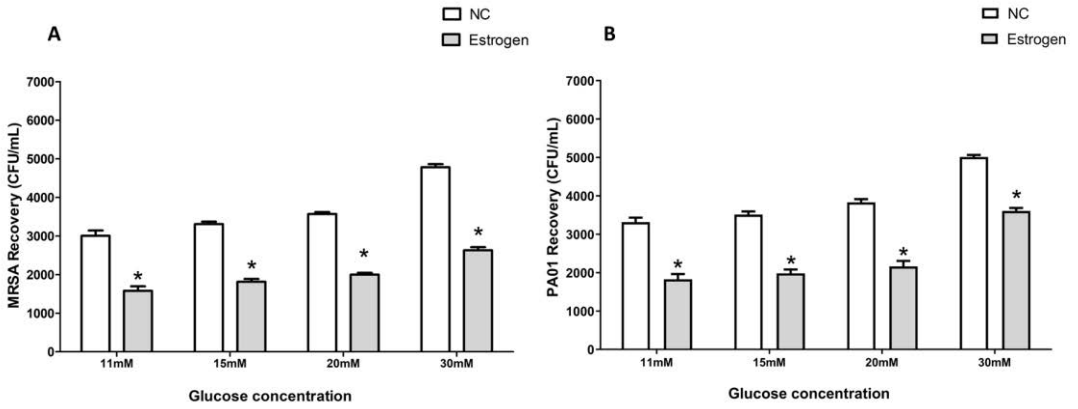

Figure 4.4 Estrogen Enhances the Clearance of MRSA (A) and PAO1 (B) by *in Vitro* M1-Macrophages.

***** Indicates significant reductions (P<0.05) in MRSA (A) and PAO1 (B) recovery following exposure of M1-like macrophages to estrogen treatment (1 x 10^{-7} M) in comparison to the corresponding untreated negative control (NC) M1-like macrophages cultured at different glucose concentrations (11 mM, 15 mM, 20 mM, and 30 mM). Data represent an average of twelve independent experiments (n = 12), with error bars indicating the standard error of the mean.

4.4.4 Effect of Estrogen on the *In Vitro* Phagocytosis of Planktonic MRSA and *P. aeruginosa* by M2 Macrophages

Building upon previous assessments of M0 and M1 macrophage-mediated phagocytosis, the effect of estrogen on planktonic MRSA and *P. aeruginosa* clearance by M2 macrophages was explored at different glucose concentrations (11 mM, 15 mM, 20 mMand 30 mM) (Figure 4.5). Estrogen (1 x 10^{-7} M) significantly ($P < 0.05$; n = 12) reduced the recovery of MRSA compared to the negative control (Figure 4.5.A), at all glucose concentrations. Similarly, the number of recovered *P. aeruginosa* was significantly ($P < 0.05$; n = 12) reduced following treatment of M2 macrophages with estrogen (1 x 10^{-7} M) in comparison with corresponding untreated negative control (NC) M2 macrophages, independent of the glucose concentration (Figure 4.5.B). Collectively, these findings indicate that estrogen stimulated the phagocytosis of planktonic MRSA and *P. aeruginosa* by M2 macrophages.

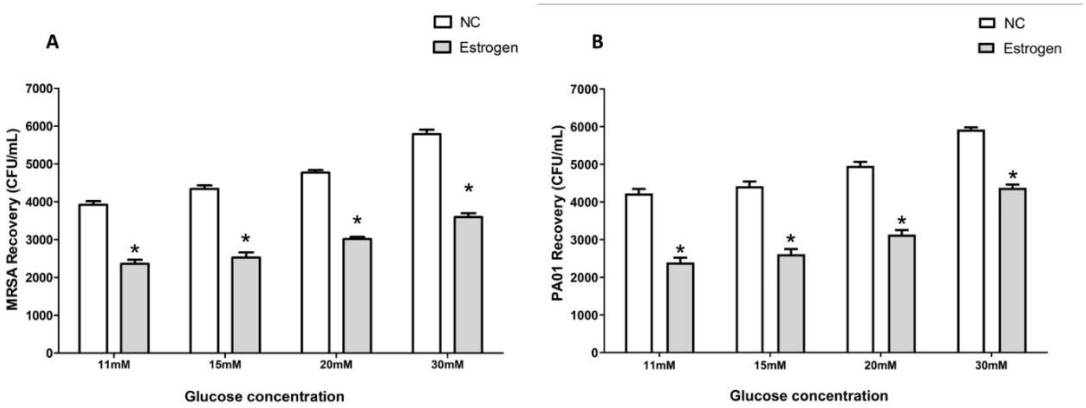

Figure 4.5 Estrogen Enhances the *in Vitro* Clearance of MRSA (A) and PA01 (B) by M2 Macrophages.

*****Indicates significant reductions (P<0.05) in MRSA (A) and PAO1 (B) recovery following exposure of M2-like macrophages to estrogen treatment (1 x 10^{-7} M) in comparison to the corresponding untreated negative control (NC) M1-like macrophages cultured at different glucose concentrations (11 mM, 15 mM, 20 mM, and 30 mM). Data represent an average of twelve independent experiments (n = 12), with error bars indicating the standard error of the mean.

4.4.5 Estrogen Enhances the *In Vitro* Phagocytosis of MRSA and *P. aeruginosa* by M1 Macrophages Compared to M0 and M2 Macrophage Subsets

This investigation compared the effect of estrogen treatment (1 x 10^{-7} M) on the phagocytic activity of M0, M1, and M2 macrophages using an *in vitro* model of DFUs. The recovery of both MRSA and *P. aeruginosa* (CFU/mL) was compared following a period of host-pathogen interaction with estrogen-exposed or untreated macrophage phenotypes cultured in increasing glucose (11 mM, 15 mM, 20 mM and 30 mM) conditions. The results highlighted differential phagocytic capacities among M0, M1, and M2 macrophages, elucidating their distinct abilities to engulf and eliminate bacteria under hyperglycaemia conditions. In the absence of estrogen treatment, M1 macrophages exhibited superior bacterial clearance compared to M0 and M2 subsets under all glucose concentrations tested (Figure 4.6). The recovery of both MRSA (A) and *P. aeruginosa* PA01 (B) was significantly lower when co-cultured with M1 macrophages, compared to co-culture with M0 or M2 macrophages ($P < 0.05$; n = 12).

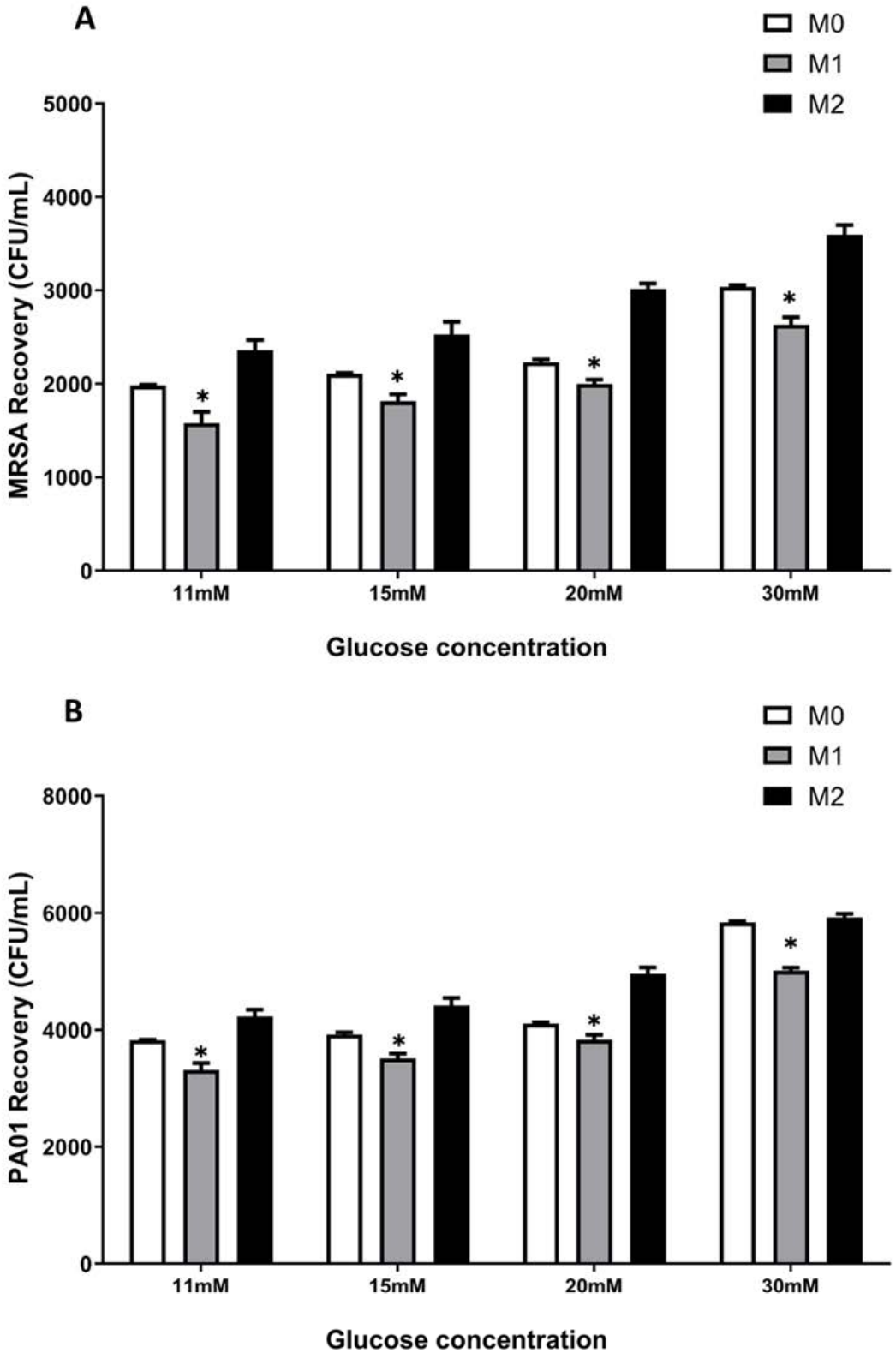

Figure 4.6 Comparative Analysis of the Phagocytosis of MRSA (A) and *P. aeruginosa* PA01 (B) by Untreated M0, M1, and M2 Macrophages.

M1 macrophages exhibited significantly (*P< 0.05, n=12) enhanced phagocytosis (reduced bacterial recovery) of both MRSA (A) and *P. aeruginosa* PA01 (B) when compared to either M0 or M2 macrophages cultured across all glucose concentrations tested. Data represent the mean bacterial recovery ± StEM.

Estrogen exposure further enhanced the phagocytic capacity of M1 macrophages (Figure 4.7), significantly reducing bacterial recovery of MRSA (A) and *P. aeruginosa* PA01 (B) compared to estrogen-treated M0 or M2 macrophages ($P < 0.05$; $n = 12$) cultured across all glucose concentrations tested.

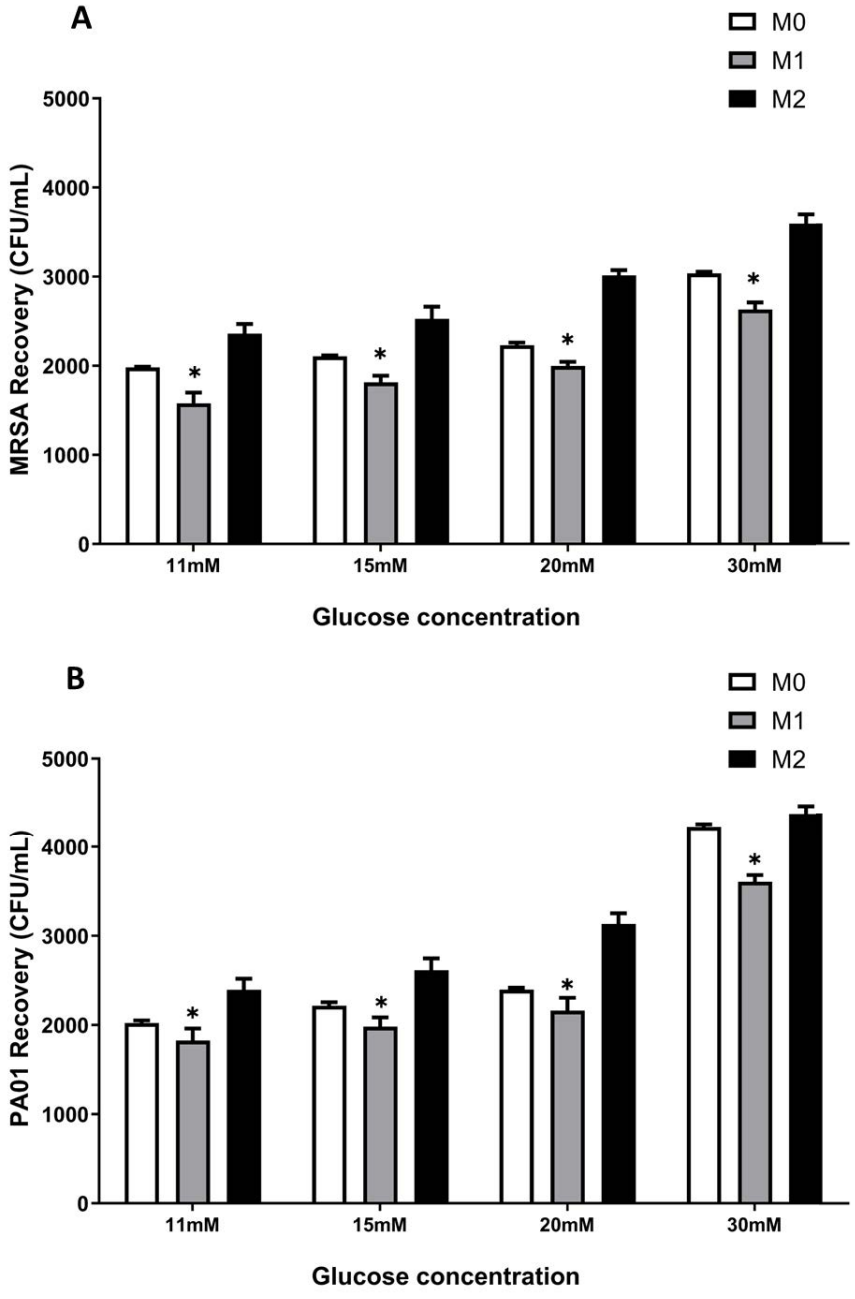

Figure 4.7 Comparative Analysis of the Phagocytosis of MRSA (A) and *P. aeruginosa* PA01 (B) by Estrogen-Treated M0, M1, and M2 Macrophages.
Estrogen stimulation (1×10^{-7} M) significantly (*$P < 0.05$, $n=12$) enhanced the phagocytosis of both MRSA (A) and *P. aeruginosa* PA01 (B) by M1 macrophages compared to M0 and M2 macrophages, as evidenced by reduced bacterial recovery following interaction with M1 macrophages across all glucose concentrations. Data represent the mean ± StEM.

4.4.6 Effect of Estrogen on the *In Vitro* Phagocytosis of MRSA and *P. aeruginosa* Biofilms by M1-Derived Macrophages

The influence of estrogen (1 x 10^{-7} M) on the clearance of MRSA and *P. aeruginosa* biofilms was investigated using M1 macrophages grown under elevated glucose conditions. Given the previous findings that highlighted M1 macrophages as the most phagocytic macrophage phenotype, the macrophage-biofilm interaction experiments were only conducted using pro-inflammatory M1 macrophages. The impact of estrogen treatment on M1-like macrophage clearance of MRSA and *P. aeruginosa* biofilms was tested across varying glucose concentrations (11 mM, 15 mM, 20 mM and 30 mM).

Findings in the first research chapter showed elevated glucose levels impede macrophage-mediated phagocytosis of bacteria. In contrast, this chapter has revealed estrogen significantly reverses the negative effect of glucose by promoting macrophage-mediated clearance of MRSA (Figure 4.8.A) and *P. aeruginosa* (Figure 4.8.B) biofilms, as indicated by the reduced MRSA and *P. aeruginosa* recovery across all glucose conditions (Figure 4.8; * P<0.05).

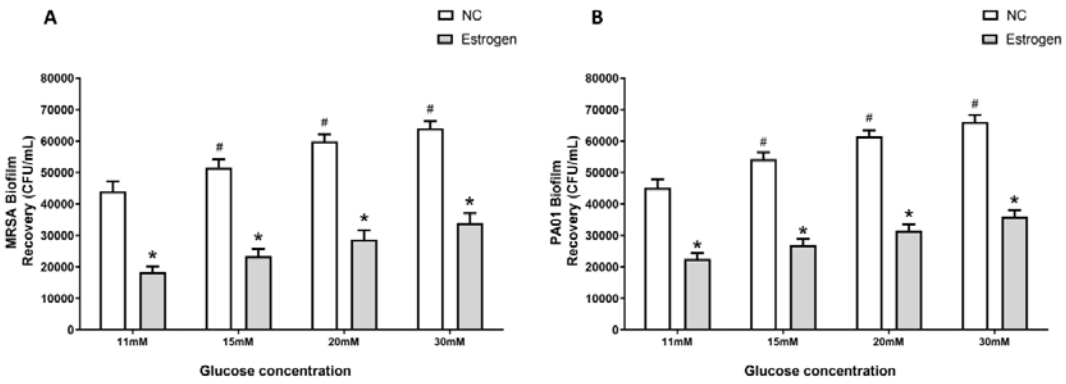

Figure 4.8 Estrogen Promotes the *In Vitro* Phagocytosis of MRSA and *P. aeruginosa* Biofilms by M1 Macrophages.
*Indicates significant reductions (P<0.05) in (A) MRSA and (B) *P. aeruginosa* biofilm recovery following exposure to estrogen treatment (1 x 10^{-7} M) of M1-like macrophages in comparison to untreated negative control (NC) M1-like macrophages cultured at different glucose concentrations (11 mM, 15 mM, 20 mM, and 30 mM). # Represents significant difference (P<0.05) between the indicated glucose group compared to 11 mM glucose. Data represent an average of twelve independent experiments (n = 12), with error bars indicating the standard error of the mean.

To further elucidate the influence of estrogen on the phagocytic clearance of biofilms, a crystal violet assay was conducted to assess the MRSA and *P. aeruginosa*

biofilm mass following co-culture with estrogen-treated or untreated negative control (NC) M1 macrophages. Estrogen treatment increased M1-mediated clearance of MRSA and *P. aeruginosa* biofilms across all glucose conditions tested, as shown by a significant ($P<0.05$, $n=12$) decrease in biofilm biomass (Figure 4.9A and Figure 4.9B) when compared to NC macrophages.

The visual intensity of crystal violet staining was also captured for MRSA (Figure 4.9C) and *P. aeruginosa* (Figure 4.9D) biofilms following co-culture with estrogen-exposed or untreated NC macrophages. There was increased density of crystal violet staining with both MRSA and *P. aeruginosa* in the presence of high glucose, indicative of enhanced biofilm adherence and formation under hyperglycaemic conditions. In contrast, the faded crystal violet intensity under all glycaemic conditions following treatment of M1 macrophages with estrogen indicated decreased MRSA and *P. aeruginosa* biofilm growth (Figure 4.9).

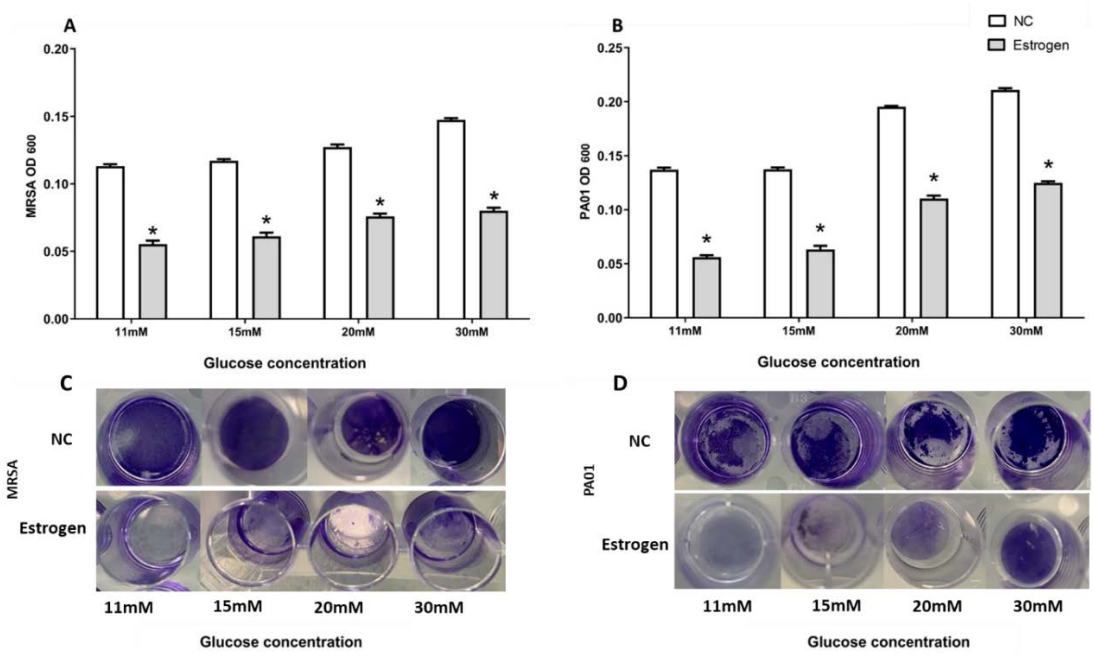

Figure 4.9 Quantification of MRSA and *P. aeruginosa* (PA01) Biofilm Formation by Crystal Violet Staining.
Estrogen increased the clearance of MRSA (A, C) and *P. aeruginosa* (B, D) biofilms as revealed by significantly (*$P<0.05$) reduced crystal violet staining (biomass) following co-culture with estrogen-treated M1 macrophages compared with untreated negative control (NC) M1 macrophages cultured at different glucose concentrations. Data in panels A and B represent the mean optical density (OD) measured at a wavelength of 600nm ± StEM of three independent experiments (n = 3).

4.4.7 Visualisation of Biofilm Internalisation

4.4.7.1 Confocal Microscopy

To visually assess the effects of high glucose environment and estrogen treatment on macrophage-mediated phagocytosis of *S. aureus* and *P. aeruginosa*, M1 macrophages were cultured in low (11 mM) or high (30 mM) glucose medium and treated with estrogen (1×10^{-7} M) prior to incubation with GFP-*S. aureus* or mCherry- *P. aeruginosa*. Macrophages were counter-stained with Alexa Fluor 647 phalloidin or FITC-CD197 antibodies as described in 2.12.1, enabling imaging and comparison of biofilm clearance by confocal microscopy (Figure 4.10). Hyperglycaemia (30 mM) significantly reduced the internalisation (uptake) of fluorescent *S. aureus* (Panels A3) and *P. aeruginosa* (Panels B3) by M1 macrophages, compared to euglycemic conditions (11 mM), Panels A1 and B1, respectively). Interestingly, estrogen stimulation (1×10^{-7} M) of M1 macrophages increased bacterial internalisation of both *S. aureus* (Panels A2 and A4) and *P. aeruginosa* (Panels B2 and B4) compared to the corresponding untreated controls (Panels A1 and A3 for *S. aureus*, Panels B1 and B3 for *P. aeruginosa*), regardless of the glucose concentration. This is evident from the increased uptake of bacteria observed within the estrogen-treated M1 macrophages.

In summary, confocal microscopy confirmed high (30 mM) glucose inhibited the clearance of *S. aureus* and *P. aeruginosa* biofilms by M1 macrophages, while estrogen treatment enhanced bacterial phagocytosis, reversing the detrimental effect of high glucose.

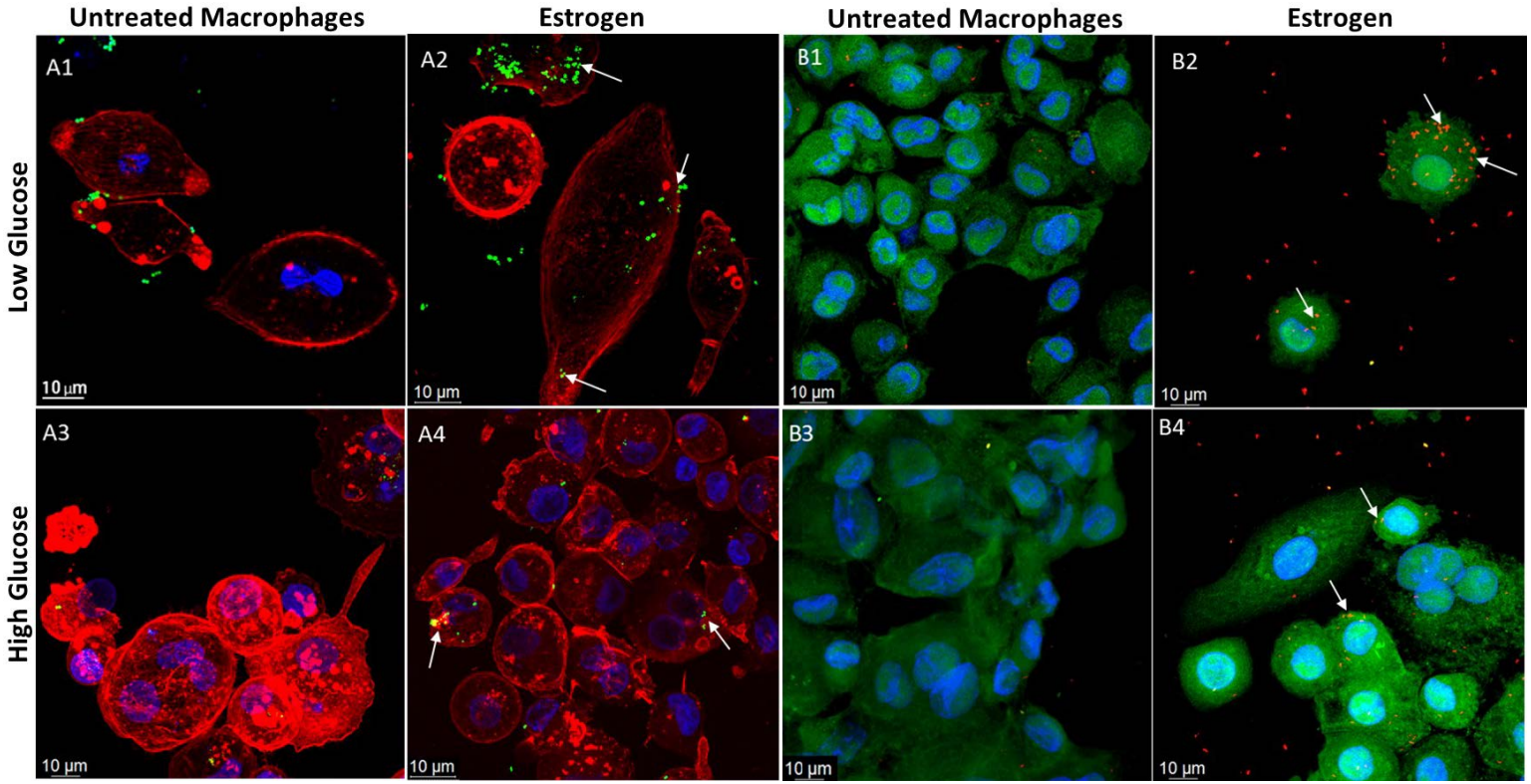

Figure 4.10 Effect of Estrogen on the Phagocytosis of GFP-*S. aureus* (A; green) and mcherry-*P. aeruginosa* (B; red) Biofilms by M1 Macrophages.
The internalisation of *S. aureus* (green) by M1 macrophages (F-actin stained red with Alexa Fluor 647 phalloidin) was enhanced by estrogen supplementation (1 x 10^{-7} M) under both 11mM (A2) and 30mM (A4) glucose conditions, compared to corresponding untreated M1 macrophages (A1 and A3, respectively). Similarly, estrogen treatment promoted the internalisation of mCherry-*P. aeruginosa* (red) by M1 macrophages (stained green with FITC anti-CD197 antibody) under both 11mM (B2) and 30mM (B4) glucose concentrations, compared to corresponding untreated M1 macrophages (B1 and B3, respectively).

4.4.7.2 Scanning Electron Microscopy

Detailed interactions between M1 macrophages and MRSA/*P. aeruginosa* bacterial biofilms were visualised using SEM as described previously in 2.2.13.2. SEM imaging enabled high-resolution views of *S. aureus* (Figure 4.11) and *P*. *aeruginosa* (Figure 4.12) biofilm uptake by M1 macrophages cultured under low (11 mM) and high (30 mM) glucose conditions, with and without estrogen treatment (Figure 4.12).

High glucose (30 mM) concentrations increased MRSA biofilm formation compared to low (11 mM) glucose, as evidenced by dense clustering of bacteria in hyperglycaemic (30 mM glucose) conditions (B1) compared to more sparsely scattered bacteria observed under euglycemic (11 mM glucose) conditions (A1). Estrogen treatment enhanced the macrophage-mediated engulfment of MRSA biofilms (A3 & B3) compared to corresponding untreated negative control M1 macrophages, even under high glucose conditions that otherwise impaired phagocytosis (A2 & B2 respectively). In particular, estrogen-treated macrophages formed more pronounced pseudopodial extensions that surrounded and internalised bacterial cells compared to untreated control macrophages. Estrogen rescued the impaired phagocytic response induced by high glucose, highlighting the potential of use of estrogen-based therapies to enhance the phagocytic function of macrophages under hyperglycaemic environments.

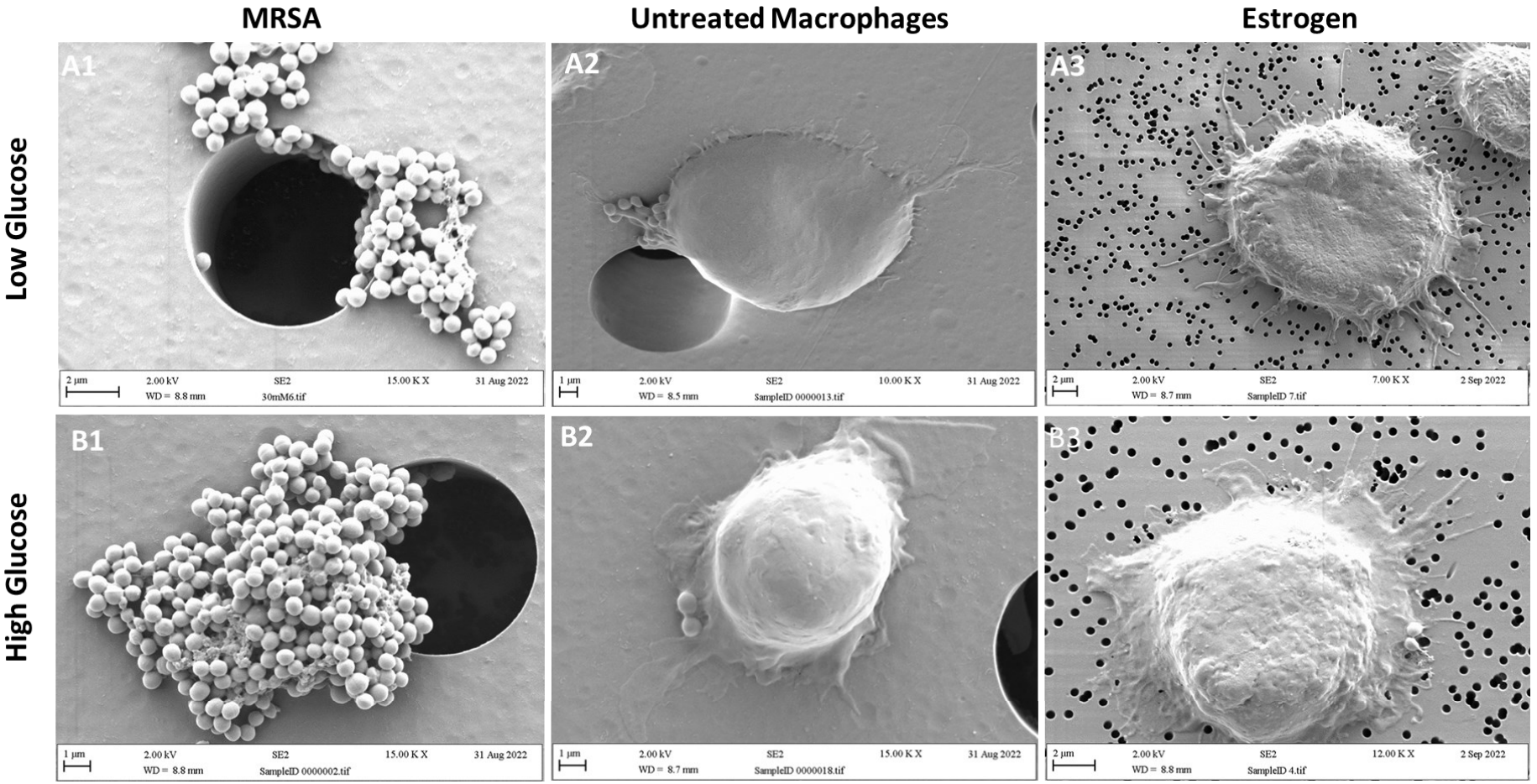

Figure 4.11 Analysis of (MRSA) Biofilm Formation and MRSA-Macrophage Interactions.

SEM images representing the influence of glucose concentration and estrogen treatment on MRSA-macrophage interactions. (A1) Low glucose conditions showed sparsely scattered bacteria, while (B1) high glucose conditions exhibited a dense clustering of bacteria. Estrogen-treated macrophages (A3, B3) demonstrated enhanced interaction compared to untreated controls (A2), particularly under high glucose conditions (B2).

Similarly, examination of *P. aeruginosa* biofilms under varying glucose concentrations revealed notable distinctions (Figure 4.12). At 30 mM glucose (Panel B1), SEM showed dense clustering of bacteria with material resembling extracellular polymeric substance (EPS) matrix, whereas at 11 mM glucose colonies appeared more sparsely dispersed with little evidence of EPS (A1).

Upon estrogen treatment, SEM analysis revealed enhanced macrophage interaction with *P. aeruginosa* biofilms (A3 & B3) compared to untreated macrophages (A2 & B2) cultured at low or high glucose conditions. Remarkably, estrogen-treated macrophages consistently exhibited pronounced pseudopodial extensions that typically facilitate bacterial encapsulation and internalisation. The findings suggest that estrogen may restore phagocytic function in M1 macrophages under hyperglycaemic environments, highlighting the potential use of estrogen-based therapies against *P. aeruginosa* biofilm infections.

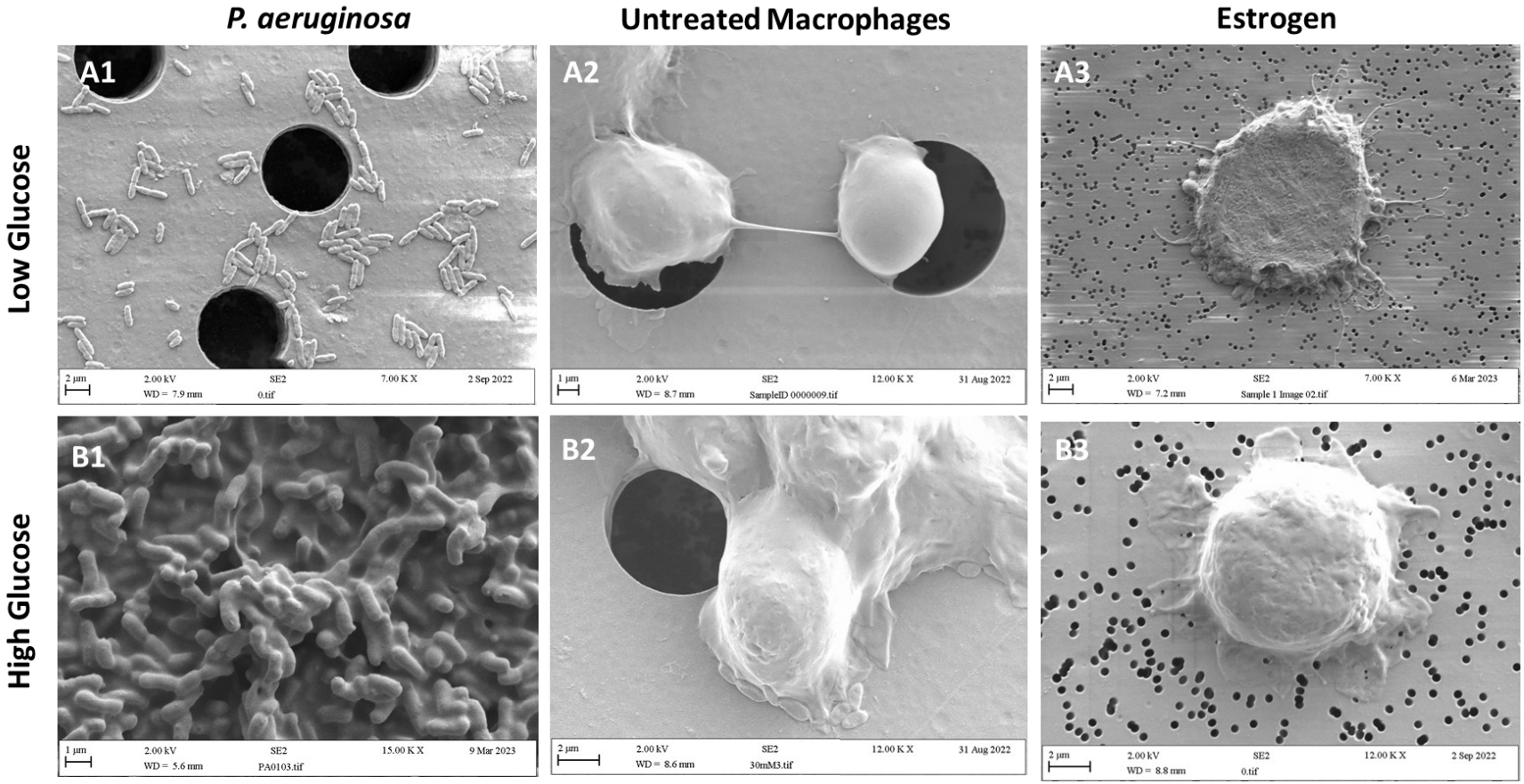

Figure 4.12 Examination of *P. aeruginosa* Biofilm Development and Macrophage Interaction.

SEM images illustrating the impact of glucose concentration and estrogen supplementation on *P. aeruginosa*- M1 macrophage interactions. (A1) Biofilms under low glucose showed dispersed bacteria, contrasting with (B1) high glucose conditions characterised by a densely packed bacteria with potential evidence of EPS matrix formation. Estrogen-treated macrophages (A3, B3) exhibited enhanced interaction compared to untreated counterparts (A2 and B2).

4.5. Discussion

Diabetic wound infection is one of the main causes of impaired wound repair in DFUs. Indeed, diabetic wounds are often infected with a variety of Gram-positive and Gram-negative bacterial species including *S. aureus* and *P. aeruginosa* (Di Domenico et al., 2020; Tkaczyk et al., 2022). Research suggests that estrogen has a significant effect on the inflammatory phase of wound healing *in vivo* (Ashcroft et al., 1999a). Topical and systemic estrogen treatments have been shown to enhance the rate of wound repair in both genders, particularly in post-menopausal females, by dampening the inflammatory response (Ashcroft and Ashworth, 2003a). In addition, estrogen deprivation has been shown to diminish the phagocytic capability of neutrophils and macrophages, thereby increasing the risk of infection and causing a delay in healing (Ashcroft and Ashworth, 2003a). Although estrogen has been shown to reverse delayed healing in the elderly, its effect on the phagocytosis of bacteria during DFIs has received limited attention to date. In particular, the potential role of estrogen in macrophage-mediated bacterial clearance in a diabetic model of inflammation has not yet been examined. Therefore, this chapter investigated the effect of estrogen supplementation on the macrophage-mediated clearance of typical wound bacteria under sustained hyperglycaemic conditions compared to phagocytosis mediated by estrogen-deprived macrophages.

In chapter 3, glucose had a significant concentration-dependent effect on impairing the clearance of MRSA and *P. aeruginosa* by monocyte-derived macrophages ($P<0.05$). This finding corroborates prior research indicating that hyperglycaemia impedes the phagocytic capacity of macrophages (Pavlou et al., 2018; Morey et al., 2019; Vance et al., 2019). Moreover, existing literature suggests that restoring normal phagocytic function can be achieved through improved glycaemic control (Lecube et al., 2011).

Initially, it was important to examine any potential direct influence of estrogen on the growth of MRSA and *P. aeruginosa in vitro*. After a 3-hour incubation with estrogen at various glucose concentrations (11, 15 mM, 20 mM and 30 mM), no

significant differences in bacterial growth rates were observed compared to bacteria cultured in the absence of estrogen. This led to the conclusion that any variations in bacterial counts during subsequent host-pathogen interaction assays would not be due to the influence of estrogen on bacterial growth but could instead be attributed to estrogen's influence on the phagocytic function of macrophages. These findings align with prior research indicating that estrogen at physiological and supraphysiological levels does not affect bacterial growth (Engelsöy et al., 2021). However, it is worth noting that estrogen at very low concentrations has been reported to enhance the growth of some bacterial species such as *Escherichia coli* (Mahdavi et al., 2013; Wang et al., 2013; Engelsöy et al., 2021; Demirel et al., 2022). However, these effects were only observed under conditions involving significantly longer exposure periods than the 3-hour incubation period used in this study (Mahdavi et al., 2013; Hammouda et al., 2023).

The impact of estrogen on the clearance of the wound-associated planktonic MRSA and *P. aeruginosa* by M0, M1 and M2 monocyte-derived macrophages was assessed. Incubating any of macrophage phenotypes with supraphysiological levels of estrogen (1×10^{-7} M) for 24 hours significantly ($P<0.05$) enhanced the clearance of both MRSA and *P. aeruginosa,* regardless of glucose concentration (Figures 4.2, 4.3 and 4.4). In line with these findings, Crompton et al. (2016) demonstrated that estrogen treatment could reverse the delay in cutaneous wound healing caused by exposure to *K. pneumoniae* LPS in a murine model. This suggested a beneficial effect of estrogen in counteracting the impairment of wound repair by bacterial factors. Li et al. (2000) provided complementary evidence, showing that 24-hour and 48-hour exposure to estrogen increases the engulfment of amyloid β-peptide (Aβ) by microglial cells, known as brain macrophages, in a time-dependent manner (Li et al., 2000) . In the same study, estrogen was shown to improve the phagocytic capabilities of brain macrophages against fluorescent *E. coli*. Indeed, estrogen significantly enhanced the clearance of *Escherichia coli* in a dose-dependent fashion in comparison with negative control untreated cells (Li et al., 2000).

Estrogen treatment significantly enhanced the clearance of MRSA and *P. aeruginosa* by monocyte-derived macrophages across all glucose concentrations,

counteracting the inhibitory effects of hyperglycaemia on bacterial phagocytosis as previously shown in chapter 3. These findings indicated that estrogen stimulates macrophage-mediated phagocytosis and may help overcome diabetes-associated impairment in bacterial clearance.

The bacterial diversity in chronic wound infections is wide and depends mainly on the wound type. Chronic wounds usually comprise both Gram-positive and Gram-negative species, including *Staphylococcus*, *Pseudomonas*, *Enterobacter*, *Streptococcus*, *Escherichia Stenotrophomonas*, and *Serratia* spp (Dowd *et al.,* 2008). One of the main limitations of the research conducted by Crompton et al. (2016), and Li et al. (2000) is their choice of bacteria. *K. pneumonia* and *E coli* are Gram-negative pathogens that can cause several infectious diseases and may infect chronic wounds. However, they are not the leading pathogens that cause wound infections in humans. MRSA and *P. aeruginosa* are the main nosocomial pathogens that are known to infect wounds, particularly DFUs. In line with previous reports, the data in this chapter confirms that estrogen promotes *in vitro* phagocytosis of both a Gram-negative and a Gram-positive wound-associated pathogen by M0, M1 and M2 macrophages.

However, within the complex real-life milieu of DFUs, bacteria frequently evade immune responses through the development of biofilm growth, a crucial component of microbial communities that is implicated in the chronicity and the persistence of DFUs (Afonso et al., 2021). Studying estrogen's effect on the phagocytosis of MRSA and *P. aeruginosa* biofilms by M1 macrophages under high glucose conditions provides further insights into diabetic wound infections and highlights possible strategies for anti-biofilm therapeutics.

M1 macrophages demonstrate greater phagocytic activity compared to M0 and M2 macrophages, mainly due to their pro-inflammatory nature and metabolic profiles. M1 macrophages are characterised by enhanced glycolytic metabolism, which supports their robust phagocytic capabilities (Hickman et al., 2023; Huo et al., 2023). In contrast, M2 macrophages, while also capable of phagocytosis, tend to focus on anti-inflammatory responses and tissue repair, leading to a comparatively lower phagocytic activity (Chakraborty et al., 2020; Hickman et al., 2023; Tacconi et

al., 2024). Having established the superior phagocytic capacity of M1 macrophages against planktonic bacteria compared to M0 and M2 subsets (Figure 4.7), further host-pathogen interaction assays in this study we conducted using M1 macrophages. Classically-activated M1 macrophages have been reported to be more phagocytic compared to resting M0 macrophages and alternatively-activated M2 macrophages *in vitro* (Tarique et al., 2015; Lam et al., 2016). The study by Lam et al. (2016) directly compared the interactions of naive, M1 and M2 macrophages with *Porphyromonas gingivalis*. They found that while M1 and M2 cells showed increased phagocytosis of *P. gingivalis* versus naive macrophages, only the M1 phenotype was able to effectively kill the internalised bacteria. This enhanced microbicidal activity of M1 cells correlated with their higher production of nitric oxide compared to M2 and naive macrophages. Furthermore, M1 macrophages responded to *P. gingivalis* infection by producing significant levels of the inflammatory cytokines TNF-α and IL-12, which were minimally induced in M2 and naive cells. The study by Tarique et al. (2015) also compared phagocytic abilities of M0, M1 and M2 macrophages using FITC-labeled *Eschericia coli*. Using flow cytometry, they found that M1 cells induced by LPS displayed enhanced phagocytosis compared to M0 and M2 cells. Additionally, interesting studies indicate that directing classical-activation of macrophages towards an M1 phenotype is an effective therapeutic strategy for promoting biofilm clearance (Hanke et al., 2013; Yu et al., 2020). Hanke et al. (2013) found that M1 macrophages exhibit greater *S. aureus* biofilm killing ability compared to alternatively activated M2 macrophages. This was observed when *S. aureus* biofilms were reported to develop immune evasion tactics which involved enhancing the alternative switch in macrophage polarisation towards an M2 phenotype, which appeared to be less phagocytic (Hanke et al., 2013). Thus, based on findings in this study, and the body of evidence confirming that M1 macrophages possess greater phagocytic and antibacterial abilities compared to M0 and M2 macrophages, further experiments in this study focused on assessing the effects of estrogen the phagocytic functions of pro-inflammatory M1 macrophages under high glucose conditions relevant to the diabetic wound microenvironment. Enhancing M1

macrophage-mediated phagocytosis represents a promising therapeutic approach for overcoming DFIs.

In line with planktonic data, novel results from the biofilm host-pathogen recovery assay demonstrated that estrogen supplementation significantly enhanced M1 macrophage-mediated clearance of both MRSA and *P. aeruginosa* biofilms at all glucose concentration (11, 15 mM, 20 mM and 30 mM) compared to NC M1 macrophages. Moreover, estrogen reversed the impaired phagocytosis caused by hyperglycaemia observed in Chapter 3.

The crystal violet staining results mirrored the results from the host-pathogen interaction assays, with M1 exposure to estrogen significantly reducing MRSA and *P aeruginosa* biofilm mass compared to the untreated negative control macrophages at all glucose concentrations. This demonstrated that estrogen was able to promote M1-mediated clearance of bacterial biofilms under hyperglycaemic conditions. These findings confirm and extend the work conducted by Souza et al. (2021) and El Mohtadi (2019), who demonstrated a significant estrogen-mediated increase in the *in vitro* phagocytic activity of macrophages against the planktonic forms of MRSA and *P. aeruginosa*. However, these studies only used planktonic models of infection. In addition, diabetic wound conditions were not investigated (El Mohtadi, 2019; Souza et al., 2021). By assessing the effects of estrogen on the clearance of biofilms formed by key wound pathogens under high glucose environments, this project addresses a significant gap in the literature and provides novel insights into immunomodulation in the complex diabetic wound milieus. This study is among the first to elucidate connections between estrogen, macrophage phagocytic function, and biofilm clearance under conditions highly relevant to DFIs.

Visualisation of host-pathogen interactions via confocal microscopy showed treatment of M1 macrophages with estrogen increases the internalisation of GFP-*S. aureus* and mCherry- *P. aeruginosa* biofilms by M1 macrophages compared to untreated control macrophages under both low and high glucose environments. SEM further confirmed impaired phagocytosis under high glucose conditions and showed estrogen-mediated enhancements in host-pathogen interactions. Notably, SEM revealed morphological responses of M1 macrophages to MRSA and *P.*

aeruginosa biofilms. M1 cells exhibited differential pseudopodial formation against these pathogens, in concordance with the hyperglycaemia-induced cytoskeletal changes seen in M1 macrophages by confocal microscopy in Chapter 3. The extension of membrane protrusions like pseudopods is a hallmark of activated macrophages and facilitates phagocytic uptake of particulate material including bacteria (Rittig et al., 1999; Rougerie et al., 2013). The SEM data showed biofilm-associated bacteria dispersed through the membrane pores and macrophages extending pseudopods following estrogen treatment, structures typically involved in bacteria entrapment (Lowry et al., 1998). Similar cytoskeletal rearrangements enabling phagocytic cup formation around bacterial targets have been reported in macrophages (Tsuboi and Meerloo, 2007; Jaganathan et al., 2022). This suggests estrogen signalling induces changes in macrophage cytoskeletal arrangements that warrants further investigation. In summary, microscopy assessments visually validated the bacterial recovery assays while providing novel insights into potential estrogen-mediated changes in actin dynamics in M1 macrophages and pathogen-specific biofilm arrangements in response to elevated glucose.

Chapter 5: Estrogen Receptor-Alpha Signalling Enhances the Phagocytosis of Bacteria by Macrophages Through Modulation of Key Mediators of Innate Immunity

5.1 Introduction

5.1.1 Estrogen Receptors

ERs are nuclear receptor proteins that play a crucial role in regulating innate immune responses and mediating the effects of estrogen in the body (Paterni et al., 2014). There are two main ER subtypes, ER-α and ER-β, which are encoded by different genes and have different physiological effects and tissue distribution (Paterni et al., 2014; Fuentes and Silveyra, 2019). Both ER-α and ER-β are expressed in monocytes and macrophages, with higher expression of ER-α compared to ER-β (Ribas et al., 2011). The presence of ERs allows estrogen to directly affect macrophage activities and functions (Lu et al., 2004; Murphy et al., 2009; Toniolo et al., 2015; Pelekanou et al., 2016). When estrogen binds to the ligand binding domain of ERs, the receptors dimerize and translocate to the nucleus, where they bind to estrogen response elements in the DNA, regulating the transcription of target genes which mediates estrogen's effects (Klinge, 2000). Research has shown that LPS, an important structural component of Gram-negative bacteria, can impact the expression and activation of both ER-α and ER-β in macrophages (Campesi et al., 2017). LPS-mediated regulation of ERs has also been shown to affect bacterial clearance by mediating the phagocytic activity of macrophages (Campesi et al., 2017).

Although some studies have shown estrogen enhances phagocytosis, especially in relation to brain microglial macrophages (Loiola et al., 2019; Yanguas-Casás et al., 2020; Aryanpour et al., 2021) and sepsis (Rettew et al., 2009; Sun et al., 2021; Zhang et al., 2021; Lakbar et al., 2023), the precise molecular mechanisms by which ER signalling modulates inflammation and phagocytosis require further elucidation.

ER agonists and antagonists can be used as tools to study the distinct functions of ER-α and ER-β. The ER-α agonist propyl pyrazole triol (PPT) and the ER-β agonist diarylpropionitrile (DPN) bind to and activate their respective receptor subtypes (Meyers et al., 2001; Thammacharoen et al., 2009; Minutolo et al., 2011) In contrast, the ER-β antagonist PHTPP (4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol) and the ER-α antagonist

TPBM (Theophylline, 8-[(benzylthio)methyl]-(7Cl,8Cl)) block receptor activity (Sun et al., 2002; Mao et al., 2008; Al-Khyatt et al., 2018). Using these selective ER agonists and antagonists provides insight into how each ER subtype uniquely contributes to regulating innate immune cell function (Mao et al., 2008).

Enhancing phagocytosis in macrophages is likely to have important implications for diabetic wound healing and the resolution of DFIs. Impaired immune function in diabetes can lead to reduced pathogen clearance, persistent inflammation, and chronic wounds. Elucidating how estrogen enhances phagocytic responses in macrophages by co-ordinately regulating innate immune receptors and inflammatory molecules may reveal therapeutic mechanisms by which ER signalling can promote wound healing.

5.1.2 Estrogen and CD14

The role of the membrane receptor CD14 in facilitating the recognition and engulfment of apoptotic cells and bacteria is well-established. CD14 acts as a co-receptor for TLR4, enabling optimal inflammatory responses to pathogens (Devitt et al., 1998; Frevert et al., 2000; Knapp et al., 2006; Zanoni and Granucci, 2013). Upon activation, CD14 triggers intracellular signalling cascades in macrophages, prominently involving the NF-κB pathway (Ciesielska et al., 2022). This leads to the production and release of proinflammatory cytokines and other inflammatory mediators which are crucial for mounting an effective immune response (Zanoni et al., 2011; Zanoni and Granucci, 2013). Furthermore, sCD14 acts as a damage-associated molecular pattern (DAMP), which interacts with PRRs, leading to pro-inflammatory cytokine and chemokine production in macrophages (Alarcón-Vila et al., 2020).

Notably, ER signalling has been shown to modulate the macrophage-mediated release of inflammatory markers such as TNF-α, IL-1β, , IL-6 and IL-23 (Villa et al., 2015; Dama et al., 2021), thereby regulating the overall inflammatory response (Monteiro et al., 2014; Villa et al., 2016). Estrogen can influence gene expression of these cytokines and alter their secretion through potential crosstalk with CD14-mediated signalling pathways (Jiang et al., 2005; Anas et al., 2010b; Yu et al., 2021).

Therefore, understanding the molecular connections between estrogen and CD14-triggered intracellular cascades may provide important insights into their immunomodulatory effects, especially in hyperglycaemic conditions. Investigating how estrogen influences CD14 signalling and downstream cytokine production in high glucose environments could shed light on the immune dysregulation in diabetes and may potential identify therapeutic targets that could lead to the resolution of inflammation and clearance of bacteria in patients with DFUs.

5.1.3 Estrogen and TREM2

TREM2 is an immune receptor expressed on the surface of macrophages and other myeloid cells. By binding to its ligands, TREM2 triggers intracellular signalling pathways that modulate cytokine production, cell proliferation, and phagocytic activity (Hsieh et al., 2009; Yao et al., 2019; Colonna, 2023).

The link between TREM2 and estrogen has been the subject of recent scientific research. It has been found that estrogen, particularly 17β-estradiol, plays an important role in controlling the activation and polarization of microglia, a significant process involved in neuroinflammation and neuroprotection (Villa et al., 2016; Thakkar et al., 2018). While the specific mechanisms are still being investigated, there is evidence that estrogen may enhance TREM2 mRNA and protein levels expression in microglial cells (Habib et al., 2013; Habib et al., 2014; Aryanpour et al., 2021). This suggests that estrogen treatment may shift microglia more towards an anti-inflammatory phenotype by upregulating TREM2 protein expression as well as modulating their pro- and anti-inflammatory cytokine expression (Habib et al., 2014; Ferrara et al., 2022). Therefore, estrogen may indirectly or directly modulate TREM2-mediated phagocytosis in the context of neuroinflammation, nevertheless more research is needed to elucidate the pathways involved in this process.

Beyond the central nervous system, TREM2 also plays a significant role in modulating macrophage function. Microglia, and macrophages in other tissues, employ numerous common processes to maintain homeostasis during inflammation, based on their antigen presentation techniques (Durafourt et al.,

2012). Both cell types share many similar characteristics, including the expression of important PRRs that recognize DAMPs or PAMPs such as LPS as well as TREM2 receptors (Durafourt et al., 2012).

As a phagocytic receptor, TREM2 has been linked with the modulation of phagocytosis, inflammation, and activation of macrophages in response to various microbial infections (Sharif and Knapp, 2008; N'Diaye et al., 2009; Dabla et al., 2022). Previous studies have shown that TREM2 is involved in internalising multiple bacterial species, such as *S. aureus*, *P. aeruginosa*, *E. coli*, *Francisella tularensis*, *Brucella abortus*, and *Mycobacterium tuberculosis* (Daws et al., 2003; N'Diaye et al., 2009; Dabla et al., 2022). Moreover, TREM-2 suppression has been shown to inhibit bacterial recognition and phagocytosis by both bone marrow–derived macrophages and non-phagocytic ovary cells (N'Diaye et al., 2009). This highlights the important effects of TREM2 on innate immune functions and bacterial clearance by phagocytic cells.

Similarly, estrogen has been shown to influence the polarization of macrophages and their phagocytic activity through binding to the ERs (Bolego et al., 2013; Toniolo, 2014; Toniolo et al., 2015; El Mohtadi, 2019; Loiola et al., 2019). Upon binding to ERs, estrogen activates the PI3K/Akt and MAPK signalling pathways, which intersect with the downstream signalling cascades of TREM2 (Gentilini et al., 2007; Yao et al., 2019; Chen et al., 2020). Therefore, estrogen may enhance the TREM2-induced activation of splenic tyrosine kinase (Syk), which is a key mediator of the potential phagocytic effects of TREM2 (Deczkowska et al., 2020; Colonna, 2023). Estrogen may also modulate TREM2 activity in macrophages by regulating intracellular calcium levels, as TREM2 signalling relies on calcium influx (Guo et al., 2002; Jairaman et al., 2022) TREM2, upon binding to ligands like bacterial components, it triggers downstream signalling cascades involving mobilization of calcium from intracellular stores as well as calcium influx across the plasma membrane (Kober and Brett, 2017; Okada et al., 2020). This calcium signalling is crucial for enabling the cytoskeletal rearrangements and inflammatory mediator production required for phagocytosis and pathogen clearance (Desai and Leitinger, 2014; Tedesco et al., 2019). Estrogen has been shown to increase cytosolic calcium

levels by modulating calcium channels and pumps (Morley et al., 1992; Zaitsu et al., 2007), so it could potentially enhance TREM2 signalling and its downstream phagocytic/inflammatory effects in macrophages by impacting calcium dynamics. Furthermore, estrogen regulates TREM2 gene expression through direct binding of estrogen response elements (EREs) to ERs in the TREM2 gene promoter region (Fumagalli et al., 2018; Essex et al., 2020).

ApoE has been identified as a ligand for the TREM2 receptor (Atagi et al., 2015; Lyu et al., 2023). Multiple studies have investigated the interaction between ApoE and TREM2 (Atagi et al., 2015; Shi and Holtzman, 2018; Kober et al., 2021; Mai et al., 2022), suggesting that the ApoE-TREM2 binding could facilitate the phagocytosis of apoptotic cells and bacteria (Yeh et al., 2016; Shi and Holtzman, 2018). Yeh et al. (2016) provided key evidence of a direct interaction between TREM2 and ApoE using immunoprecipitation and confocal microscopy analyses. The immunoprecipitation assays demonstrated that TREM2 was able to physically bind ApoE. Moreover, confocal microscopy imaging revealed co-localization of ApoE and TREM2 on the surface of microglial cells, indicating these molecules cluster together in close proximity. This co-clustering suggests ApoE may act as a ligand that binds and activates TREM2 signalling (Yeh et al., 2016). Additional research has shown that the TREM2-ApoE interaction activates intracellular signalling pathways that control cytoskeletal rearrangements required for the phagocytic uptake of microorganisms (Takahashi et al., 2005; Zhou et al., 2018). Together, these findings indicate that TREM2 binding to ApoE may regulate phagocytic signalling in macrophages. Current evidence indicates that estrogen may directly or indirectly control the TREM2-mediated phagocytic functions in macrophages, but more research on the molecular mechanistic interactions is needed, especially in the context of hyperglycaemia. The effects of estrogen and TREM2-ApoE axis in macrophage phagocytosis have not been studied in hyperglycaemic conditions. Therefore, investigating how estrogen and TREM2-ApoE signalling influences the innate immune function of macrophages cultured under elevated glucose environments may provide insights into immune system dysregulation and impaired wound healing in diabetes and DFIs.

5.2 Aim and Objectives

5.2.1 Aim

The aim of this chapter was to investigate the regulation of inflammatory signalling pathways by estrogen under hyperglycaemic conditions, with particular focus on CD14 and TREM2-ApoE signalling in M1 macrophages during the phagocytosis of MRSA and *P. aeruginosa* using an *in vitro* model of a DFI.

5.2.2 Objectives

- To determine the effect of estrogen on the expression of keys inflammatory mediators including TNF-α, IFN-β and NO.
- To examine nuclear translocation of NF-κB pathway to investigate downstream signalling pathways involved in modulating inflammation.
- To analyse the influence of estrogen on TREM2, CD14 and ApoE protein expression by M1 macrophages under high glucose conditions
- To determine which ER isoform(s) mediate the effects of estrogen on the phagocytosis of bacteria under hyperglycaemic conditions using selective ER-α and ER-β agonists/antagonists.

5.3 Methods

5.3.1 Cell Culture

An *in vitro* model of diabetes was generated using the human monocytic cell line U937 as described in 2.3. The cells were cultured under aseptic conditions at 37 °C and 5% CO_2 in RPMI-1640 CM. To model the hyperglycaemic conditions found in diabetes, U937 monocytes were grown in CM supplemented with increasing glucose concentrations of 15, 20 and 30 mM as described in 2.1.3. The negative control (NC) was U937 monocytes grown in CM containing maximal euglycemic levels (11 mM) of glucose observed in random glucose tests. The monocytes ($1x10^6$ cells/mL) were subsequently differentiated into M0 macrophage-like cells after treatment with 50 ng/mL PMA for 24 hours. After differentiation, the macrophages

were polarized towards an M1 pro-inflammatory phenotype by stimulation with 100 ng/mL LPS and 20 ng/mL IFN-γ in either CM or GS medium as described in 2.5.

5.3.2 Estrogen Receptor Activation and Inhibition

To investigate the specific pathways mediated by estrogen in enhancing the antibacterial functions of M1 macrophages, M1 macrophages were treated with estrogen receptor (ER) agonists or antagonists. For ER activation, M1 macrophages were stimulated with the ER-α agonist PPT (1 x 10^{-6} M)(Frasor et al., 2003) or the ER-β agonist DPN (1 x 10^{-6} M)(Bansal and Chopra, 2014) in CM or GS medium and incubated for 24 hours at 37 °C and 5% CO_2.

For ER inhibition, M1 macrophages were pre-treated with the ER-β antagonist PHTPP (1 x 10^{-6} M) (Kim et al., 2013) or ER-α antagonist TPBM (1 x 10^{-6} M) (Kim et al., 2013; Singh, 2017)in CM or GS medium and incubated for 24 hours at 37 °C and 5% CO_2. Cells were then stimulated with 1 x 10^{-7} M estradiol for an additional 24 hours in CM or GS medium.

5.3.3 Enzyme-Linked Immunosorbent Assay (ELISA)

The secreted levels of the inflammatory cytokines TNF-α and IFN-β, as well as the soluble proteins sCD14, sTREM2, and ApoE were measured by ELISA from supernatants of M1 macrophages cultured under increasing glucose conditions following the manufacturers' instructions as described in 2.9.

5.3.4 NO Assay

Classically-activated M1 macrophages cultured under increasing glucose levels were treated with or without estrogen for 24 hours. NO production was measured in cell culture supernatants using the Griess assay as described in 2.10. NO concentration released by the macrophages was calculated from the absorbance at 540nm by interpolation from a standard curve of sodium nitrite.

5.3.5 Flow Cytometry

Flow cytometry was used to assess the effects of estrogen on the cell surface expression of mCD14, mTREM2, and ApoE in M1 macrophages following ER agonism/blockade. M1 macrophages were labelled with fluorochrome-conjugated antibodies specific for mCD14 (FITC anti-CD14 or APC anti-CD14), mTREM2 (AlexaFluor 647 anti-TREM2), or ApoE (FITC anti-ApoE). Flow cytometry was then used to quantify the cell surface expression of these proteins by acquiring 10,000 events on a MACSQuant Analyzer and analysing the data with FlowJo software to determine the percentage (%) of positive cells and the MFI for each surface marker.

5.3.6 Confocal Microscopy

Upright confocal microscopy (STELLARIS 5, Leica, UK) was used to visualise the effects of estrogen and ERs agonists/antagonists on NF-κB/p65 activation and nuclear translocation in M1 macrophages cultured under euglycemic or hyperglycaemic conditions as described in 2.12.2.

5.4 Results

5.4.1 The Influence of Estrogen on TNF-α, IFN-β and NO Production by M1 Macrophages Under Hyperglycaemic Conditions

The effect of estrogen ($1x10^{-7}$ M) supplementation on TNF-α, IFN-β, and NO production (Figure 5.1) by M1 monocyte-derived macrophages was compared to untreated negative control (NC) macrophages under different glucose conditions (11 mM, 15mM, 20mM and 30mM).

As shown in Chapter 3, increasing glucose levels resulted in differential modulation of these inflammatory markers in M1 macrophages. TNF-α production increased with elevated glucose concentration, while IFN-β and NO levels were reduced under hyperglycaemic conditions compared to levels measured in M1 NC macrophages.

As illustrated in Figure 5.1 A, estrogen had no significant effect on NO production at all glucose concentrations ($P>0.05$). Similarly, there was no significant difference in

IFN-β levels (Figure 5.1B) between estrogen-treated and untreated M1 macrophages across all glucose concentrations (P>0.05). However, estrogen supplementation significantly reduced TNF-α levels (Figure 5.1.B) compared to the untreated NC across all glucose concentrations (P<0.001). In summary, estrogen selectively decreased TNF-α production by M1 macrophages but did not influence NO or IFN-β levels compared to the untreated NC (Figure 5.1).

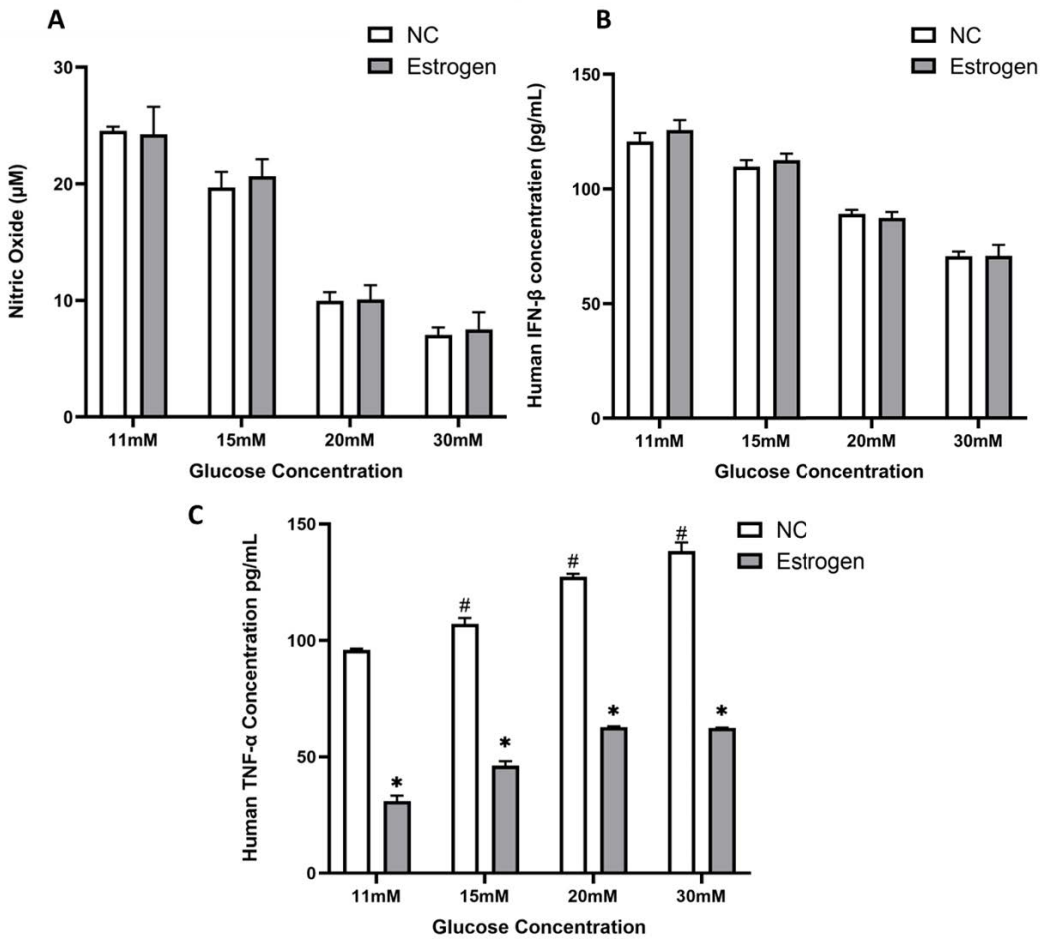

Figure 5.1 Effect of Estrogen on the Secretion of Key Inflammatory Mediators from M1 Macrophages.
Estrogen had no significant impact on either NO (A) or IFN-β (B) secretion from M1 macrophages compared to the corresponding untreated NC (*P>0.05, n=6). However, TNF-α secretion from M1 macrophages significantly decreased (*P<0.001, n=6) following treatment with estrogen ($1x10^{-7}$ M) across all glucose concentrations (C) compared to the corresponding untreated NC. Error bars represent the StEM. # Indicates significant differences (P<0.05) compared to 11mM NC.

To elucidate whether the anti-inflammatory effects of estrogen are mediated through ER-α or ER-β, or both, TNFα secretion was measured following treatment with selective ERs agonists and antagonists. As shown previously, estrogen

significantly decreased TNFα levels compared to the estrogen-deprived corresponding NC macrophages. Blocking ER-α with TPBM followed by estrogen treatment abrogated the effects of estrogen, with significantly higher TNFα production detected compared to estrogen treatment alone across all glucose concentration ($P<0.05$). In contrast, blocking ER-β with PHTPP followed by estrogen treatment failed to significantly ($P>0.9999$) reverse the effect of estrogen alone on TNF-α secretion across all glucose concentrations tested.

In concordance with these findings, the ER-α agonist PPT significantly ($P<0.001$) decreased TNF-α production to levels similar to those observed with estrogen treatment, with no significant difference between them ($P>0.05$). Conversely, the ER-β agonist DPN did not significantly alter TNF-α levels secreted by M1 macrophages compared to the corresponding untreated negative control (NC) ($P>0.05$). Together, these results implicate ER-α, but not ER-β, in mediating the observed anti-inflammatory effects of estrogen on TNFα production by M1 macrophages.

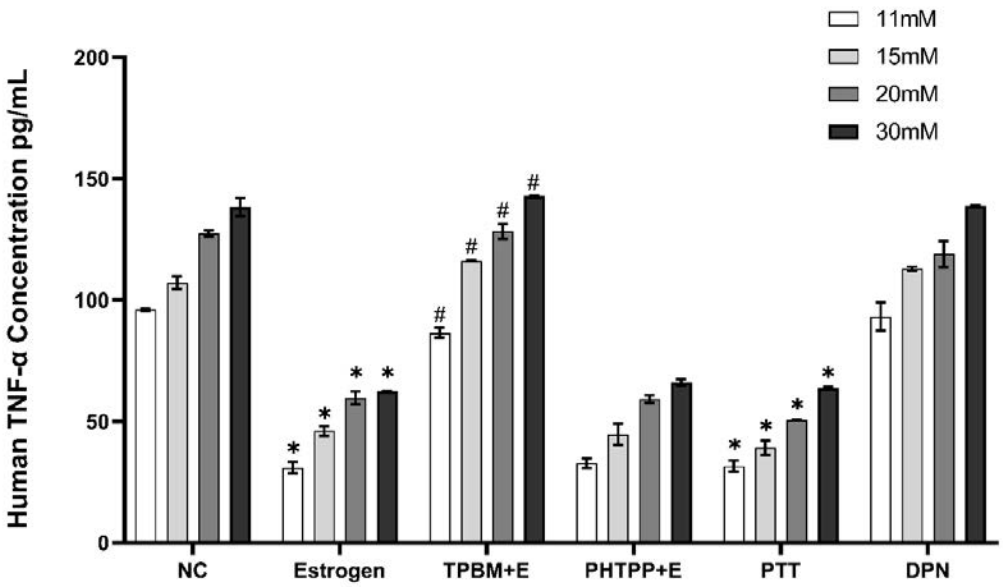

Figure 5.2 ER-α Activation Decreases the Production of TNF-α by M1 Macrophages. Estrogen (E) and the selective ER-α agonist (PTT) significantly reduced the secretion of TNF-α by M1 macrophages compared to the corresponding untreated NC. Blockade of ER-α with TPBM reversed the effects of estrogen on TNF-α secretion by M1 macrophages, with no significant differences observed in TNF-α levels compared to corresponding untreated NC, regardless of glucose concentration. In contrast, blockade of ER-β with PHTPP did not impact the ability of estrogen to reduce TNF-α secretion. DPN did not significantly reduce TNF-α secretion compared to the NC. Data represents an average of n = 3 experiments. * Indicates significant differences ($P<0.05$) compared to corresponding NC. # Indicates significant differences ($P<0.05$) compared to M1 macrophage treated with estrogen alone. Error bars represent the StEM.

5.4.2 Estrogen Dampens Hyperglycaemia-Induced Inflammation by Suppressing NF-κB Nuclear Translocation

The nuclear translocation of the transcription factor NF-κB serves as a key molecular readout of inflammatory responses, including the inflammatory phase of wound repair. NF-κB binds to kappa enhancer elements and induces expression of inflammatory mediators (Iacobazzi et al., 2023). Therefore, tracking the intracellular distribution of NF-κB, which is a key player in hyperglycaemia-induced inflammation (Cheng et al., 2023), provides a critical insight into the inflammatory signalling and phagocytic functions of M1 macrophages in this study.

NF-κB p65 localisation in M1 macrophages was measured under low (11 mM) or high (30 mM) glucose conditions, with or without estrogen treatment ($1x10^{-7}$ M), by confocal microscopy as detailed in 2.1.11.2 (Figure 5.3.A and Figure 5.3.B). The influence of estrogen on NF-κB p65 nuclear-cytoplasmic partitioning was quantified by computational analysis of microscopy images using ImageJ software (Figure 5.3.C and Figure 5.3.D). The nuclear: cytoplasmic ratio of RelA (NF-κB) was plotted (C) along with total cellular RelA fluorescence intensity (D). Panels A and B illustrated that NF-κB p65 (red) is present in both nuclear and cytoplasmic compartments in untreated NC macrophages, with enriched nuclear localisation under high glucose conditions as established in Chapter 3. In contrast, estrogen-treated macrophages displayed a marked reduction of nuclear red fluorescence, indicative of decreased NF-κB p65 nuclear translocation. As a control, LPS-treated macrophages were included, as LPS is a potent activator of the NF-κB pathway (Nomura, 2001; Hobbs et al., 2018). LPS treatment significantly (##<0.001) increased NF-κB nuclear translocation and total cellular NF-κB levels in macrophages under both low and high glucose concentrations. Importantly, estrogen treatment significantly reduced this LPS-induced NF-κB activation, suggesting estrogen can inhibit the inflammatory response triggered by LPS and hyperglycaemia in macrophages via inhibition of NF-κB signalling. Quantitative image analysis using ImageJ software confirmed these microscopic observations. The nuclear: cytoplasmic ratio of NF-κB (Figure 5.3.C) and the total cellular NF-κB fluorescence intensity (Figure 5.3.D) were quantified to determine the effect of estrogen treatment on M1 macrophages cultured under 11 mM or 30 mM glucose conditions. The results showed that treatment with estrogen significantly decreased the nuclear localisation (n=12, 11mM and 30mM, $P<0.05$) and total fluorescence level of NF-κB ($P<0.05$), compared to the corresponding untreated NC. Together, these findings demonstrate that estrogen inhibits hyperglycaemia-induced NF-κB activation and nuclear translocation in pro-inflammatory M1 macrophages.

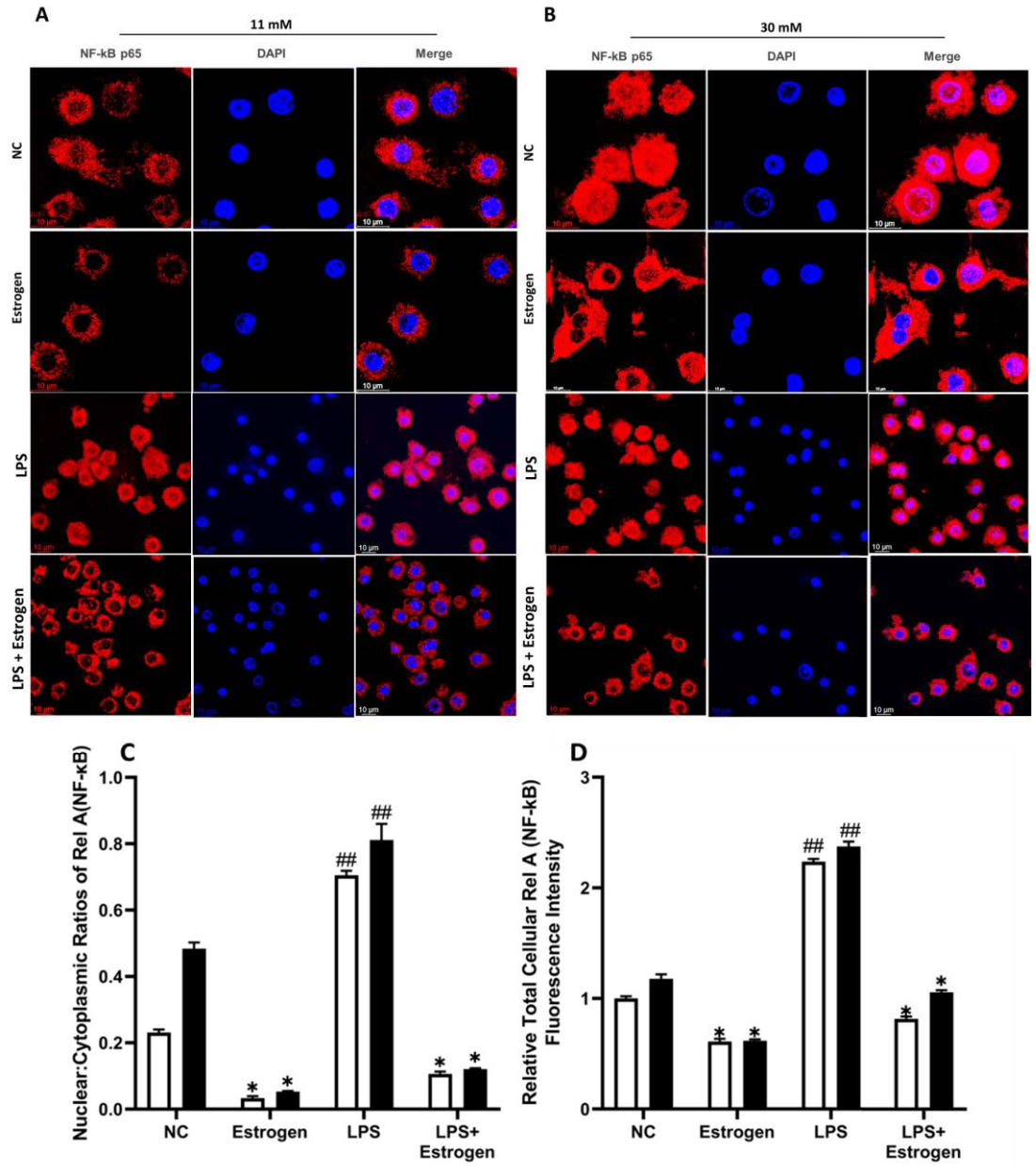

Figure 5.3 Estrogen Decreases Hyperglycaemia-Induced NF-κB p65 Nuclear Translocation in M1 Macrophages

(A,B) Estrogen reduced NF-κB p65 (red) nuclear translocation at 11mM and 30mM glucose. LPS increased nuclear translocation which was reduced by estrogen co-treatment. Nuclei: DAPI (blue). (C) Nuclear:cytoplasmic NF-κB p65 ratio confirming estrogen reduced nuclear translocation at 11mM and 30mM glucose. LPS increased nuclear translocation (##P<0.001) which was reduced by estrogen (*P<0.05). (D) Estrogen reduced total NF-κB levels at both glucose concentrations (*P<0.05). LPS significantly increased total NF-κB (##P<0.001) which was attenuated by estrogen. *P<0.05 vs untreated control**.**Error bars represent the StEM. Scale bars represent 10 µm.

Subsequently, ER-α and ER-β signalling was interrogated in relation to NF-κB p65 nuclear translocation in M1 macrophages under conditions of euglycemic (11 mM) and hyperglycaemic (30 mM) conditions. Confocal microscopy analysis revealed that estrogen supplementation suppressed nuclear localization of NF-κB p65 (red) compared to the cytoplasmic fraction, irrespective of glucose concentration (Figure 5.4).

Blocking ER-α with TPBM prior to estrogen treatment significantly (Figure 5.4; $P<0.01$) reversed the inhibitory effects of estrogen, resulting in higher levels of NF-κB p65 accumulating in the nucleus. In concordance with this finding, the ER-α agonist PPT significantly ($P<0.001$) decreased p65 nuclear translocation to levels comparable to estrogen treatment. In contrast, the ER-β agonist DPN did not significantly ($P>0.05$) reverse the inhibitory effects of estrogen on NF-κB p65 translocation. In addition, blocking ER-β with PHTPP prior to estrogen treatment did not significantly alter NF-κB p65 nuclear translocation compared to estrogen treatment alone at either glucose concentration. Collectively, these findings indicate that the suppressive effects of estrogen on NF-κB p65 nuclear translocation in M1 macrophages are mediated through ER-α, and not ER-β signalling, under both euglycemic and hyperglycaemic conditions.

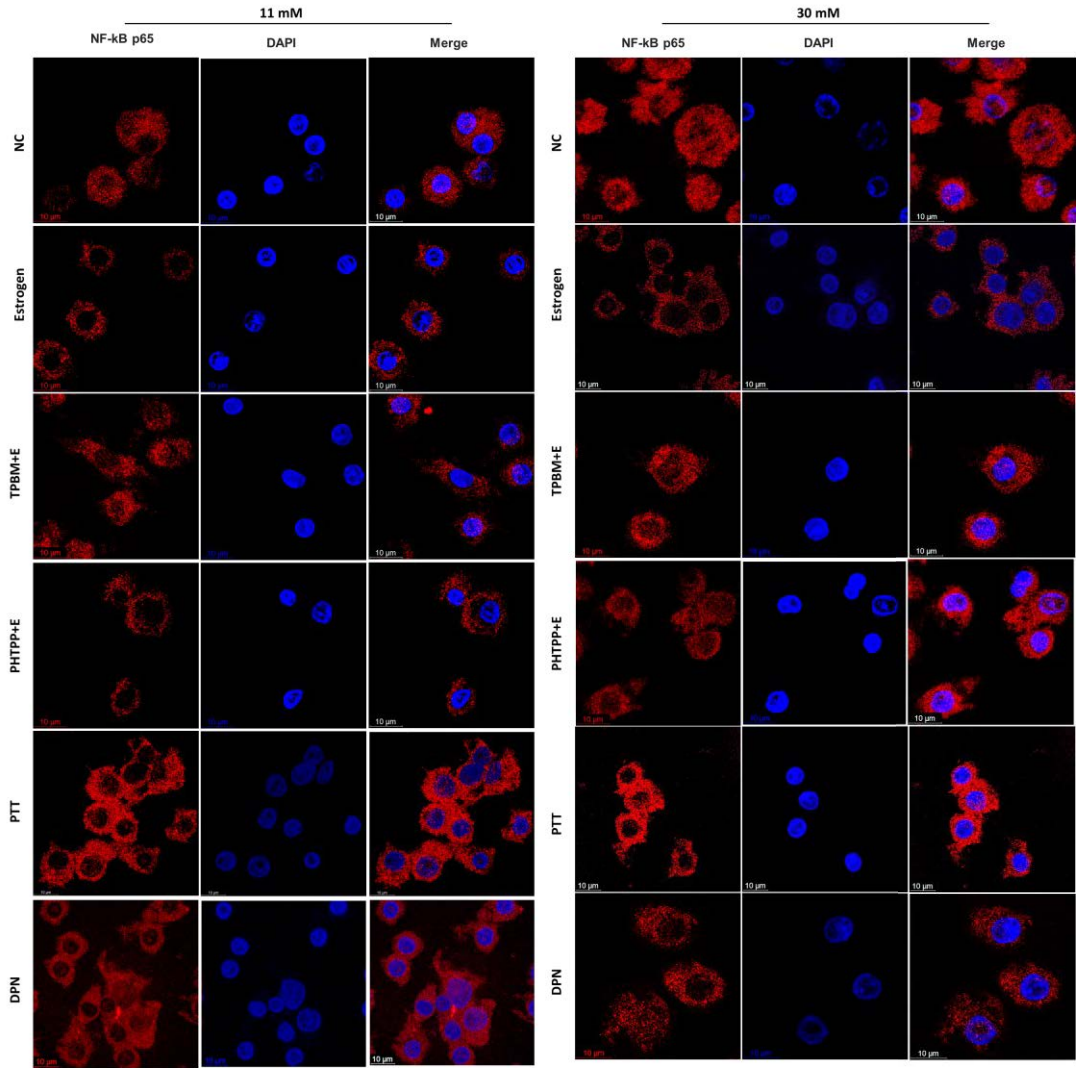

Figure 5.4 Estrogen Inhibits NF-κB p65 Nuclear Translocation in M1 Macrophages via specific binding to the ER-α rather than ER-β.
Estrogen and the ER-α agonist PPT reduced NF-κB p65 nuclear translocation in M1 macrophages compared to NC cells at both 11mM (A) and 30mM (B) glucose concentrations. The ER-β antagonist PHTPP followed by estrogen treatment did not reverse the inhibitory effects of estrogen on p65 nuclear translocation. In contrast, the ER-α antagonist TPBM reversed the effects of estrogen, resulting in increased p65 nuclear accumulation at similar levels to those observed in NC cells cultured under both glycaemic conditions. The ER-β agonist DPN did not inhibit p65 nuclear translocation. Cells were stained with the p65 subunit of NF-κB (red), nuclei with DAPI (blue) and captured at 100X magnification. Scale bars represent 10 µm.

The impact of ER agonism and antagonism on NF-κB p65 nuclear-cytoplasmic ratio was measured by analysis of microscopy images using ImageJ software (Figure 5.5.A and 5.5.B). The nuclear: cytoplasmic ratio of RelA (NF-κB) is plotted (A) together with total cellular RelA fluorescence intensity (B). Treatment with estrogen or the ER-α agonist PPT significantly ($P<0.0001$) decreased the nuclear localisation (i.e. the nuclear: cytoplasmic ratio) and total cellular levels of NF-κB p65, in comparison with

untreated NC cells. Treatment with the ER-β antagonist PHTPP prior to estrogen exposure did not significantly ($P>0.05$) reverse the inhibitory effects of estrogen on either p65 nuclear translocation or total cellular levels of cellular RelA. In contrast, the ER-α antagonist TPBM reversed the effects of estrogen, resulting in increased p65 nuclear localization and total cellular levels that were similar to those observed in untreated NC macrophages. However, the ER-β agonist DPN had no significant effect on NF-κB p65 nuclear translocation or total cellular RelA levels compared to those measured in untreated NC macrophages.

Together, these novel results indicate that the inhibitory effects of estrogen on NF-κB p65 nuclear translocation and total cellular levels are specifically mediated through binding to ER-α, while ER-β signalling appears to play no role in regulating this pathway under the conditions investigated.

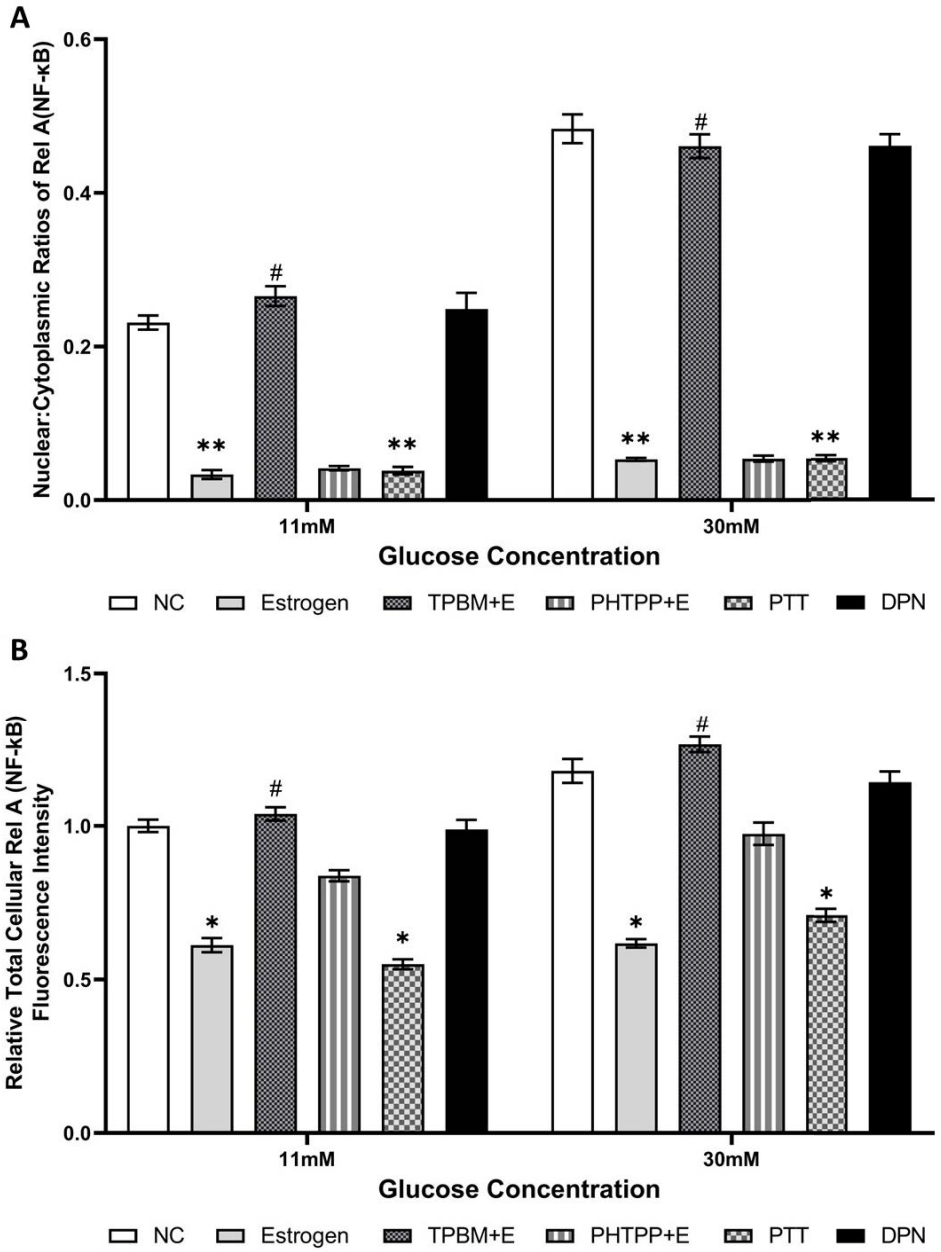

Figure 5.5 The Effect of Selective Activation of ERs on NF-κB Nuclear Translocation under Low versus High Glucose Conditions.

Estrogen inhibits NF-κB p65 nuclear translocation and total cellular levels via ER-α signalling under both euglycemic and hyperglycaemic conditions. (A) Nuclear: cytoplasmic ratio of NF-κB p65 translocation following selective stimulation or blockade of ER-α and ER-β. Estrogen and the ER-α agonist PPT significantly reduced p65 nuclear translocation compared to negative control (NC) macrophages cultured at either 11mM or 30mM glucose. The ER-β antagonist PHTPP did not reverse the inhibitory effect of estrogen whereas the ER-α antagonist TPBM significantly reversed the action of estrogen, increasing p65 nuclear levels similar to those observed in untreated NC macrophages. (B) Relative total cellular NF-κB p65 fluorescence intensity following ER modulation. Estrogen and PPT treatment decreased total cellular p65 levels compared to NC at both glucose concentrations. PHTPP did not reverse the inhibitor activity of estrogen, whereas TPBM significantly blocked the effect of estrogen. *P<0.01, **P<0.001 indicate significant differences compared to the corresponding NC. and # Indicates significant differences (P<0.001) compared estrogen treatment alone. Error bars represent the StEM.

5.4.3 Estrogen Reduces CD14 Expression under Hyperglycaemic Conditions.

5.4.3.1 The Effect of Estrogen on CD14 Protein Levels.

The effect of estrogen on mCD14 expression levels in M1 macrophages was investigated in this study. Classically-activated monocyte-derived M1 macrophages were treated with 17β-estradiol (1×10^{-7} M) for 24 hours under euglycemic (11 mM) or hyperglycaemic (30 mM) glucose conditions. mCD14 levels were then measured by flow cytometry as described in section 2.8.3.

Estrogen significantly ($p < 0.0001$, n=6) decreased mCD14 expression on M1 macrophages compared to untreated controls under both euglycemic (11 mM) and hyperglycaemic (30 mM) conditions (Figures 5.6). The histograms in Figure 5.6.A demonstrate a leftward shift in mCD14 fluorescence intensity, indicating a reduced receptor expression in estrogen treated M1 cells. Quantification of the flow cytometry data revealed a decrease in the percentage of mCD14+ cells from 22.1 ± 3.1% in NC macrophages to 15.7 ± 2.3% in estrogen-treated macrophages at 11 mM glucose. Similarly, estrogen reduced the mCD14+ population from 45.7 ± 2.5% to 19.1 ± 3.6% at 30 mM glucose.

In addition to its expression on the macrophage cell surface, CD14 can also be cleaved and secreted into the extracellular environment as a sCD14. To determine the effect of estrogen on sCD14 secretion, the conditioned media from the estrogen-treated M1 macrophages was collected prior to quantifying sCD14 levels by ELISA. In alignment with previous results highlighted in Chapter 3, exposure of macrophages to high (30mM) glucose resulted in an approximate 2-fold increase in sCD14 secretion compared to macrophages cultured under euglycemic (11mM) glucose (Figure 5.6). Intriguingly, compared to the untreated NC, estrogen stimulation significantly ($P<0.0001$, n=6) decreased sCD14 secretion by M1 cells cultured under both euglycemic (11 mM) and hyperglycaemic (30 mM) conditions (Figure 5.6.C).

Overall, these findings demonstrate that estrogen reduces the secretion of both mCD14 and sCD14 by M1 macrophages, independent of glycaemic status. Downregulation of both membrane-bound CD14 receptors and the extracellular release of soluble CD14 by M1 is likely to contribute to the observed effects of estrogen on inflammation and bacterial clearance.

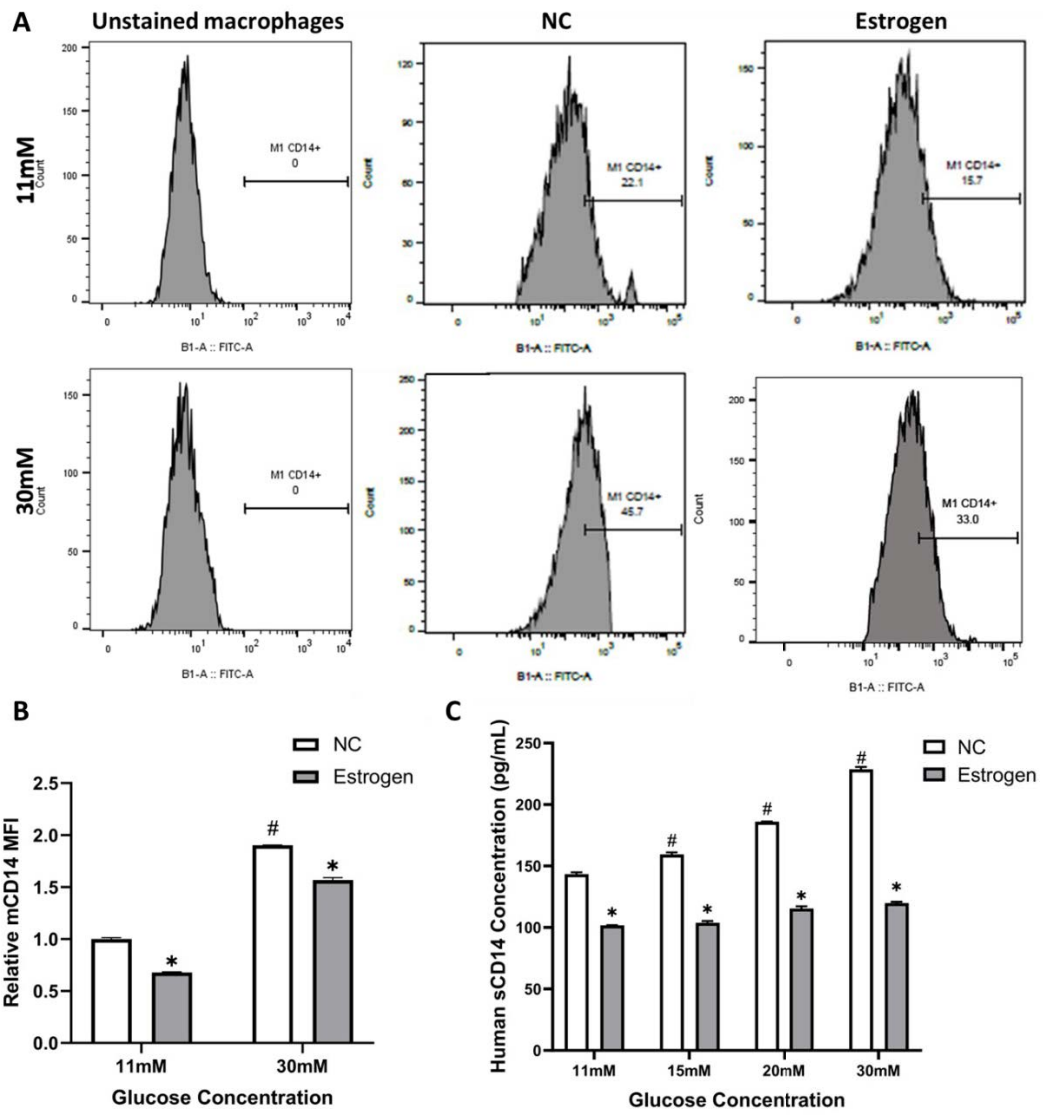

Figure 5.6 Estrogen Reduces *In Vitro* CD14 Expression in M1 Macrophages.
Monocyte-derived M1 macrophages were cultured in the presence of euglycemic (11mM) or hyperglycaemic (30 mM) glucose concentrations prior to treatment with or without estrogen (1 x 10^{-7} M). (A) Flow cytometry histograms showed a lower proportion of M1 macrophages expressing mCD14 following treatment with estrogen. (B) Quantitative analysis of mCD14 MFI indicated a significant decrease in mCD14 surface expression in estrogen-treated macrophages compared to untreated NC macrophages at both glucose concentrations. Flow cytometry data represent an average of n = 6 experiments. (C) ELISA analysis showed treatment of M1 macrophages with estrogen significantly decreased the secretion of sCD14 by M1 macrophages compared to untreated negative control (NC) macrophages at both glucose concentrations. Data represent an average of n=6 experiments (*: P<0.0001). Significant differences compared to the hyperglycaemic 30mM conditions are indicated (# P<0.001), Error bars represent the StEM.

The CD14 expression experiments were repeated using agonists and antagonists of ER-α and ER-β to determine which ER proteins estrogen was acting through to mediate CD14 expression. Flow cytometry analysis revealed that the ER-α agonist PPT mirrored the effect of estrogen, significantly reducing mCD14 expression compared to untreated NC at both 11mM and 30mM glucose ($P<0.001$). Similarly, ELISA showed PPT significantly decreased sCD14 levels compared to untreated NC macrophages ($P<0.001$). However, blocking ER-α with TPBM prior to estrogen treatment significantly ($P<0.001$) reversed the inhibitory effects of estrogen, increasing mCD14 back to similar levels observed in untreated NC macrophages. Conversely, when ER-β was blocked with PHTPP prior to estrogen treatment, there was no significant difference ($P>0.05$) in mCD14 or sCD14 compared to estrogen treatment alone at either glucose level. The ER-β agonist DPN also had no significant effect on either mCD14 ($P>0.05$) or sCD14 ($P>0.05$) expression compared to untreated NC macrophages. Taken together, these results demonstrate that the inhibitory effects of estrogen on CD14 expression at all glucose environments are mediated through selective binding to ER-α rather than ER-β.

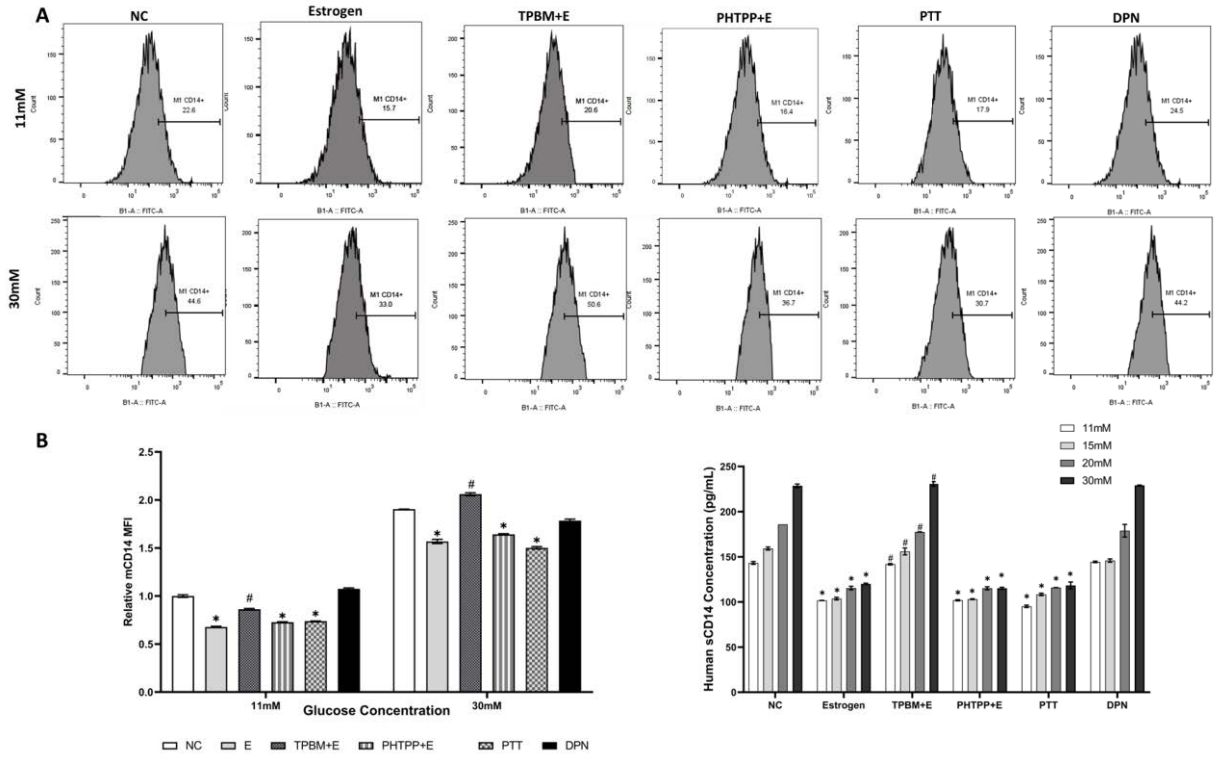

Figure 5.7 Estrogen Inhibits CD14 Expression in M1 Macrophages Through Activation of ER-α.

M1 macrophages were cultured in euglycemic (11mM) or hyperglycaemic (15, 20 and 30 mM) glucose prior to either ER activation with estrogen, PTT or DPN, or ER blockade with TPBM or PHTPP. (A) Flow cytometry histograms illustrating decreased cell surface expression of mCD14 in macrophages following treatment with estrogen or PTT. (B) Quantitative mCD14 MFI analysis confirmed a significant reduction in mCD14 levels following treatment with estrogen or PTT at all glucose concentrations. ER blockade with TPBM but not PHTPP was able to significantly (#; $P<0.001$) reverse the effect of estrogen treatment, increasing mCD14 levels back to those observed in the corresponding untreated NC. (C) ELISA analysis showing that estrogen and PTT significantly reduced sCD14 secretion compared to the corresponding untreated NC. TPBM reversed this effect when given with estrogen (#; $P<0.05$), while PHTPP did not significantly alter sCD14 levels compared to estrogen alone. The ER-β agonist DPN had no significant effect on mCD14 or sCD14 expression. Data are represented as means ± StEM (n=6). *: $P<0.05$ compared to untreated. #: $P<0.05$ compared to estrogen treatment.

5.4.4 Estrogen Increases TREM2 and ApoE Expression under Hyperglycaemic Conditions.

5.4.4.1 The Effect of Estrogen on TREM2 Expression in M1 Macrophages.

The potential influence of estrogen on TREM2 protein expression in M1 macrophages cultured under euglycemic (11mM) or hyperglycaemic (30mM) glucose conditions was subsequently investigated. TREM2+ cells were gated, and histograms highlighting mTREM2 expression were plotted comparing estrogen-treated cells (red histogram) to untreated NC cells (blue histogram). Estrogen treatment induced a right shift in the mTREM2 fluorescence intensity indicating increased mTREM2 expression in estrogen treated M1 macrophages compared to untreated NC macrophages (Figure 5.8.A). Quantitative analysis of the MFI data revealed that estrogen significantly increased mTREM2 expression by over 50% compared to untreated NC cells at both 11mM and 30mM glucose conditions ($P<0.0001$) (Figure 5.8.B). Histogram analysis confirmed that the entire population of estrogen-treated macrophages expressed mTREM2, as reflected by the uniform right shift with no residual population at baseline expression levels. Together, these results demonstrated that estrogen enhances mTREM2 expression in M1 macrophages under both euglycemic and hyperglycaemic conditions. ELISA analysis further demonstrated that estrogen significantly ($P<0.0001$) increased the secretion of sTREM2 levels in comparison to untreated NC macrophages cultured under either glycaemic condition (Figure 5.8.C). Collectively, these findings showed estrogen upregulates both cell surface levels of mTREM2 and secreted levels of sTREM2 by M1 macrophages, irrespective of glucose levels.

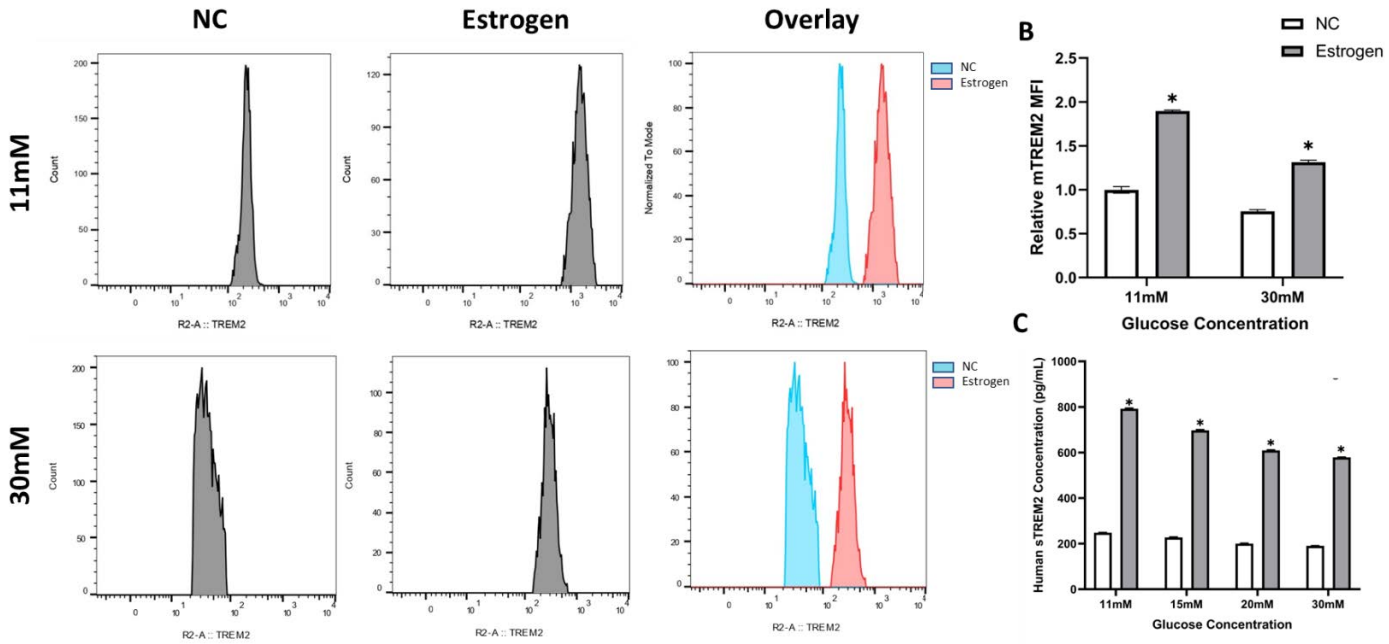

Figure 5.8 Estrogen Increases TREM2 Expression in M1 Macrophages.

Monocyte-derived M1 macrophages were cultured in the presence of euglycemic (11mM) or hyperglycaemic (15mM, 20mM, 30mM) glucose concentrations prior to treatment with or without estrogen (1 x 10^{-7} M). (A) Flow cytometry histograms showed an increased proportion of M1 macrophages expressing mTREM2 following estrogen treatment compared to untreated negative control (NC) macrophages cultured under both euglycemic and hyperglycaemic conditions. (B) Quantitative analysis of mTREM2 MFI indicated estrogen significantly increased mTREM2 surface compared to untreated macrophages, regardless of glucose concentration. Flow cytometry data represent an average of n=6 experiments. (C) ELISA analysis showed treatment of M1 macrophages with estrogen significantly increased the secretion of sTREM2 compared to untreated NC macrophages at all glucose concentrations (11 mM, 15 mM, 20 mMor 30mM). Data represent an average of n=6 experiments. *: P<0.001 compared to corresponding untreated condition. Error bars represent StEM.

The TREM2 expression experiments were repeated using agonists and antagonists of ER-α and ER-β to determine which ER proteins estrogen was acting through to mediate TREM2 expression. Flow cytometry and ELISA analysis was used to quantify the membrane-bound and soluble TREM2 protein levels respectively. Flow cytometry analysis showed that the ER-α agonist PTT significantly ($P<0.0001$) increased mTREM2 levels, provoking similar effects to estrogen when compared with the NC macrophages cultured at 11mM ($P=0.003$) and 30mM ($P=0.001$) glucose concentrations. Similarly, ELISA revealed PPT significantly upregulated sTREM2 secretion compared to untreated NC macrophages ($P<0.05$). However, blocking ER-α with the antagonist TPBM prior to estrogen treatment significantly reversed the stimulatory effects of estrogen on mTREM2 ($P<0.0001$) and sTREM2 ($P<0.0001$) when compared to estrogen treatment alone. When ER-β was blocked with PHTPP prior to estrogen treatment, mTREM2 and sTREM2 levels did not significantly differ compared to estrogen treatment alone at either glucose concentration. In addition, the ER-β agonist DPN did not significantly affect mTREM2 ($P>0.05$) or sTREM2 ($P>0.05$) expression compared to untreated NC macrophages cultured at either glucose concentration. Collectively, these results indicate that estrogen acts through ER-α activation, and not ERβ, to stimulate both membrane-bound and soluble TREM2 protein expression in M1 macrophages, irrespective of glycaemic conditions (Figure 5.9).

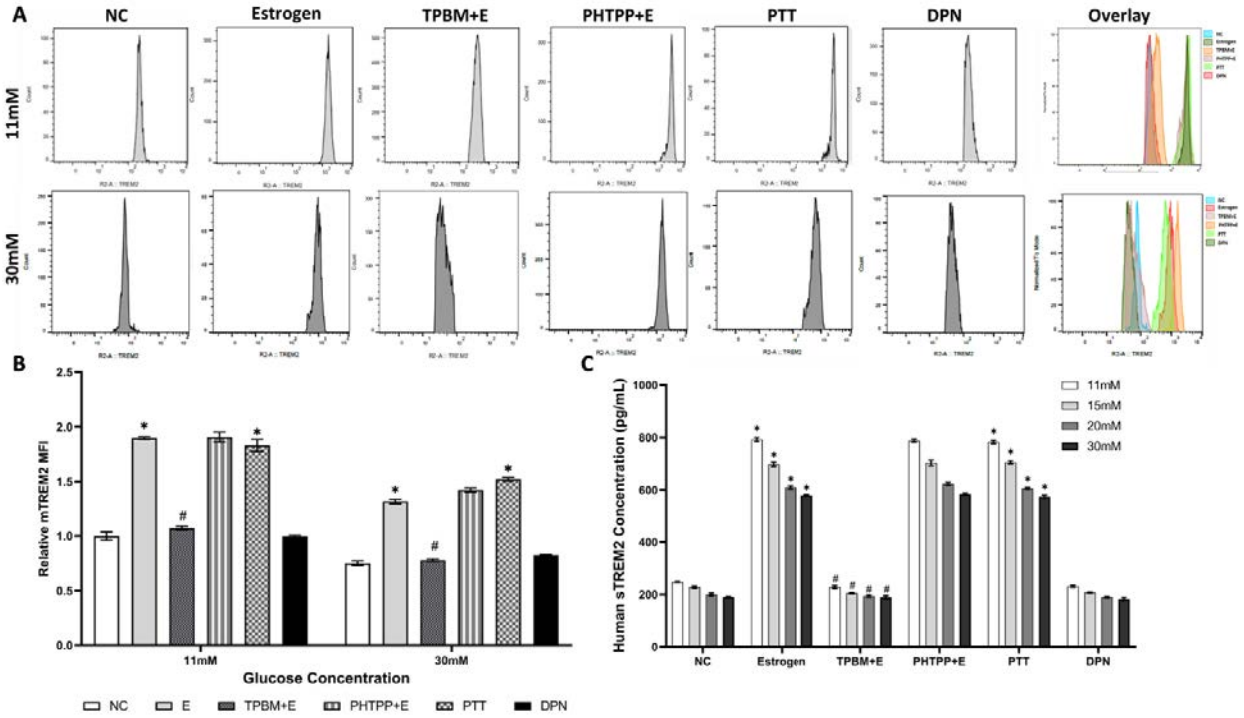

Figure 5.9 Estrogen Upregulates TREM2 Expression in M1 Macrophages Through binding to the ER-α

M1 macrophages were cultured in euglycemic (11mM) or hyperglycaemic (15mM, 20mM, 30mM) glucose prior to either ER activation with estrogen, PPT or DPN, or ER blockade with TPBM or PHTPP. (A) Flow cytometry histograms showed an increased proportion of cells expressing mTREM2 following treatment with estrogen or the ER-a agonist PPT compared to untreated NC cells. (B) Quantitative analysis of mTREM2 MFI indicated estrogen and PPT, but not the ER-β agonist DPN, significantly increased mTREM2 levels at all glucose concentrations. The ER-α antagonist TPBM significantly reversed the stimulatory effects of estrogen on mTREM2 (#; P<0.001) at both glucose levels whereas the ER-β antagonist PHTPP had no effect on estrogen-induced mTREM2 levels. (C) ELISA analysis showed estrogen and PPT significantly increased sTREM2 secretion, whereas the ER-β agonist DPN had no significant effect on compared to untreated NC macrophages across all glucose conditions. The antagonist TPBM significantly negated the stimulatory effect of estrogen on sTREM2 secretion (#; P<0.001) whereas the PHTPP had no effect on estrogen-induced sTREM2 levels. Data represent means ± SEM (n=6). *: P<0.001 compared to untreated. #: P<0.001 compared to estrogen treatment.

Given estrogen induced TREM2 expression, it was hypothesised that estrogen may enhance the phagocytosis of bacteria by M1 macrophages via the ApoE-TREM2 signalling axis in M1 macrophages. Thus, the effect of estrogen on ApoE, a key natural ligand for TREM2 (Krasemann et al., 2017), was also investigated. Monocyte-derived M1 macrophages were cultured under euglycemic and hyperglycaemic conditions, and treated with or without estrogen, prior to quantification of intracellular and secreted ApoE protein levels by flow cytometry and ELISA respectively. Evaluating ApoE regulation under hyperglycaemic conditions is highly relevant given the link between diabetes and impaired macrophage-mediated phagocytosis via dysregulated ApoE-TREM2 signalling (Graham et al., 2016).

The MFI of intracellular ApoE significantly increased in estrogen-treated macrophages compared to untreated NC macrophages ($P<0.0001$) at both glucose concentrations (Figure 5.10.B). Similarly, estrogen treatment significantly ($P<0.0001$) increased secreted levels of ApoE from M1 macrophages grown under both glucose concentrations compared to corresponding untreated NC macrophages (Figure 5.10.C). Together, these findings showed estrogen elevates intracellular and secreted levels of ApoE, suggesting the TREM2/ApoE signalling may play a substantial role in mediating the positive effects of estrogen on the phagocytic function of M1 macrophages, promoting bacterial clearance under euglycemic and hyperglycaemic conditions.

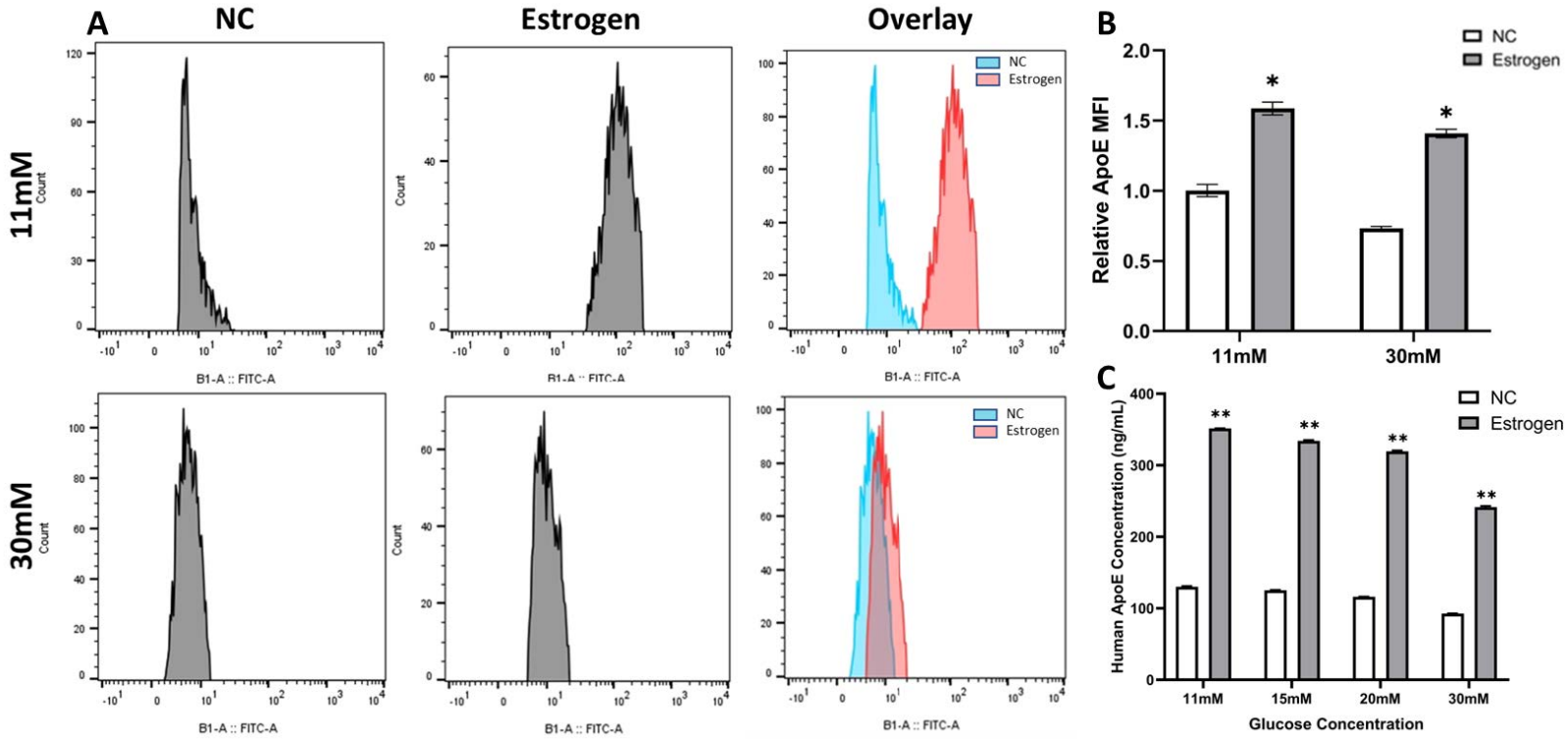

Figure 5.10 Estrogen Upregulates ApoE Expression in M1 Macrophages *in vitro*.

M1 macrophages were cultured under increasing glucose concentrations (11, 15, 20, and 30mM) before treatment with or without estrogen (1 x 10^{-7} M) for 24 hours. (A) Flow cytometry histograms demonstrated elevated intracellular ApoE expression following estrogen treatment compared to untreated negative control (NC) cells under both 11mM and 30mM glucose conditions. (B) Quantitative analysis of the ApoE MFI confirmed that estrogen significantly increased ApoE levels compared to the NC (n=6, * P<0.0001). (C) ELISA analysis further showed that estrogen increased the secreted ApoE levels compared to untreated M1 macrophages across all glucose conditions (11, 15, 20, and 30mM) (n=6, **P<0.001). Error bars represent the standard error of the mean (StEM).

The ApoE expression experiments were repeated using agonists and antagonists of ER-α and ER-β to determine which ER proteins estrogen was acting through to media ApoE expression (Figure 5.11). Flow cytometry analysis (Figures 5.11.A and 5.11 B) was used to quantify cellular ApoE levels. Consistent with previous findings, the ER-α agonist PPT increased the percentage of ApoE+ cells at both 11mM (99.4±1.1% versus 22±3.1% in the untreated NC) and 30mM glucose (40±3.4% versus 16±2.3% in the untreated NC) in a similar manner to estrogen. The ER-α antagonist TPBM significantly ($P<0.0001$) blocked the effects of estrogen on cellular ApoE, whereas the ER-β antagonist PHTPP had no significant ($P>0.05$) effect on intracellular ApoE levels compared to estrogen treatment alone at either glucose level. Consistent with this finding, the ER-β agonist DPN had no significant influence on intracellular ApoE levels compared to untreated NC macrophages ($P>0.05$).

ELISA analysis (Figure 5.11.C) showed estrogen and the ER-α agonist PPT significantly ($P<0.05$) increased secreted ApoE levels compared untreated NC macrophages at both glucose concentrations. Consistent with this finding, the ER-α antagonist TPBM negated the stimulatory effect of estrogen on secreted ApoE ($P<0.05$). In contrast, DPN had no significant effect on secreted ApoE levels compared to untreated NC macrophages ($P>0.05$), and PHTPP failed to significantly reverse the effect of estrogen on secreted ApoE ($P>0.05$).

Collectively, these findings indicate that estrogen upregulates both intracellular and secreted levels of ApoE through selective activation of ER-α rather than ER-β in M1 macrophages (Figure 5.11).

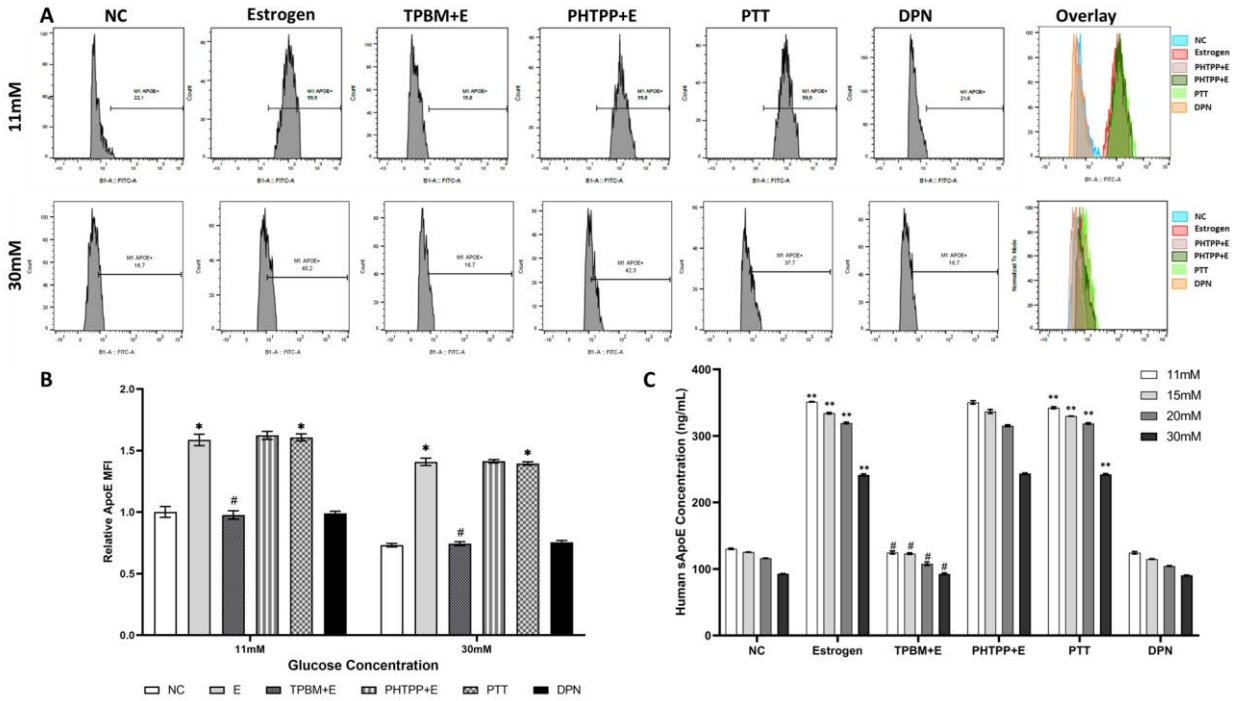

Figure 5.11 Estrogen Increases ApoE Expression in M1 Macrophages Through binding to the ER-α.

M1 macrophages were cultured in 11mM or 30mM glucose prior to either ER activation or blockade with TPBM or PHTPP. (A) Flow cytometry histograms showed an increased proportion of cells expressing ApoE following treatment with estrogen or PPT compared to untreated cells at both glucose levels. (B) Quantitative analysis of ApoE MFI indicated estrogen or PPT significantly increased cellular ApoE levels compared to the untreated NC macrophages cultured at 11mM and 30mM glucose. The ER-α antagonist TPBM reversed the stimulatory activity of estrogen on intracellular ApoE levels (#; $P<0.05$) whereas ER-β blockade with PHTPP was unable to significantly reverse the effects of estrogen on cellular ApoE levels. (C) ELISA analysis showed estrogen and PPT treatment significantly increased secreted ApoE levels compared to NC macrophages cultured under euglycemic or hyperglycaemic conditions. The ER-β agonist DPN did not affect secreted ApoE expression under euglycemic or hyperglycaemic conditions. TPBM, but not PHTPP, negated the estrogen-induced stimulation of secreted ApoE (# $P<0.05$). Data are represented as means ± StEM (n=6). * $P<0.05$, ** $P<0.001$ compared to untreated NC macrophages. # $P<0.05$ compared to macrophages treated with estrogen alone.

5.5 Discussion

This chapter provided evidence of key signalling pathways through which estrogen may be mediating innate immune responses, particularly the stimulation of phagocytic functions in M1 macrophages cultured under euglycemia or hyperglycaemia.

Estrogen was shown to reverse the detrimental effects of prolonged hyperglycaemia on key inflammatory mediators; TNF-α, IFN-β, and NO released by M1 macrophages. Estrogen induced a significant decrease in TNF-α protein secretion by M1 macrophages under euglycemic and hyperglycaemic conditions. TNF-α is well known for its role in DFU pathology, including promoting excessive inflammation and impairing wound healing (Kaiser and Polk, 1997; Xu et al., 2013). The reported estrogen-mediated reduction in TNF-α secretion is consistent with research linking estrogen with suppressed inflammatory responses (Lai et al., 2009; Plackett et al., 2015; Zhou et al., 2016; Zhuo et al., 2022). Several studies have explored this across various cell types and experimental models. In their 2013 study, (Liu et al.) demonstrated that estrogen suppress TNF-α production by inhibiting p38 MAPK phosphorylation in mouse bone marrow-derived macrophages (BMMs). This provided more evidence that estrogen signalling may act as a brake on inflammatory processes. Building on this, Brown et al. (2010) investigated whether similar effects occurred in the central nervous system. Indeed, they found estrogen suppressed inflammatory mediator release, including TNF-α, from brain lysate. This anti-inflammatory role extended estrogen's influence to neuroinflammatory conditions. Further support came from Zhuo et al. (2022), who focused on murine macrophages - a cell type highly relevant to the present study. They reported that estrogen potently inhibited the production of TNF-α, IL-6 and IL-1β in these cells by upregulating the IRE1α-XBP1 signalling axis. Their work highlighted macrophages as a key target of estrogen's anti-inflammatory actions.

Multiple human and animal studies have demonstrated the regulation of pro-inflammatory cytokine production by estrogen in various diseases and contexts . Schröder et al. (1995) found a higher mortality from sepsis in males compared to

premenopausal females that was attributed to the downregulation of TNF-α expression by circulating estrogen in premenopausal females during sepsis-driven inflammation. Other reports have shown estradiol inhibits TNF-α expression in RAW 264.7 murine macrophages (Srivastava et al., 1999; Tomaszewska et al., 2003; Kang et al., 2005) and human THP-1 macrophages (An et al., 1999; Capellino et al., 2005). The present findings are in line with the previously stated literature, demonstrating that estrogen attenuates hyperglycaemia-induced TNF-α expression in U937 -derived M1 macrophages.

Selective manipulation of ER-α and ER-β activation or blockade was performed to identify the ER subtype(s) through which estrogen was acting to regulate innate immune functions in M1 macrophages. Since nuclear ERs are expressed in M1 macrophages, estrogen may directly influence macrophage function through binding to the ER-α and/or ER-β (Villa et al., 2015). Results from this study implicate a key role for ER-α activation in mediating the effects of estrogen in M1 macrophages. These results are consistent with prior research studies highlighting the predominant role of ER-α in mediating estrogen's protective effects during inflammation, particularly in the cardiovascular system (Brouchet et al., 2001). Moreover, a recent study by Shu et al. (2022) reported that ER-α knockdown in female mice leads to increased M1 macrophage activation and elevated expression of inflammatory cytokines, including TNF-α.

Limited studies have assessed the crosstalk between ERs and TNF-α activity and their signalling pathways in the context of diabetes. This is particularly relevant, as estrogen signalling can lead to distinct cell-specific results, highlighting the complexity of estrogen-ER interactions and their implications during inflammation in different physiological contexts (De Gendt and Verhoeven, 2012). A study conducted by Kahlert et al. (2000) demonstrated that estrogen induces the insulin-like growth factor 1 (IGF-1) receptor pathway via binding to the ER-α *in vitro*. Notably, the IGF-1 signalling pathway was shown to downregulate the expression and secretion of TNF-α by immune cells, including macrophages (Álvarez et al., 2007; Sukhanov et al., 2007). This suggests that the observed estrogen-mediated decline in TNF-α expression in M1 macrophages may be mediated, at least in part,

through the estrogen-induced activation of the IGF-1 receptor pathway via activation of ER-α. However, it is important to note that some contrasting findings have been reported in different disease models and experimental settings such as the upregulation of TNF-α expression in breast cancer cells via the binding of estrogen to ER-α (Lee and Nam, 2008; To et al., 2014).

While estrogen significantly reduced TNF-α levels, no significant effects were observed on IFN-β production by M1 macrophages under both euglycemic or hyperglycaemic conditions. The effect of estrogen on IFN-β levels appears to be context-dependent, influenced by multiple factors such as cell type, experimental conditions, duration of treatment, and contribution from estrogen-independent pathways (Nagarajan et al., 2005). Studies conducted by (Tasker et al., 2014; Tasker et al., 2016) demonstrated that estrogen induces IFN-β expression in monocyte-derived macrophages, which in turn protects against HIV infections (Tasker et al., 2014). Additionally, Singh et al. (2021) found that estrogen treatment significantly increases the expression of IFN-stimulated genes (ISGs) in healthy females, suggesting a role for estrogen in the induction of IFN-β. Conversely, Panchanathan et al. (2015) showed estrogen activated type I IFN signaling and increased IFN-β in murine bone marrow cells, while Pazos et al. (2012) reported decreased IFN-β levels during influenza infection in pregnant mice with high estrogen. Adding another layer, Tabibzadeh et al. (1989) showed that estrogen inhibited IFN-β and IL-6 production by endometrial stromal cells.

In the current study, estrogen also had no significant effects on NO production under euglycemic or hyperglycaemic conditions. This suggests that estrogen may not alter the activity of inducible nitric oxide synthase (iNOS), the enzyme responsible for NO generation (McNeill et al., 2015). The literature reveals conflicting reports on estrogen's ability to modulate iNOS expression and NO levels, with the outcomes appearing to be cell type-dependent (Chen et al., 1999; Stefano et al., 2003; Karpuzoglu and Ahmed, 2006; Liu and Wang, 2013). Several studies demonstrate estrogen can directly enhance NO production by promoting vasodilation and improving blood flow in endothelial cells (Chen et al., 1999; Nevzati et al., 2015), Additionally, physiological concentrations of 17β-estradiol stimulate

NO release from nerve cells via ERβ (Stefano et al., 2003). In contrast, other evidence suggests estrogen may inhibit NO production in certain contexts. Liu and Wang (2013) found estrogen suppressed NO production primarily through the p38 MAPK pathway in lipopolysaccharide-stimulated bone marrow-derived macrophages. However, You et al. (2003) reported opposing effects, demonstrating physiological estrogen concentrations increased NO release and iNOS expression in rat peritoneal macrophages. This variability in estrogen's effects on NO metabolism likely stem from cell type-specific differences and nuances in the experimental conditions employed across studies.

To elucidate the mechanistic pathways involved in the estrogen-mediated regulation of inflammatory markers, such as TNF-α, during hyperglycaemia, the role of the transcription factor NF-κB was investigated. NF-κB drives the expression of inflammatory mediators in response to stimuli (Gasparini and Feldmann, 2012). Findings in this study reveal that estrogen reduces NF-κB nuclear translocation in M1 macrophages as well as the total fluorescent levels of NF-κB, particularly under high glucose conditions, when compared with untreated controls. The decreased nuclear translocation of NF-κB observed in estrogen-treated M1 macrophages is indicative of reduced NF-κB activation, which in turn dampens the excessive inflammatory response via gene transcription of inflammatory mediators, particularly TNF-α (Wang et al., 2014).

To further explain the mechanisms underlying estrogen's anti-inflammatory effects, LPS was used as a control to activate the TLR4 pathway (Maeshima and Fernandez, 2013), a key initiator of the NF-κB signalling cascade and subsequent expression of inflammatory cytokines like TNF-α (Soares et al., 2010; Maeshima and Fernandez, 2013; Hobbs et al., 2018). LPS treatment significantly increased NF-κB nuclear translocation and total cellular NF-κB levels in M1 macrophages under both euglycemic and hyperglycaemic conditions. Importantly, estrogen co-treatment markedly attenuated this LPS-induced NF-κB activation, suggesting that estrogen can effectively suppress the TLR4-mediated inflammatory response triggered by LPS. These findings are consistent with previous studies demonstrating estrogen's ability to modulate the TLR4 signalling pathway and associated inflammatory

responses in macrophages. Meng et al. (2023) found that direct activation of ERα inhibited the TLR4 signalling pathway and subsequent inflammatory response in macrophages. Additionally, Villa et al. (2015) showed that activation of intracellular ERα by estrogen reduced the pro-inflammatory phase and facilitated the resolution of inflammation in macrophagic cells by regulating the SOCS3 and STAT3 signalling pathways. These studies support our observations that estrogen can attenuate LPS-induced NF-κB activation and the downstream inflammatory cascade. However, it is important to note that the role of estrogen in modulating TLR4-mediated inflammation is complex and may depend on various factors, including the duration of exposure and the specific experimental conditions. For instance, Calippe et al. (2008) found that chronic exposure to estrogen enhanced the LPS-induced production of pro-inflammatory mediators like IL-12 and TNF-α in macrophages, through activation of ERα. This suggests that estrogen can potentially promote TLR4-triggered inflammation under certain circumstances. Furthermore, Rettew et al. (2009), demonstrated that estrogens augment cell surface TLR4 expression on murine macrophages and regulate sepsis susceptibility *in vivo*. The removal of endogenous estrogen in mice resulted in reduced production of pro-inflammatory cytokines like IL-6 and TNF-α in response to LPS challenge, associated with decreased expression of TLR4 and the LPS-binding protein LBP1. This indicates that physiological levels of estrogen can augment TLR4 expression and the inflammatory response to LPS. These findings highlight the complexity of estrogen's role in regulating TLR4-mediated inflammation and suggest that the effects of estrogen may be context-dependent, influenced by factors such as duration of exposure, concentration, and the specific cellular environment.

Furthermore, the present study examined the involvement of both ER-α and ER-β in regulating NF-κB p65 nuclear translocation in classically-activated M1 macrophages. Notably, the results suggest that estrogen-mediated inhibition of NF-κB p65 nuclear translocation is specifically mediated through activation of ER-α rather than ER-β. Several published studies support these findings including the work of Ghisletti et al. (2005) who demonstrated that estradiol inhibits p65 nuclear translocation in RAW 264.7 cells, and Murphy et al. (2010) who reported that estrogen inhibits LPS-

induced increases in nuclear p65 levels in human macrophages. In addition, Ghisletti et al. (2005) also found that estrogen reduces p65 intracellular transport to the nucleus through selective binding to the ER-α and not ER-β in brain macrophages extracted from ERα-/ ERβ-null mice. Furthermore, multiple studies have confirmed that ER-α is necessary for the modulation of anti-inflammatory activity of estrogen in inflammatory pathologies (Dubal et al., 2001; Ashcroft et al., 2003; Polanczyk et al., 2003; Vegeto et al., 2003; Garidou et al., 2004).

Collectively, these findings highlight the inhibition of NF-κB translocation as an important mechanism through which estrogen promotes bacterial clearance and dampens inflammatory responses in M1 macrophages. Consequently, localised suppression of NF-κB activity via topical estrogen supplementation or more specifically ER-α agonism may be a potential therapeutic avenue to alleviate excessive hyperglycaemia-induced inflammation in DFUs.

Given the increased susceptibility to bacterial infections observed under hyperglycaemia and the potential role of estrogen in immune regulation, the effect of estrogen on the expression of key receptor proteins and ligands was investigated in M1 macrophages. The cell surface receptor CD14 was initially investigated due to its critical role as an essential PRR for innate immunity (Gregory, 2000). Multiple lines of evidence support a mechanistic link between estrogen signalling and regulation of CD14 expression/function in phagocytic cells like macrophages.

Estrogen significantly decreased the levels of both mCD14 and sCD14 in M1 macrophages compared to the untreated control, independent of glycaemic status. This corroborates with previous studies demonstrating direct transcriptional regulation of CD14 by estrogen in THP-1 and microglial cells (Polari et al., 2018; Fehrenbach et al., 2021). Vegeto et al. (2004) also found that estrogen inhibits the LPS-mediated increase in CD14 levels in RAW 264.7 macrophages. However, they reported no effect on LPS-induced CD14 in microglia, highlighting the context-dependent nature of estrogenic responses. The presence of estrogen receptors in these immune cells allows for direct transcriptional control of CD14 levels (Cutolo et al., 2004). Additionally, estrogen exhibits well-known anti-inflammatory actions, reducing production of pro-inflammatory cytokines like TNF-α (Vegeto et al., 2004;

Dimitrijević et al., 2013; Villa et al., 2015) that are partly regulated by CD14 signalling cascades (Dentener et al., 1993; Kawai et al., 2000; Q. Yang et al., 2016; Pepe et al., 2017; Dama et al., 2021). Therefore, a potential mechanism of action underlying these findings could involve the ability of estrogen to dampen inflammation by reducing TNF-α cytokine production, leading to further suppression of the proinflammatory response via decreased CD14 expression. Furthermore, evidence indicates estrogen can augment cell surface expression of innate immune receptors like TLR4 on macrophages (Rettew et al., 2009; Rettew et al., 2010; Fehrenbach et al., 2021). As a co-receptor for TLR4/LPS recognition (Park and Lee, 2013; Raby et al., 2013), CD14 levels/functions may be co-ordinately regulated. Lastly, estrogen targets multiple intracellular mediators like NF-κB and kinases that transduce signals emanating from pattern recognition receptors and CD14 pathway (Srivastava et al., 1999; Srivastava et al., 2001; Valverde and Parker, 2002). The observed reduction in mCD14 expression in M1 macrophages by estrogen will most likely result in suppression of downstream signalling due to the diminished availability of CD14 membrane receptors at the cell surface. The CD14/TLR pathway has been well described in the literature, and includes transcription factors such as NF-κB and kinases such as mitogen-activated protein kinase (MAPK) that become activated and induce immune responses (Srivastava et al., 1999; Brubaker and Gay, 2000; Srivastava et al., 2001; Valverde and Parker, 2002).

The combination of direct transcriptional control, cytokine-mediated indirect effects, and potential co-regulation of associated receptors allows estrogen to influence CD14 expression, and downstream signalling in a multi-faceted manner in phagocytic cells like macrophages.

Although the mechanism of sCD14 release from cells is not completely understood, it has been reported to involve endocytosis of mCD14 (Durieux et al., 1994). Thus, a decrease in endocytosis of mCD14 may contribute to the reduced release of sCD14 by M1 macrophages observed in this study. Soluble CD14 itself plays important roles in innate immune responses and inflammatory processes. As a soluble receptor, sCD14 can bind to LPS and other pathogen-associated molecules, facilitating their

transfer to immune cells like macrophages and potentiating inflammatory signalling cascades (Kitchens and Thompson, 2005). Elevated sCD14 levels have been implicated in the exacerbation of inflammatory conditions such as infections, rheumatoid arthritis, and atherosclerosis (Anker et al., 1997; Nareika et al., 2008; Teixeira et al., 2021).

Conversely, by reducing sCD14, estrogen could dampen excessive inflammation driven by high glucose levels (Nareika et al., 2008; Sanjurjo et al., 2023) as well as sCD14-mediated recognition of pathogen components (Kitchens and Thompson, 2005). This aligns with estrogen's well-established anti-inflammatory properties, which are mediated in part through suppression of pro-inflammatory cytokines like TNF-α, as also observed in this study (Vegeto (Vegeto et al., 2004; Dimitrijević et al., 2013; Villa et al., 2015). Interestingly, sCD14 has also been reported to act as an acute phase protein and anti-inflammatory mediator, as its levels correlate with C-reactive protein and IL-6, under certain contexts (Beers et al., 2020; Ichise et al., 2020). By modulating sCD14 levels, estrogen may fine-tune the balance between its pro- and anti-inflammatory roles during an immune response.

Overall, the estrogen-mediated reduction in sCD14 levels, coupled with decreased mCD14 expression, likely contributes to dampening excessive inflammation triggered by CD14-dependent pattern recognition. This could serve as a protective mechanism in hyperglycaemic and/or infectious contexts. Investigating the involvement of ER-α and ER-β in regulating CD14 protein expression revealed that downregulation of mCD14 and sCD14 is mediated through activation of ER-α under both euglycemic or hyperglycaemic conditions. These findings align with previous research highlighting the role of ER-α in mediating the anti-inflammatory effects of estrogen. For instance, Pepe et al. (2017) and Shu et al. (2022) observed increased TNF-α release in ER-α-deficient macrophages, underscoring the importance of ER-α in regulating inflammatory responses. Additionally, Douin-Echinard et al. (2008) demonstrated the key role of ER-α, but not ERβ, in proinflammatory cytokine regulation.

The TREM2/ApoE signalling pathway has been shown to enhance phagocytosis and inhibit pro-inflammatory cytokine secretion in microglia (Hsieh et al., 2009; Leri et

al., 2023). Activation of the TREM2 pathway is dependent on the binding of the ligand ApoE to the TREM2 receptor (Krasemann et al., 2017; Li et al., 2021). Furthermore, TREM2 has been demonstrated to activate the SYK/PI3K/AKT pathways involved in phagocytosis, while suppressing inflammation through NF-κB activation downstream of PKC (Peng et al., 2010; Yao et al., 2019; Chen et al., 2020). Thus, the involvement TREM2 and ApoE play in the beneficial effects of estrogen on the phagocytosis of bacteria was elucidated in this study.

The effect of estrogen on the expression of TREM2, and its ligand, ApoE, in M1 macrophages was subsequently investigated. Estrogen significantly increased the expression of both mTREM2 and sTREM2 in M1 macrophages, irrespective of glucose concentrations. Similarly, estrogen significantly enhanced the protein levels of both intracellular and secreted ApoE by M1 macrophages, countering the suppressive impact of hyperglycaemia on ApoE levels. Moreover, the estrogen-induced increase in TREM2 and ApoE expression was mediated through ER-α. These findings are supported by Wang et al. (2006) who reported that ApoE mRNA and protein expression increases in response to ER-α activation in brain macrophages.

However, other studies suggest the relationship between estrogen and TREM2 expression may be more complex. Essex et al. (2022) showed that female osteoclasts (a macrophage subtype) carrying the TREM2R47H/+ variant exhibited decreased sensitivity to estrogen signalling, indicating crosstalk between TREM2 and estrogen receptor signalling pathways in these cells. Furthermore, Eren et al. (2023) found that in Alzheimer's disease patient-derived macrophages, female patient cells showed a higher fold increase in TREM2 mRNA compared to male cells. Collectively, these results suggest that the effects may depend on factors such as sex, genetic variants like TREM2R47H, and macrophage phenotype.

In conclusion, estrogen appears to restore the TREM2/ApoE immunomodulatory axis following the hyperglycaemia-induced inhibition of TREM2/ApoE expression in M1 macrophages. Increased expression of both TREM2 and ApoE following treatment with estrogen has been widely reported (L. Zhao et al., 2012; Ratnakumar et al., 2019; Aryanpour et al., 2021) Moreover, research on cholesterol metabolism during menopause provides supporting evidence for a mechanistic link between

estrogen and the TREM2/ApoE signalling pathway, suggesting that estrogen may regulate cholesterol transport and immune homeostasis through crosstalk with the TREM2/ApoE pathway (Colton et al., 2005; Hussain et al., 2015).

The findings of this chapter highlight intriguing connections between the beneficial role of estrogen (ER-α activation) in maintaining immune homeostasis under hyperglycaemic conditions and several key mediators (TNF-α, NF-κB, CD14, TREM2, and ApoE) of bacterial phagocytosis and inflammation. TNF-α is a major pro-inflammatory cytokine that plays a crucial role in the pathogenesis of chronic inflammatory diseases, including impaired wound healing (Xia and Triffitt, 2006; Xu et al., 2013). Additionally, the transcription factor NF-κB is a central regulator of inflammatory responses, governing the expression of numerous genes involved in immune cell activation, cytokine production, and antimicrobial defences (Iacobazzi et al., 2023). Studies indicate that CD14 and TREM2 play complementary roles in facilitating phagocytosis, exhibiting distinct localisation patterns at the plasma membrane and contributing uniquely to the phagocytic cup organisation (Painter et al., 2015; Vorselen et al., 2022). This suggests potential crosstalk between CD14 and TREM2/ApoE in mediating estrogen's effects on phagocytic function. Further interrogation of the pathways through which estrogen might mediate innate immune responses could provide insights for the development of novel therapeutic strategies to regulate those pathways in DFU patients with a DFI.

Chapter 6: Deciphering the Crosstalk Between Estrogen and CD33/TREM2 Signalling

6.1 Introduction

6.1.1 CD33/TREM2 Signalling

CD33 and TREM2 are transmembrane receptors expressed on myeloid cells, including monocytes and macrophages, and play an important role in regulating innate immune responses including phagocytosis (Crocker et al., 2012; Yao et al., 2019). CD33, also known as Siglec-3, is an inhibitory receptor that contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic domain (Taylor et al., 1999; Crocker, 2002; Crocker et al., 2007). Upon ligand binding, CD33 activates phosphatases such as SHP-1 and SHP-2, which subsequently dephosphorylate the key signalling molecule Syk, critically involved in initiating phagocytic cup formation through regulation of cytoskeleton remodelling. By suppressing Syk activity, CD33 signalling inhibits this initial step of the phagocytosis process (Taylor et al., 1999; Ulyanova et al., 1999; Paul et al., 2000). Additionally, SHP-1 can dephosphorylate and inhibit PI3K, which is a downstream effector of the phagocytic signalling cascade. PI3K plays a crucial role in coordinating later stages of phagosome formation and maturation by regulating GTPases like Rac and Cdc42 (Alan et al., 2013; Poku et al., 2023). Therefore, by targeting both the upstream kinase Syk and the downstream effector PI3K, CD33 signalling significantly disrupts the phagocytosis process, ultimately impairing the macrophage's ability to efficiently engulf and degrade bacterial pathogens. In contrast, TREM2 is an activating receptor that associates with the adaptor protein DAP12, containing an immunoreceptor tyrosine-based activation motif (ITAM) (Tanzi, 2015; Yeh et al., 2017). TREM2 activation triggers multiple signalling cascades, including the PI3K and p38MAPK pathways, ultimately promoting phagocytosis, inflammation, and other cellular functions (Hsieh et al., 2009; Kawabori et al., 2015; Yao et al., 2019; Chen et al., 2020) (Figure 6.1).

Interestingly, both CD33 and TREM2 exhibit an opposing relationship in regulating macrophage phagocytosis. Studies have demonstrated that CD33 signalling, mediated by SHP-1, can inhibit TREM2-induced phagocytosis in macrophages (Griciuc et al., 2019). This inhibitory effect is achieved through the

dephosphorylation and inactivation of PI3K, a crucial downstream effector of TREM2 signalling (Cuevas et al., 1999; Schlam et al., 2015).

In the context of DFUs, the balance between CD33 and TREM2 signalling pathways may play a crucial role in the clearance of bacteria by macrophages, which leads to improved inflammatory responses and enhanced wound repair in DFIs (Geerlings and Hoepelman, 1999; Hodgson et al., 2015). Dysregulation of this balance could impair the phagocytic functions of innate immune cells, particularly macrophages, which leads to persistent bacterial colonisation and impaired wound healing processes (Aitcheson et al., 2021). Additionally, it has been reported that CD33 expression is decreased in macrophages of individuals with diabetes (Gonzalez et al., 2012). This may potentially alter overall CD33/TREM2-mediated responses and contribute to dysregulated inflammation and/or bacterial clearance observed in DFIs. Thus, a detailed study investigating, the interplay between CD33-TREM2 in macrophages cultured under hyperglycaemia is warranted. Understanding the interconnection between these signalling pathways may highlight avenues for potential therapeutic intervention that can promote immune responses and wound healing in diabetic patients.

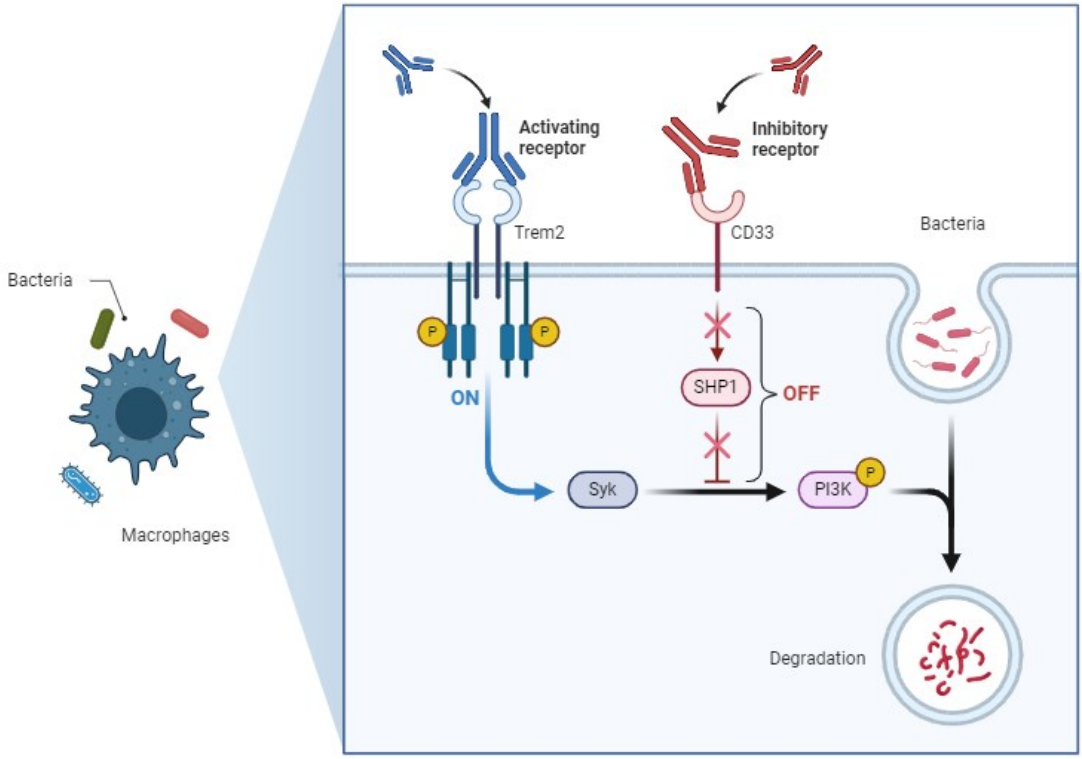

Figure 6.1 The Proposed Crosstalk Between CD33 and TREM2 in Macrophage-Mediated Phagocytosis.
CD33 and TREM2 have opposing actions on activation of macrophage-mediated immune responses, including phagocytosis. Figure created with Biorender.com and is based on published pathways (Chan et al., 2015; Griciuc et al., 2019; Tan et al., 2023).

6.2 Aim and Objectives

6.2.1 Aim

The aim of this investigation was to investigate the influence of CD33 and TREM2/ApoE signalling pathways on the clearance of bacteria and secretion of TNF-α by M1 macrophages cultured under hyperglycaemic conditions.

6.2.2 Objectives

- Determine the effect of CD33-knockout (CD33KO) on the phagocytic activity and secretion of TNF-α by M1 macrophages cultured under hyperglycaemic conditions.
- Assess the influence of estrogen stimulation on bacterial phagocytosis, TNF-α production and TREM2/ApoE expression in CD33KO M1 macrophages cultured under hyperglycaemic conditions.

- Investigate the potential crosstalk between CD33 and TREM2, with or without estrogen-stimulation in M1 macrophages cultured under hyperglycaemic conditions.
- Examine the effect of estrogen supplementation on the expression of mCD33 and sCD33 in M1 macrophages cultured under hyperglycaemic conditions.
- Compare the effect of estrogen supplementation on bacterial phagocytosis by CD33KO M1 macrophages with bacterial clearance observed in wildtype M1 macrophages cultured under hyperglycaemic conditions.

6.3 Methods

6.3.1 Cell Culture

An *in vitro* model of diabetes was generated using CD33+ U937 monocytes and CD33-knockout U937 monocytes, as previously described in 2.1.3. Cells were cultured under aseptic conditions at 37 °C and 5% CO_2, and monocytes were differentiated into M0 macrophage-like cells over 72 hours using PMA at a concentration of 50 ng/mL as described in 2.5. After differentiation, macrophages were polarized towards an M1 pro-inflammatory phenotype by stimulation with LPS at 100 ng/mL and IFN-γ at 20 ng/mL in either CM or GS (15, 20, and 30mM) medium as described in 2.6.

6.3.2 *In vitro* Host-Pathogen Interaction Experiments

The CD33+ and CD33-knockout (CD33KO) M1 macrophages were treated with or without estrogen (1×10^{-7} M) for 24 hours, as detailed in 2.1. Following estrogen stimulation, macrophages were co-incubated with planktonic MRSA or *P. aeruginosa* for 3 hours to allow host-pathogen interaction to occur. The bacterial recovery was subsequently quantified using the methods outlined in 2.11.3.1.

6.3.3 Enzyme-Linked Immunosorbent Assay (ELISA)

Secreted levels of TNF-α and soluble protein levels of CD33, TREM2, and ApoE were measured in supernatant from M1 glucose-stimulated macrophages via ELISA as described in 2.9.

6.3.4 Flow Cytometry

Flow cytometry analysis was employed to evaluate the influence of hyperglycaemia, estrogen and CD33 knockout on the cell surface expression of CD33 (mCD33) and TREM2 (mTREM2) and intracellular levels of ApoE in M1 macrophages cultured under euglycemic (11 mM) and hyperglycaemic (30 mM) conditions. Cells were labelled with fluorochrome-conjugated antibodies specific for mCD33 (APC anti-CD33), mTREM2 (AlexaFluor 647 anti-TREM2), and ApoE (FITC anti-ApoE) as described in 2.8.3 and 2.8.4. Subsequently, 10,000 events were acquired on a MACSQuant Analyzer to determine the percentage of positively-stained cells and the MFI associated with each surface marker. The quantitative flow cytometry data was analysed using FlowJo software to compare marker expression across the different treatments and glycaemic conditions.

6.3.5 Confocal Microscopy

Confocal microscopy was employed to visualise the co-localisation of mCD33 and mTREM2 receptors on M1 macrophages under euglycemic and hyperglycaemic conditions, with or without estrogen (1×10^{-7} M) treatment. To achieve this, the classically-activated M1 macrophages were cultured on glass coverslips according to the protocol described in 2.5 and 2.6. The M1 macrophages were fixed using 4% paraformaldehyde for 10 minutes at room temperature, prior to incubation with (APC)-conjugated anti-human CD33 antibody and an Alexa Fluor 488-labeled anti-TREM2 antibody for 1 hour at room temperature as outlined in 2.12.3. For confocal microscopy studies, the CD33 antibody was employed at a dilution of 1:800, while the Alexa Fluor 488-conjugated anti-TREM2 antibody was utilized at a dilution of 1:500. Subsequently, the cells were counterstained with DAPI for 5 minutes to visualize the nuclei. The M1 macrophages were washed twice and mounted onto

microscope slides for imaging on a Stellaris confocal microscope system at magnification of X100.

6.4 Results

6.4.1 CD33 Knockout Enhances the *In Vitro* Phagocytic Capacity of M1 Macrophages

The effect of CD33 on the phagocytosis of both planktonic MRSA and *P aeruginosa* colonies by CD33KO M1 macrophages cultured in CM or GS medium was investigated in this study. Across all the glucose concentrations tested, CD33 knockdown significantly decreased the recovery of both MRSA ($P<0.0001$; $n=12$) and *P. aeruginosa* ($P<0.0001$; $n=12$) compared to recovery levels observed in wildtype M1 macrophages. This suggests that CD33 knockout enhances the clearance of bacteria by M1 macrophages, independent of glucose concentration (Figure 6.2).

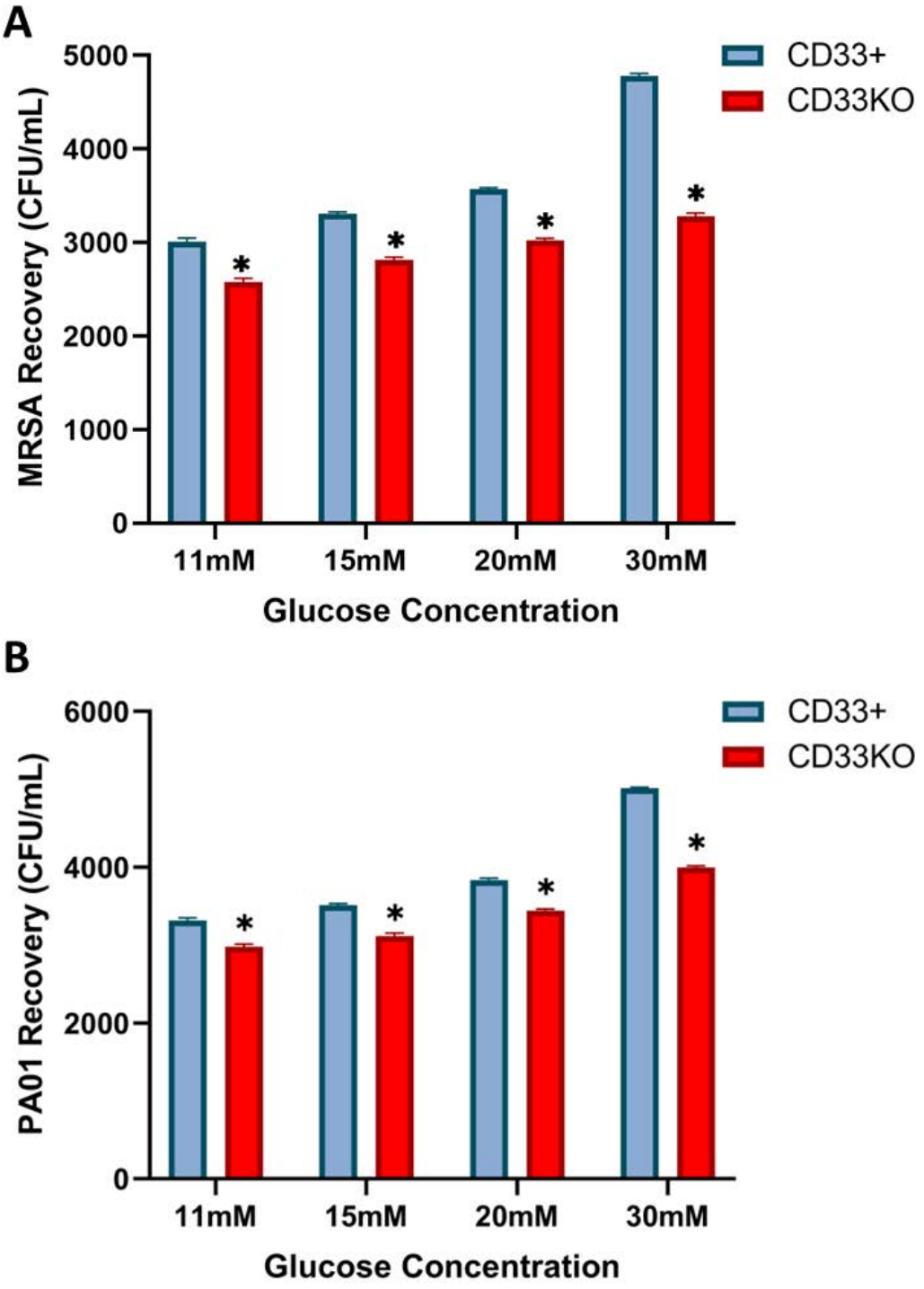

Figure 6.2 CD33 Knockout Enhances the In Vitro Phagocytosis of MRSA (A) and *P. aeruginosa* (B) by Classically-Activated M1 Macrophages.
Data are presented as an average of twelve independent experiments (n = 12). * Indicates significant differences (P<0.0001) in MRSA (A) or *P. aeruginosa* (B) recovery following co-culture with CD33 knockout M1 macrophages compared to wildtype U937-derived M1 macrophages cultured under a range of glucose concentrations (11 mM, 15 mM, 20 mMand 30 mM). Error bars represent the StEM.

6.4.2 CD33 Knockout Induces TNF-α Production by M1 Macrophages

TNF-α production by CD33 knockout M1 macrophages was compared to wildtype CD33+ M1 macrophages cultured in a range of glucose concentrations (11, 15, 20, and 30mM). CD33 knockdown significantly increased TNF-α levels compared to wildtype CD33+ M1 macrophages at all glucose concentrations ($P<0.05$). Moreover, the levels of secreted TNF-α showed a stepwise increase as the glucose concentration was elevated (11mM versus 15mM, 15mM versus 20mM, 20mM versus 30mM). following CD33 knockdown (Figure 6.3).

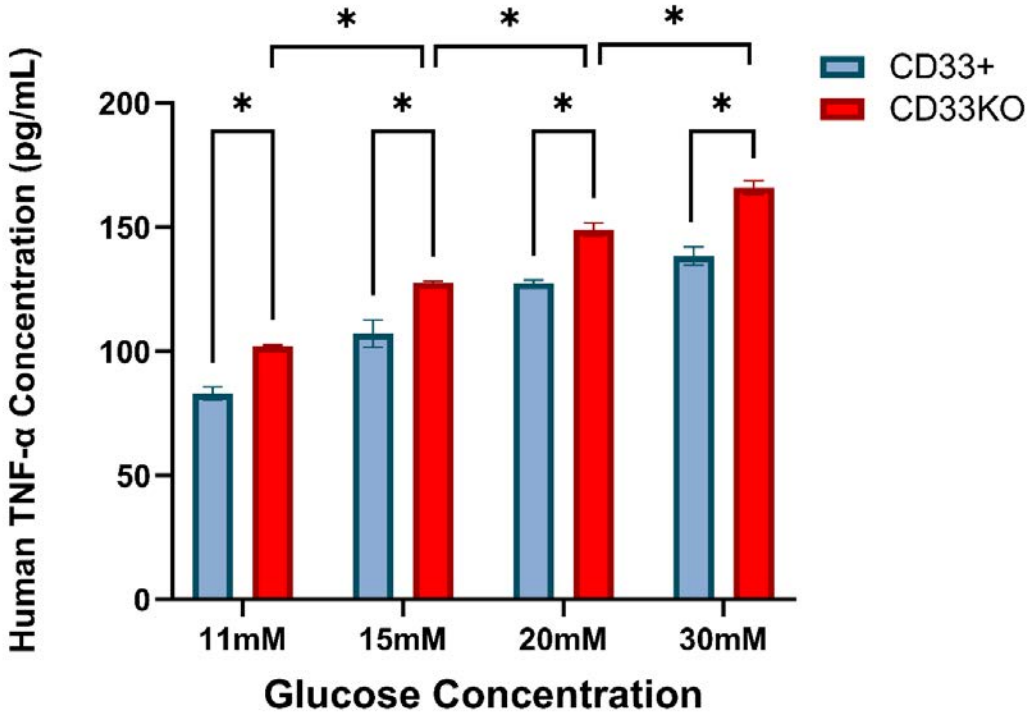

Figure 6.3 The Effect of CD33 Knockout on TNF-α Secretion Under Increasing Glycaemic Conditions.
TNF-α secretion significantly increased in glucose concentration-dependent manner in CD33 Knockout M1 macrophages cultured under increasing glucose levels (11, 15, 20, and 30mM) compared to wildtype M1 CD33+ macrophages (*$P<0.001$, n=4). Error bars represent the StEM.

6.4.3 CD33 Knockout Reduces TREM2 and ApoE Expression in M1 Macrophages

While CD33 and TREM2 may have opposing roles in macrophage-mediated phagocytic function, there is some evidence suggesting potential crosstalk between CD33 and TREM2 pathways (Griciuc et al., 2013). Multiple studies have shown that CD33 receptor levels can modulate TREM2-mediated signalling pathways,

suggesting interplay between immune responses induced by these receptors (Chan et al., 2015; Griciuc et al., 2019).

To investigate the potential interplay between CD33 and TREM2 pathways, flow cytometry analysis was employed to evaluate the impact of CD33 knockout on TREM2 expression in M1 macrophages cultured under euglycemic (11mM) and hyperglycaemic (30mM) conditions. TREM2+ cells were gated following staining with Alexa Fluor 488 anti TREM2 antibody as described in 2.8.3, and TREM2 expression histograms were plotted comparing the marker expression between CD33 knockout M1 macrophages (blue histogram) and wildtype CD33+ M1 macrophages (red histogram). CD33 knockout induced a left shift in the TREM2 curve in comparison to wildtype M1 cells, suggesting a reduction in mTREM2 expression (Figure 6.4.A). Quantitative analysis of the MFI data revealed that CD33 knockout significantly decreased mTREM2 expression compared to wildtype M1 macrophages under euglycemic conditions (11mM; $P = 0.0041$), but not under hyperglycaemic conditions (30mM; $P > 0.9999$) (Figure 6.4.B). Notably, glucose conditions did not appear to significantly influence the relative mTREM2 expression within the CD33KO cells ($P = 0.9995$), with CD33KO effectively having the same detrimental effect on mTREM2 levels as hyperglycaemia, regardless of the actual glycaemic conditions.

Furthermore, quantitative ELISA analysis provided complementary evidence, showing that CD33 knockout led to decreased levels of sTREM2 secretion by M1 macrophages cultured under both euglycemic and hyperglycaemic glucose conditions when compared to wildtype CD33+ M1 macrophages (Figure 6.4.C).

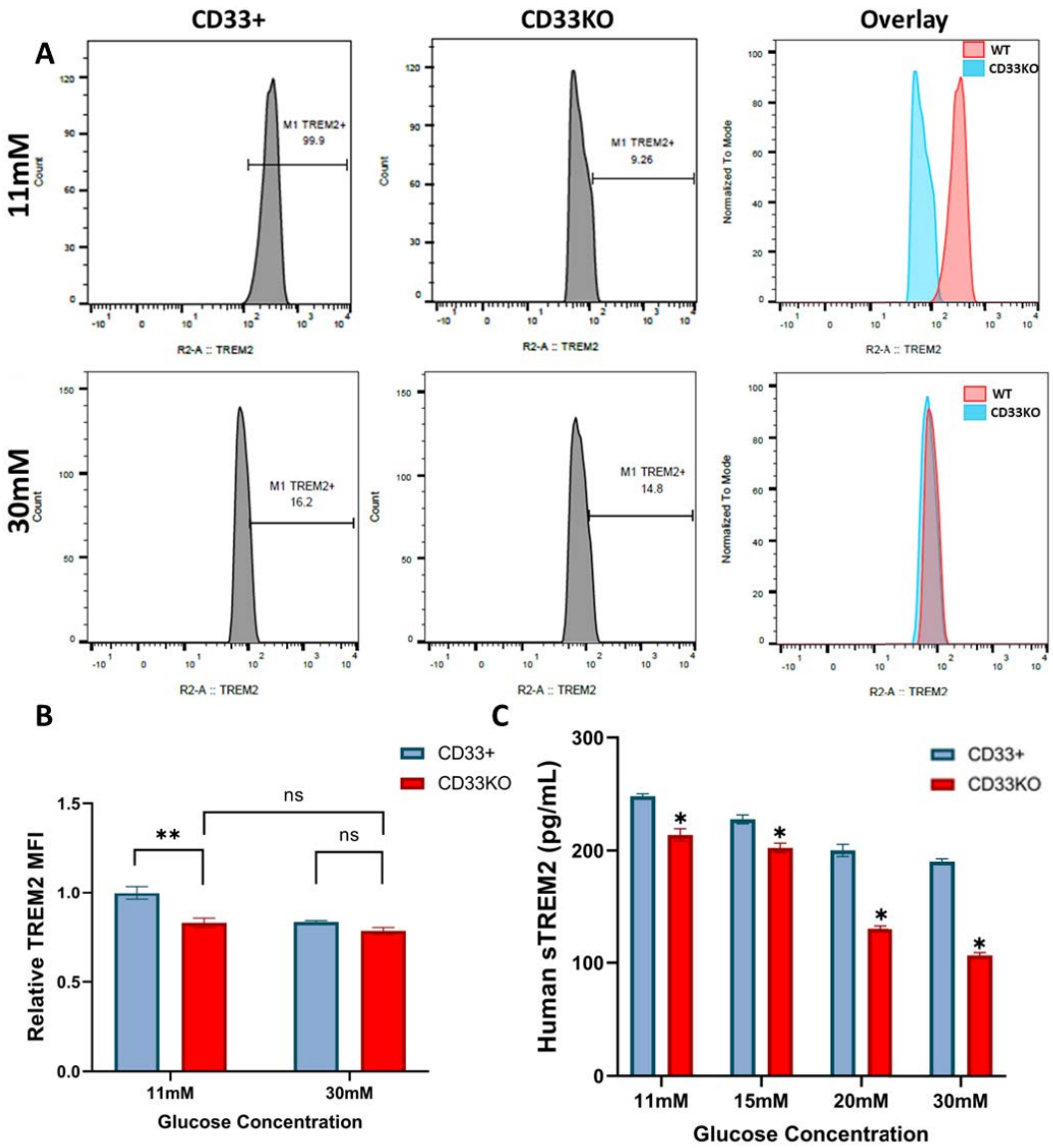

Figure 6.4 CD33 Knockout Reduces *in Vitro* TREM2 Expression by M1 Macrophages.
(A) Flow cytometry histograms comparing the TREM2 expression levels between CD33 knockout and wildtype M1 macrophages cultured under 11mM and 30mM glucose. (B) Relative mTREM2 MFI was significantly reduced in CD33KO M1 macrophages compared to CD33+ M1 macrophages cultured under glycaemic (11 mM) glucose conditions. CD33KO did not have any significant impact on mTREM2 expression in M1 macrophages cultured under hyperglycaemic (30 mM) glucose levels. (C) ELISA analysis showed a significant decrease in sTREM2 levels in CD33KO M1 macrophages compared to CD33+ M1 macrophages at all glucose concentrations (11, 15, 20, and 30mM). Results represent an average of 6 independent experiments (n=6). *Indicates significant differences between CD33KO and corresponding CD33+ M1 macrophages (P<0.001). Error bars represent the StEM.

Similar to the TREM2 findings, CD33 knockdown led to a downregulation of ApoE levels compared to CD33+ M1 macrophages, as evidenced by both flow cytometry and ELISA (Figure 6.5). Under euglycemic conditions (11mM glucose), CD33 knockout resulted in an approximate 50% reduction (21.5% in CD33KO and 10.7% in CD33+ cells) in the number of cells expressing ApoE (Figure 6.5.A). Intriguingly, the CD33-mediated decrease in ApoE expression in CD33KO M1 macrophages was less pronounced under hyperglycaemic conditions, with ApoE levels reducing from 13.6% to 8.78%, representing an approximate 35% reduction. The overlay histograms further illustrate this difference, where the histogram for CD33KO M1 macrophages (red) showed a more substantial left shift compared to the histogram for wildtype CD33+ M1 macrophages (blue) at 11mM glucose, indicating a greater reduction in ApoE expression under euglycemic conditions. Additionally, the relative ApoE MFI quantification confirmed a significant ($P<0.01$) decrease in intracellular ApoE levels upon CD33 knockout at both 11mM (~50% decrease) and 30mM (~30% decrease) glucose concentrations compared to wildtype CD33+ M1 macrophages (Figure 6.5.B).

ELISA analysis revealed CD33 knockdown led to a marked reduction in secreted ApoE levels across all glucose concentrations (11, 15 mM, 20 mM and 30 mM) compared to wildtype CD33+ M1 macrophages (Figure 6.5.C).

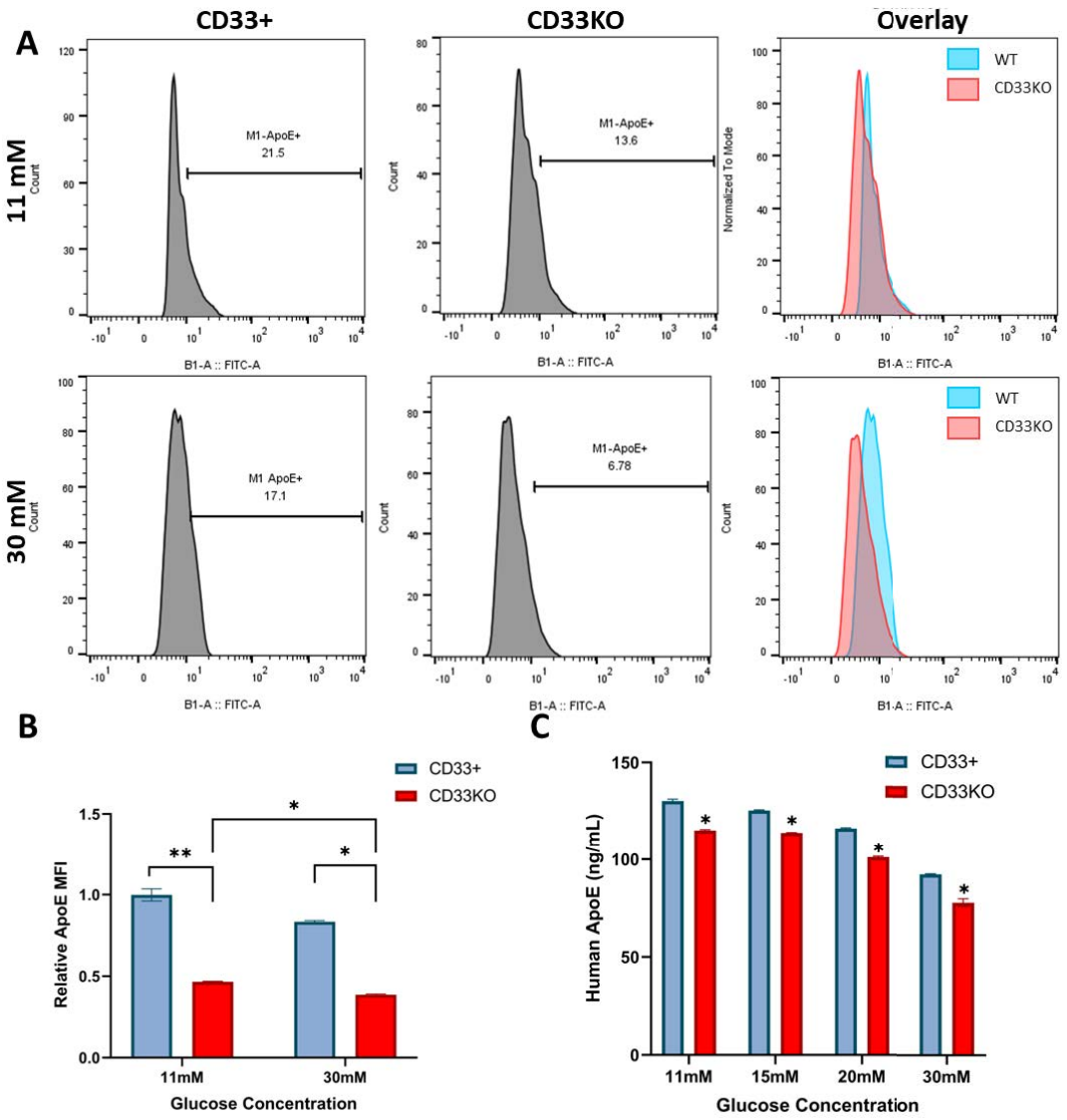

Figure 6.5 CD33 Knockout Reduces *in Vitro* ApoE Production by M1 Macrophages.
(A) Intracellular ApoE levels were significantly decreased in CD33 knockout (CD33KO) M1 macrophages in comparison with wildtype CD33+ M1 macrophages cultured under 11mM or 30mM glucose conditions. (B) Quantitative analysis showed ApoE MFI significantly decreased in CD33KO M1 macrophages compared to CD33+ M1 macrophages cultured under 11mM or 30mM glucose conditions. (C) ELISA analysis showed reduced ApoE secretion by CD33KO M1 macrophages compared to CD33+ M1 macrophages under 11mM or 30mM glucose conditions. Data represent average of 6 independent experiments (n = 6). * Indicates significant differences between CD33KO and corresponding CD33+ M1 macrophages ($P<0.01$). Error bars represent the StEM.

6.4.4 Estrogen Modulates Innate Immune Responses in CD33KO Cells

6.4.4.1 The Effect of Estrogen on CD33-Mediated Phagocytosis of MRSA and *P. aeruginosa* by M1 Macrophages

Both estrogen and knockout of CD33 was previously shown to enhance the phagocytic activity of M1 macrophages *in vitro*. Therefore, in this study the effect of estrogen on the phagocytosis of MRSA and *P. aeruginosa* by CD33KO M1 macrophages cultured under increasing (11 mM, 15 mM, 20 mMand 30 mM) glucose concentration (Figure 6.6). Estrogen (1×10^{-7} M) significantly reduced the recovery of MRSA (Figure 6.6.A) and *P. aeruginosa* (Figure 6.6.B) in CD33KO M1 macrophages across all glucose concentrations tested (**: $P < 0.0001$; $n = 12$), indicating an estrogen-mediated enhancement of the bacterial clearance.

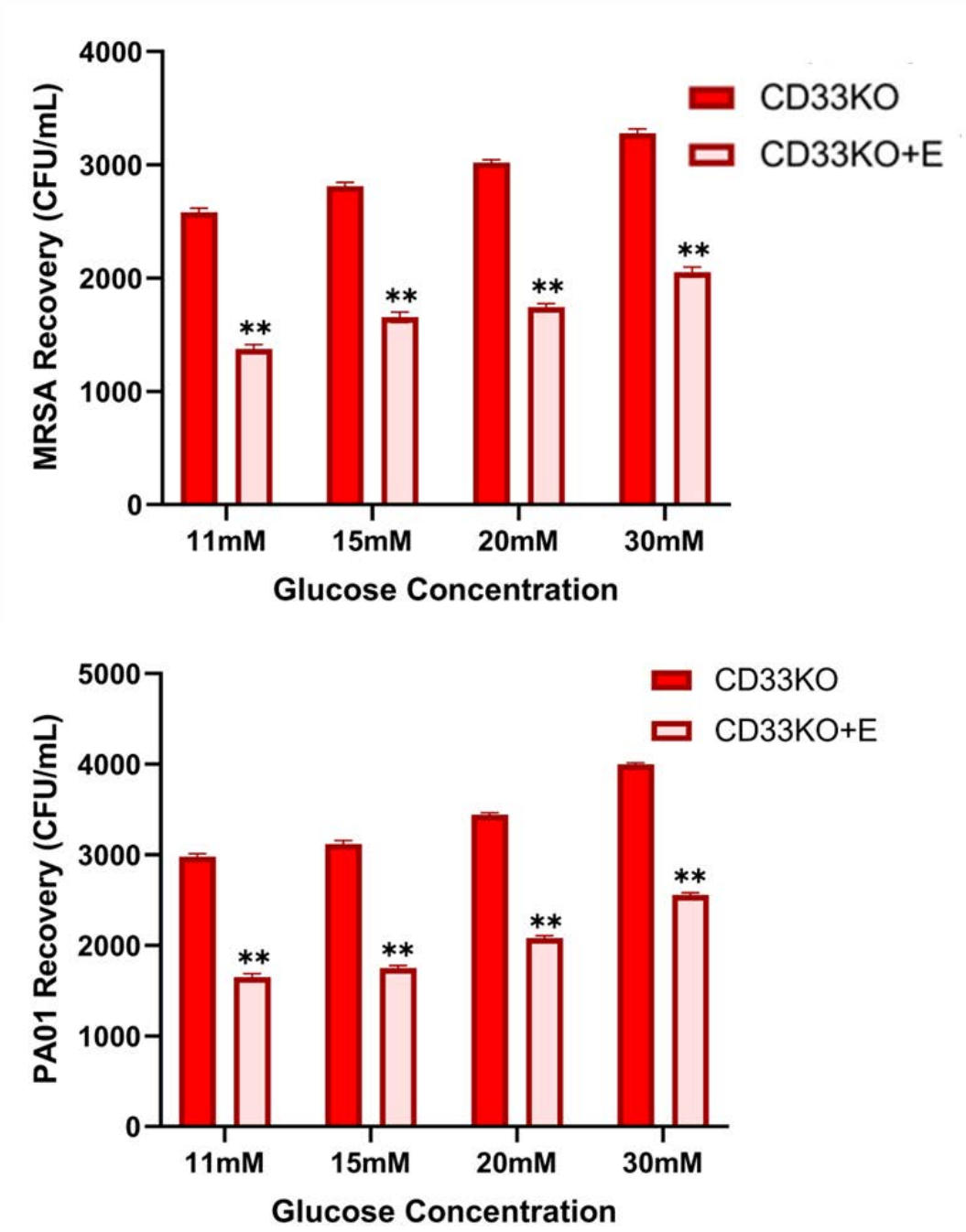

Figure 6.6 Estrogen Enhances the Clearance of MRSA (A) and *P. aeruginosa* (B) by CD33 knockout M1 Macrophages.

Estrogen (1 x 10^{-7} M) significantly (**: P<0.001) reduced the recovery of MRSA and *P. aeruginosa* by CD33 knockout (CD33KO) M1 macrophages compared to corresponding untreated CD33KO M1 macrophages cultured under increasing concentrations (11 mM, 15 mM, 20 mMor 30 mM) of glucose. Data represent an average of twelve independent experiments (n = 12). Error bars represent the StEM.

6.4.4.2 Estrogen Reduces TNF-α Production in CD33 Knockout M1 Macrophages.

Previous findings demonstrated that CD33 knockdown in M1 macrophages led to an increased secretion of the pro-inflammatory cytokine TNF-α, particularly under hyperglycaemic conditions (Figure 6.3). Thus, this study investigated the effect of estrogen treatment on TNF-α secretion by CD33KO M1 macrophages cultured under increasing (11 mM, 15 mM, 20 mM and 30 mM) glucose concentrations. Estrogen (1 x 10^{-7} M) significantly reduced TNF-α levels in CD33 knockout M1 macrophages compared to corresponding untreated CD33KO M1 macrophages across all glucose concentrations tested (Figure 6.7).

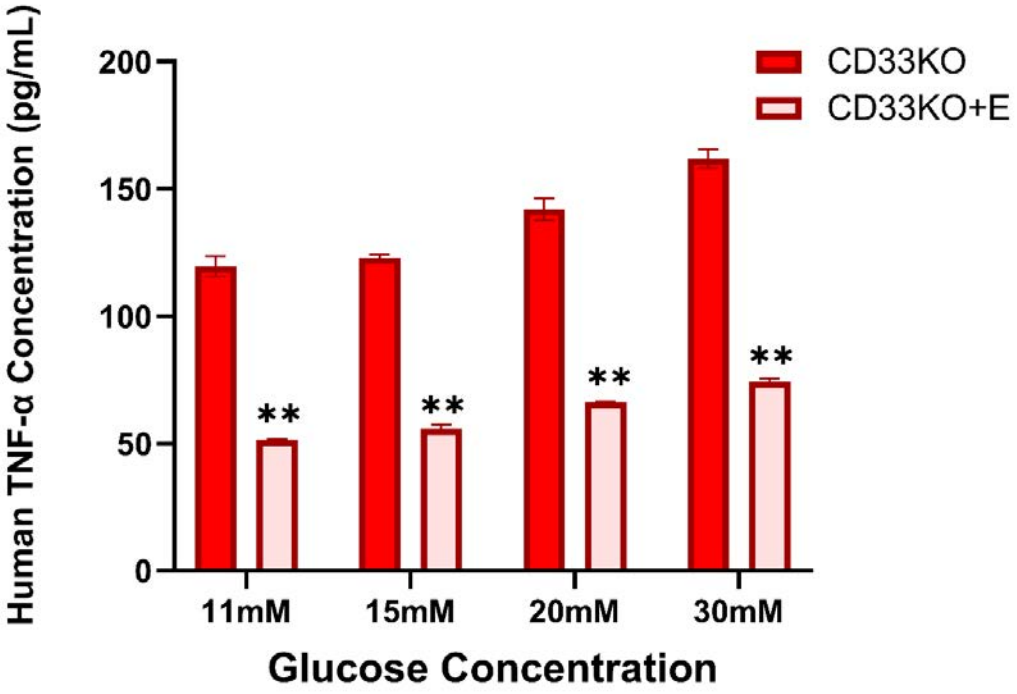

Figure 6.7 Estrogen Reduces TNF-α Secretion in CD33 Knockout M1 Macrophages.
Estrogen treatment (1 x 10^{-7} M) of CD33KO M1-derived macrophages resulted in a significant (**: P<0.001) decrease in TNF-α secretion compared to untreated CD33KO M1 macrophages cultured under increasing glucose concentrations (11 mM, 15 mM, 20 mMand 30 mM). Data represent an average of six independent experiments (n = 6). Error bars represent the StEM.

6.4.4.3 Effect of Estrogen on TREM2/ApoE Signalling in CD33 Knockout Macrophages

Previous results in this chapter reported that CD33KO enhances the phagocytic capacity and inflammatory responses in M1 macrophages, potentially through

modulation of the TREM2 signalling pathway. This effect was particularly observed when CD33 suppression led to a significant downregulation of both mTREM2 and sTREM2 levels in CD33KO macrophages, suggesting a regulatory role of CD33 in maintaining TREM2 levels. Findings in chapter 5 also demonstrated that TREM2 and ApoE expression is modulated by estrogen. Thus, given the important role of TREM2 in mediating phagocytosis and inflammatory responses in macrophages, this study investigated the effect of estrogen on TREM2 and ApoE expression in CD33KO M1 macrophages. Examining the effect of estrogen on ApoE expression in CD33 knockout cells could provide valuable translatable insights into the potential interplay between hyperglycaemia and the CD33/TREM2-ApoE axis during the age-related decline in estrogen.

Flow cytometry analysis revealed upregulation (> 50%) of mTREM2-positive staining in estrogen-treated CD33KO M1 macrophages compared to untreated CD33KO M1 macrophages. Estrogen-treated CD33KO M1 macrophages (red histogram) exhibited a rightward shift in the TREM2 fluorescence intensity curve, indicative of higher mTREM2 levels, relative to untreated CD33KO M1 macrophages (blue histogram). Quantitative analysis of the MFI data confirmed a significant increase in mTREM2 expression upon estrogen stimulation of CD33KO M1 macrophages at both glucose levels ($P<0.001$) (Figure 6.8). ELISA analysis demonstrated that estrogen treatment also resulted in elevated sTREM2 levels secreted from CD33KO M1 macrophages compared to untreated CD33KO M1 macrophages (Figure 6.8.C). Collectively the observed increase in both mTREM2 and sTREM2 levels suggests estrogen can reverse the suppression of TREM2 expression in M1 macrophages caused by CD33 knockout, restoring TREM2 levels to those observed in wildtype CD33+ M1 macrophages.

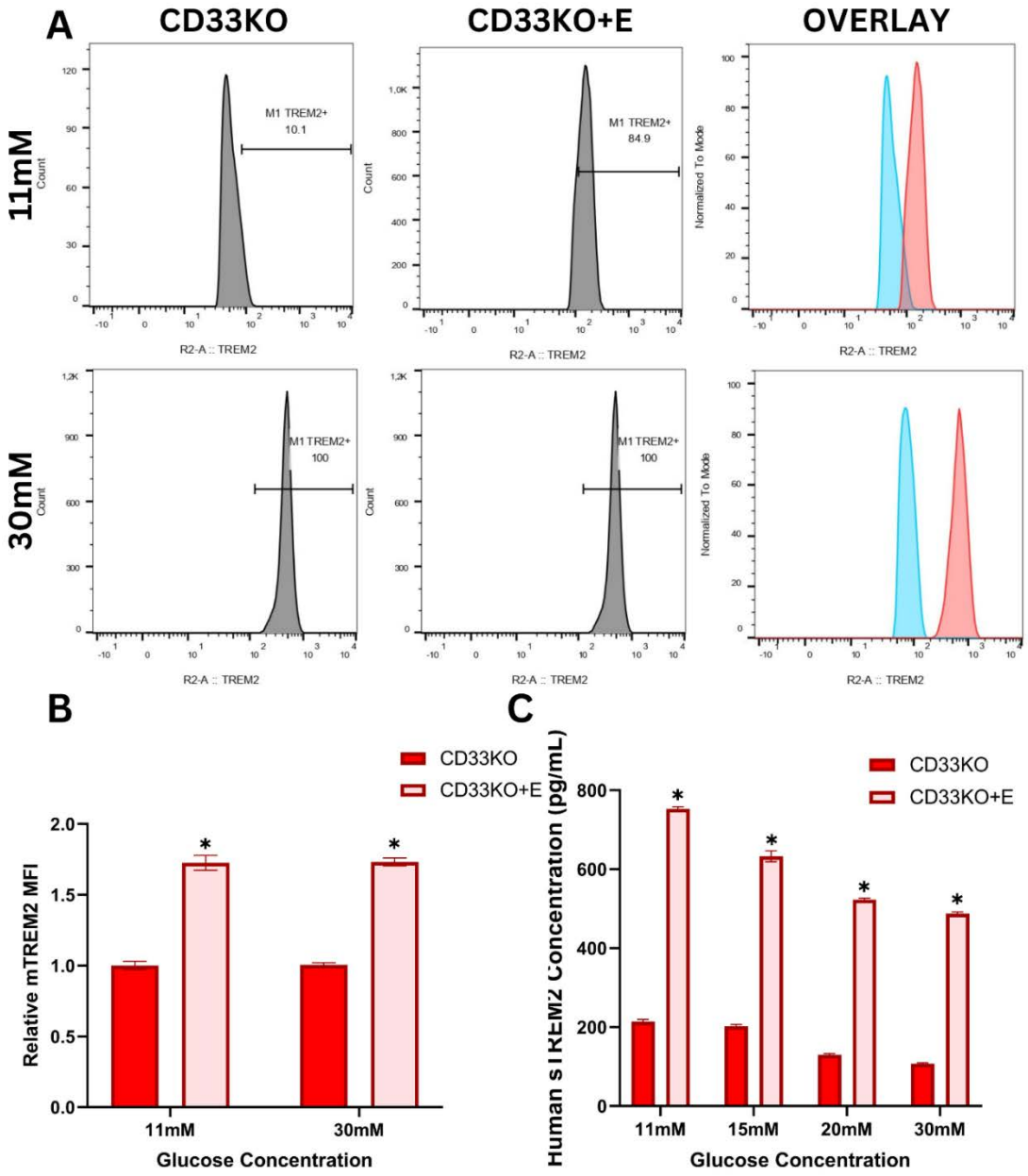

Figure 6.8 Estrogen Upregulates TREM2 in CD33KO M1 Macrophages.
Estrogen significantly increased TREM2-positive staining (A) and mTREM2 MFI (B) in CD33KO M1 macrophages compared to untreated CD33KO M1 macrophages cultured under 11mM and 30mM glucose conditions. ELISA analysis demonstrated a significant increase in sTREM2 secretion (C) from estrogen-treated CD33KO M1 macrophages compared to untreated CD33KO M1 macrophages cultured under all glucose conditions tested. Data represent an average of six independent experiments (n=6). * Indicates significant differences between estrogen-treated and corresponding untreated CD33KO M1 macrophages ($P<0.001$). Error bars represent the StEM.

Similar to the TREM2 findings, estrogen treatment led to an upregulation of cellular and secreted ApoE levels in CD33KO macrophages compared to untreated CD33KO controls, as assessed by flow cytometry and ELISA analyses, respectively (Figure 6.8.A-C).

Under euglycemic conditions (11mM glucose), estrogen treatment resulted in a significant increase (31.9% in CD33KO and 42.3% in estrogen treated-CD33KO cells) in the number of cells expressing ApoE (Figure 6.5A). Intriguingly, the estrogen-mediated increase in ApoE expression in estrogen treated CD33KO M1 macrophages was more pronounced under hyperglycaemic conditions, with ApoE levels increasing from 6.65% to 25.9%, the value increased by approximately 3-fold. The overlay histograms further illustrate this difference, where the histogram for estrogen treated CD33KO M1 macrophages (blue) showed a more substantial right shift compared to the histogram for CD33KO M1 macrophages (red) at 30mM glucose, indicating a greater increase in ApoE expression under hyperglycaemic conditions. Additionally, the relative ApoE MFI quantification confirmed a significant ($P<0.001$) increase in ApoE expression in CD33KO M1 macrophages following estrogen treatment at both 11mM and 30mM glucose concentrations compared to untreated CD33KO macrophages (Figure 6.5B).

ELISA analysis revealed a marked increase in secreted ApoE levels across all glucose concentrations (11, 15 mM, 20 mM and 30 mM) in estrogen treated CD33KO M1 macrophages compared to untreated CD33KO macrophages (Figure 6.5C).

These findings suggest that estrogen can modulate not only TREM2 levels but also ApoE expression in CD33KO macrophages, with estrogen-induced elevation of TREM2/ApoE potentially influencing downstream signalling pathways and immune functions.

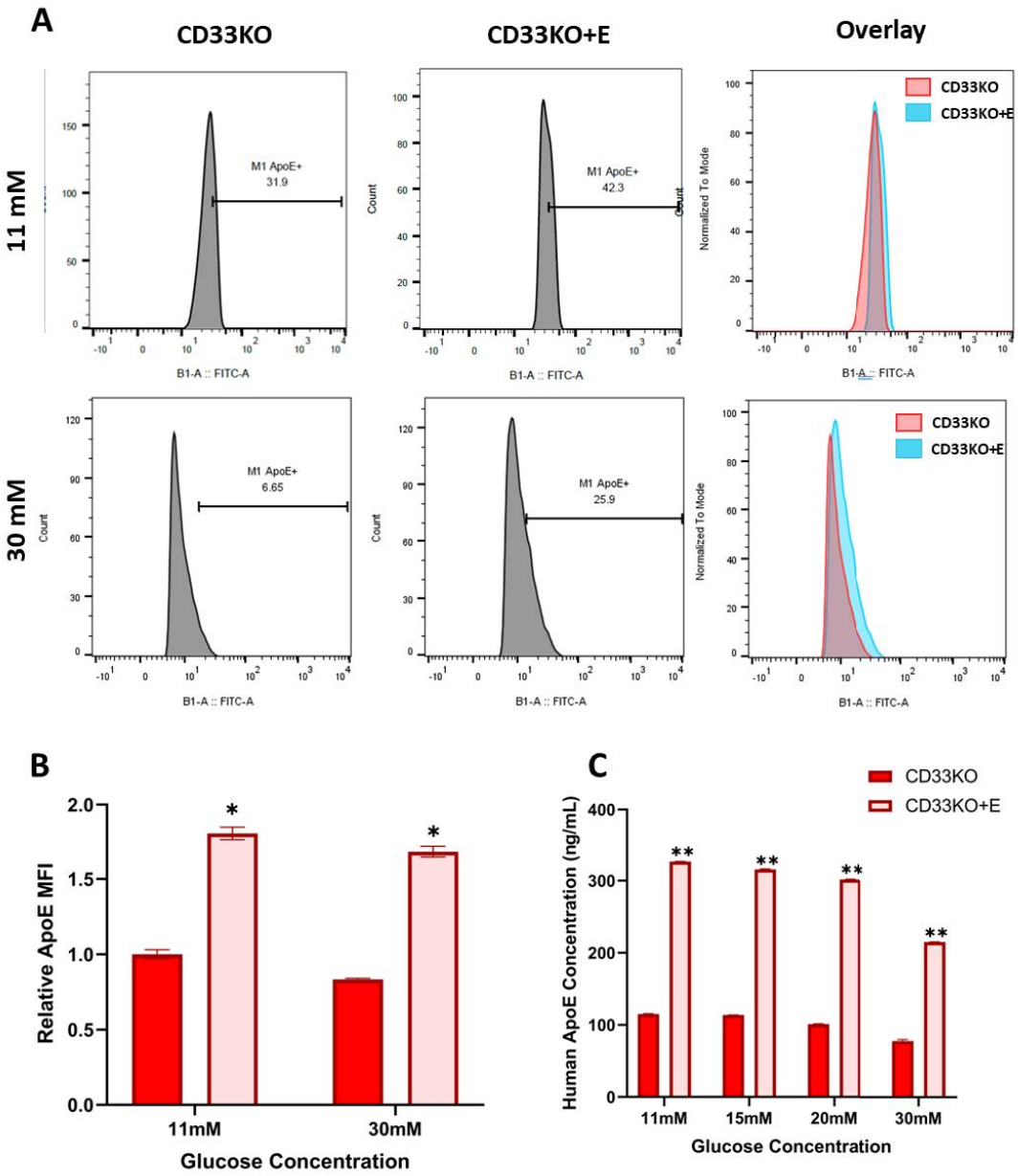

Figure 6.9 Estrogen Upregulates ApoE in CD33 Knockout M1 Macrophages.
Estrogen significantly increased intracellular ApoE-positive staining (A) and ApoE MFI (B) in CD33KO M1 macrophages compared to untreated CD33KO M1 macrophages cultured under 11mM and 30mM glucose conditions. ELISA analysis demonstrated a significant increase in ApoE secretion (C) from estrogen-treated CD33KO M1 macrophages compared to untreated CD33KO M1 macrophages cultured under all glucose conditions tested. Data represent an average of six independent experiments (n=6). * Indicates significant differences between estrogen-treated and corresponding untreated CD33KO M1 macrophages ($P<0.001$). Error bars represent the StEM.

6.4.4.4 Estrogen Restores TREM2/ApoE Levels in CD33 Knockout Cells.

Results in Chapter 5 demonstrated that estrogen upregulates TREM2 and ApoE levels in wildtype CD33+ macrophages. The effect of estrogen on TREM2 and ApoE expression in wildtype CD33+ and CD33KO M1 macrophages was compared to determine whether estrogen can restore TREM2 and ApoE expression in CD33KO M1 macrophages to levels seen in wildtype CD33+ M1 macrophages cultured under euglycemic (11mM) and hyperglycaemic (30mM) conditions. Analysis of flow cytometry results revealed that while estrogen (1×10^{-7} M) treatment substantially restored mTREM2 levels in CD33KO M1 macrophages cultured in 11mM glucose (95.5% vs 93.3% in CD33+) and 30mM glucose (100% vs 15.3% in CD33+). between CD33+ and CD33KO cells under both glucose conditions following estrogen treatment (Figure 6.10.B). Interestingly, although hyperglycaemia (30mM glucose) reduced absolute/overall levels of mTREM2 compared to euglycemic (11mM) conditions (93.3% at 11mM vs 15.3% at 30mM), estrogen treatment appears to enhance mTREM2 expression to a greater extent under hyperglycaemic (30mM) conditions compared to euglycemic (11mM) conditions in CD33KO cells. These findings suggest the interplay between CD33 and estrogen-mediated regulation of mTREM2 is influenced by glycaemic levels, with the restorative activity of estrogen increasing under hyperglycaemic conditions.

For the sTREM2 levels (Figure 6.10.C). While estrogen treatment (CD33KO+E) led to higher secreted TREM2 levels compared to wildtype (CD33+) cells across all glucose concentrations, the increase was not substantially higher under hyperglycaemic conditions compared to euglycemic conditions. at all glucose concentrations tested.

Collectively, these findings confirm that estrogen treatment (1×10^{-7} M) fully restored mTREM2 and sTREM2 levels in CD33KO M1 macrophages to levels observed in estrogen treated M1 CD33+ macrophages and even increase TREM2 expression and secretion to levels higher than in untreated CD33+ wildtype cells.

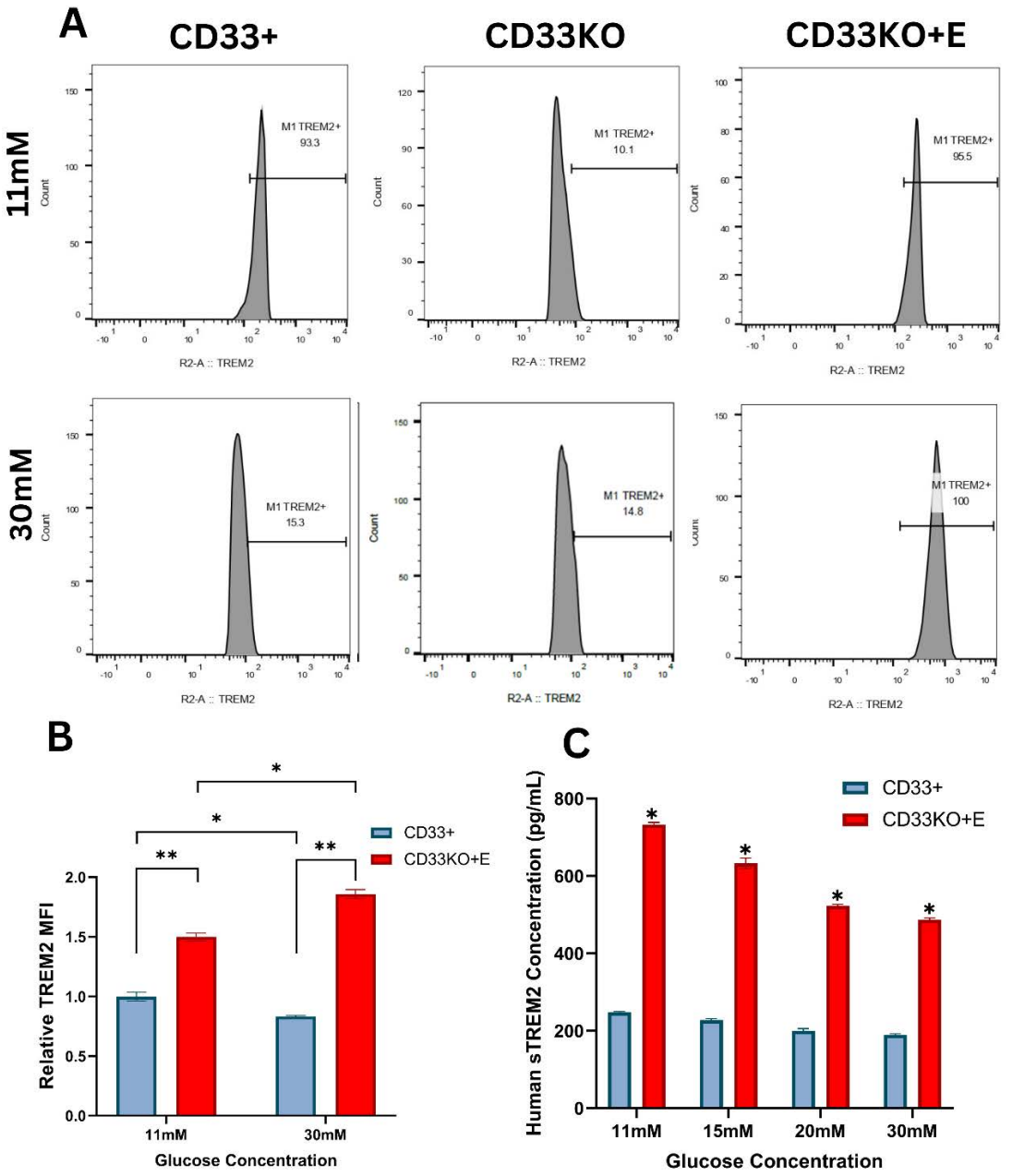

Figure 6.10 Estrogen Restores TREM2 Expression in CD33 Knockout M1 Macrophages.
(A) Flow cytometry histograms comparing mTREM2 expression levels in CD33+ (control), CD33KO (control), and estrogen-treated CD33KO M1 macrophages cultured in 11mM and 30mM glucose. CD33KO M1 macrophages showed reduced baseline mTREM2 levels compared to CD33+ cells. Estrogen treatment partially restored mTREM2 levels in CD33KO M1 macrophages. (B) Quantitative analysis of mTREM2 MFI showing estrogen-treated CD33KO M1 macrophages expressed significantly elevated levels of TREM2 compared to CD33+ M1 macrophages cultured in 11mM and 30mM glucose. (C) ELISA analysis showed estrogen treatment restored sTREM2 levels in CD33KO M1 macrophages under all glucose conditions tested. Data represent an average of six replicates (n = 6). * Indicates significant differences between CD33KO+E M1 macrophages and corresponding wildtype CD33+ M1 macrophages ($P<0.001$). Error bars represent the StEM.

Similar to TREM2 results, Estrogen Restores ApoE Expression in CD33 Knockout M1 Macrophages. Flow cytometry results revealed that CD33KO M1 macrophages exhibited reduced baseline ApoE expression compared to CD33+ controls under both glucose conditions (13.6% vs 21.5% at 11mM; 6.78% vs 17.1% at 30mM). Estrogen treatment (1×10^{-7} M) significantly increased ApoE cellular levels in CD33KO M1 macrophages cultured in 11mM glucose from 13.6% to 42.3%, exceeding CD33+ control levels (21.5%) (Figure 6.11.A). Similarly, under high glucose conditions (30mM glucose), estrogen treatment enhanced ApoE levels in CD33KO cells from 6.78% to 25.3%, surpassing CD33+ control levels (17.1%).

Similarly, under high glucose conditions (30mM glucose), baseline ApoE expression was reduced in both CD33+ and CD33KO cells compared to normal glucose (11mM) conditions. Estrogen treatment significantly increased ApoE levels ($P<0.05$; Figure 6.11.B) in CD33KO M1 macrophages cultured under high glucose conditions.

Furthermore, ELISA analysis demonstrated that estrogen treatment (1×10^{-7} M) increased secreted ApoE levels from CD33KO M1 macrophages under all glucose concentrations tested (11mM, 15mM, 20mM, and 30mM). The estrogen-treated CD33KO M1 macrophages showed significantly ($P<0.05$) higher secreted ApoE levels compared to CD33+ controls across all glucose concentrations (Figure 6.11.C).

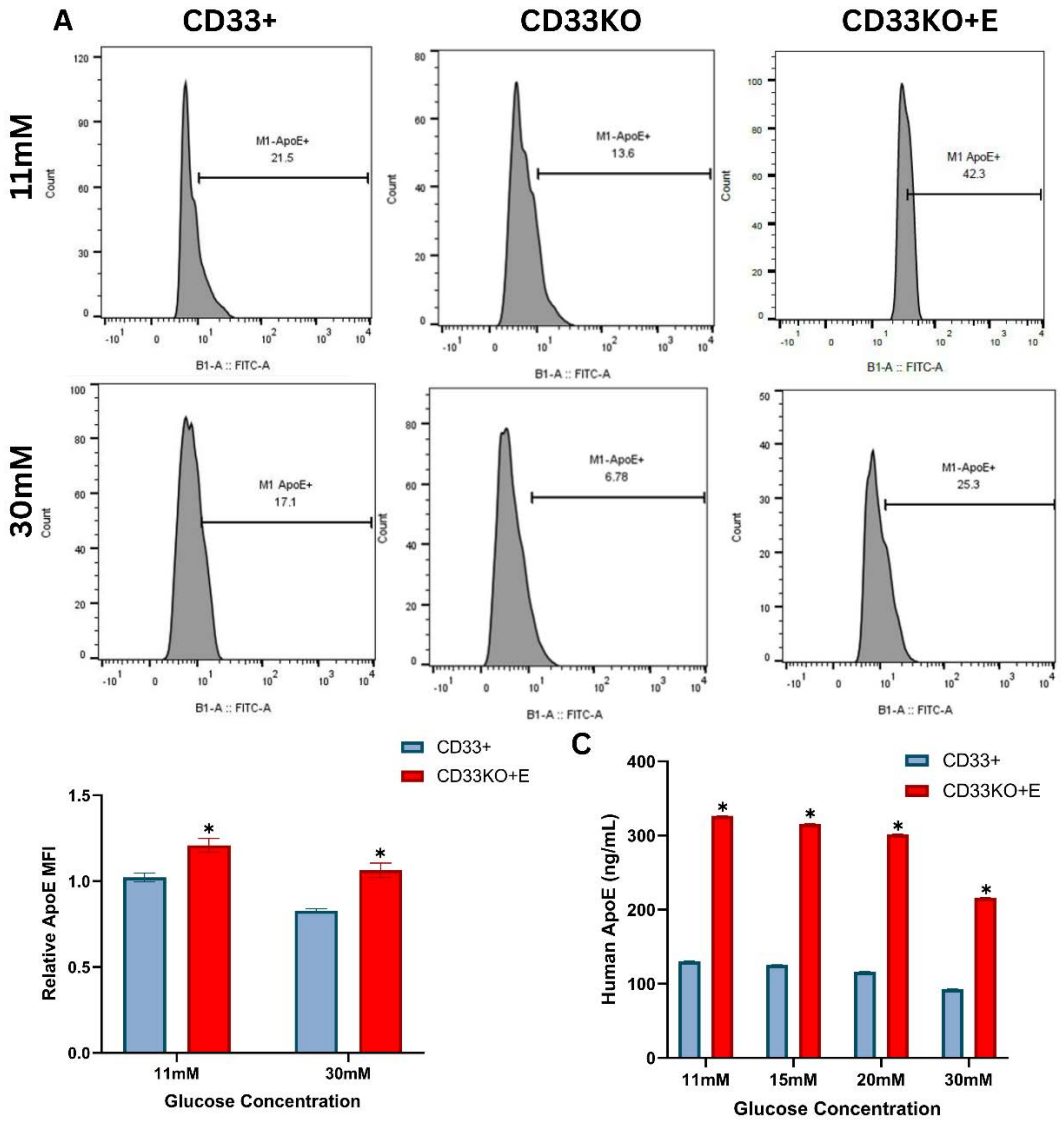

Figure 6.11 Estrogen Restores ApoE Expression in CD33 Knockout M1 Macrophages.
(A) Flow cytometry histograms comparing ApoE expression levels in CD33+ (control), CD33KO (control), and estrogen-treated CD33KO M1 macrophages cultured in 11mM and 30mM glucose. CD33KO M1 macrophages showed reduced baseline ApoE levels compared to CD33+ cells. Estrogen treatment enhanced intracellular ApoE levels in CD33KO M1 macrophages above CD33+ control levels. (B) Quantitative analysis of intracellular ApoE MFI showing estrogen-treated CD33KO M1 macrophages expressed significantly elevated levels of ApoE compared to CD33+ controls cultured in 11mM and 30mM glucose. (C) ELISA analysis showing estrogen treatment significantly increased ApoE secretion by CD33KO M1 macrophages under all glucose conditions tested (11mM, 15mM, 20mM, and 30mM) to levels exceeding those observed in CD33+ controls. (n = 6). * Indicates significant differences between estrogen-treated CD33KO M1 macrophages and CD33+ controls ($P<0.001$). Error bars represent the StEM.

6.4.5 Estrogen Modulation of CD33 proteins

Having established estrogen and CD33 signalling interacts with regulation of TREM2 and ApoE levels, this investigation assessed whether estrogen could directly modulate the expression levels of mCD33 and sCD33 in macrophages. Flow cytometric analysis demonstrated that estrogen did not significantly ($P>0.05$) change M1 macrophage expression levels of either mCD33 or sCD33 compared to untreated negative control (NC) M1 macrophages (Figure 6.12.A-D). Flow histograms (Figure 6.12.A) indicate that estrogen treatment had no effect on the levels of mCD33 expression in M1 macrophages compared to untreated negative control (NC) cells cultured under either 11mM or 30mM glucose conditions. Quantitative analysis of the MFI data (Figure 6.12.B) corroborated these findings, showing no significant differences ($P>0.05$) in mCD33 expression between estrogen-treated and NC M1 macrophages across both glucose concentrations.

ELISA experiments assessed total cellular CD33 expression (Figure 6.12.C) and sCD33 levels (Figure 6.12.D) in estrogen-treated and untreated NC M1 macrophages cultured across a range of glucose concentrations (11, 15 mM, 20 mM and 30 mM). Estrogen had no significant effect ($P>0.05$) on either cellular CD33 or secreted CD33 levels compared to untreated controls, regardless of the glucose concentration. These results from both flow cytometry and ELISA assays consistently demonstrate that estrogen treatment does not significantly alter the expression of CD33 in M1 macrophages under varying glycaemic conditions.

These findings suggest that while estrogen can modulate the CD33-TREM2/ApoE axis, it does not affect expression of CD33 itself. Thus, the observed interplay between estrogen and the CD33-TREM2/ApoE signalling pathways appears to involve estrogen and CD33 influencing TREM2 and ApoE levels, but the effects of estrogen are not mediated through changes in CD33 receptor levels.

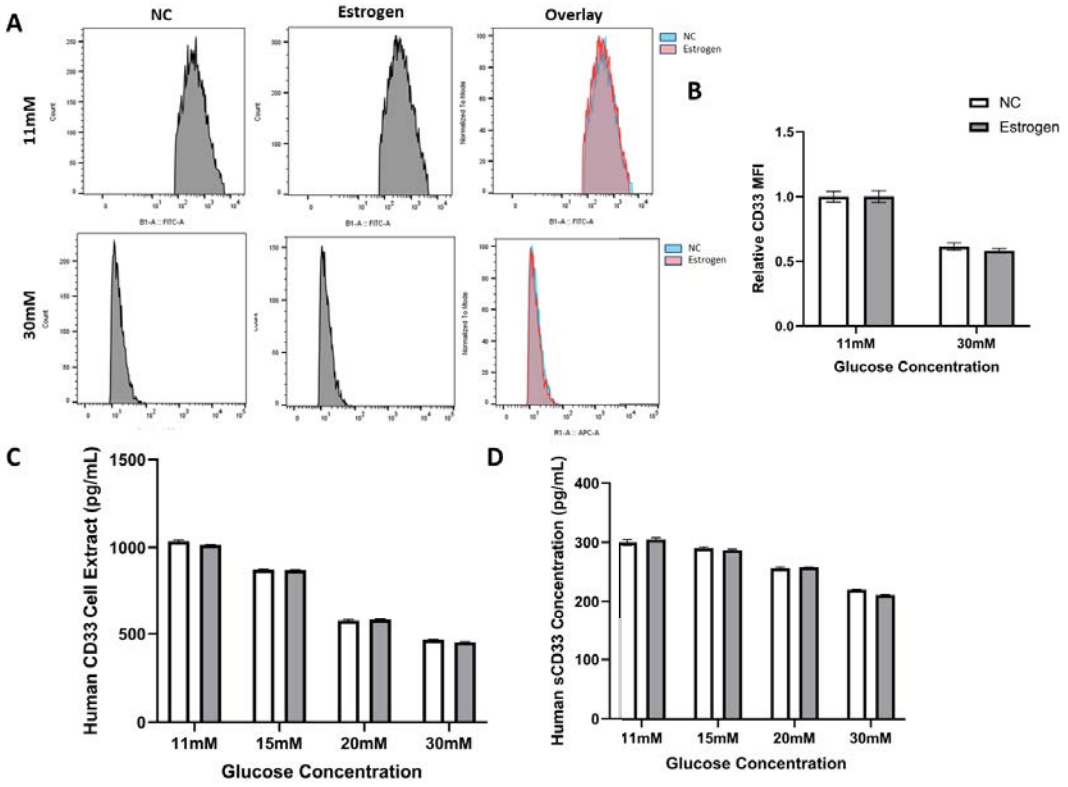

Figure 6.12 Effect of Estrogen on CD33 Expression in M1 Macrophages
(A) Flow cytometry histograms indicate that estrogen had no effect on levels of expression of mCD33 in M1 macrophages compared to untreated negative control (NC) M1 macrophages cultured under 11mM or 30mM glucose conditions. (B) Quantitative analysis of the MFI data from flow cytometry corroborates these findings, showing estrogen treatment did not significantly ($P>0.05$) alter the expression of mCD33 in M1 macrophages compared to NC M1 macrophages cultured in 11mM or 30mM glucose. (C, D) ELISA experiment assessed mCD33 (C) and sCD33 (D) levels in estrogen treated and untreated NC M1 macrophages across a range of glucose concentrations (11, 15 mM, 20 mM and 30 mM). Estrogen treatment had no significant ($P>0.05$) effect on mCD33 or sCD33 levels, regardless of glucose concentration. Data represent an average of 6 independent experiments (n=6).

6.4.6. Spatial Distribution and Co-localisation of mCD33 and mTREM2 on the Surface of M1 Macrophages

The cellular localisation of mCD33 and mTREM2 in M1 macrophages was investigated using confocal microscopy. M1 macrophages grown at euglycemic (11mM) and hyperglycaemic (30mM) glucose concentrations were prepared as described in 2.3. Cells were then incubated with or without estrogen (1×10^{-7} M) prior to fixation with 4% paraformaldehyde in DPBS for 10 minutes. Cells were stained with APC anti-human CD33 antibody and Alexa fluor 448 anti TREM2 antibody, as described in 2.12.3. Confocal microscopy was used to capture images

at 100× objective. The co-localisation of markers is highlighted in yellow (within merged overlay images).

Under euglycemic conditions (11mM glucose), mCD33 and mTREM2 exhibited a moderate degree of co-localisation (yellow) on the surface of M1 macrophages, suggesting potential cross-talk or interaction between the receptors. Intriguingly, the co-localisation of mCD33 and mTREM2 was reduced at hyperglycaemic (30mM) glucose levels, with both markers displaying a more diffuse and separated distribution pattern. Notably, both mCD33 and TREM2 expression on the surface of untreated M1 macrophages exhibited a less intense signal under hyperglycaemic compared to euglycemic conditions. Estrogen visibly restored the co-localisation of mCD33 and mTREM2 at the cell surface of M1 macrophages cultured at 11mM or 30mM glucose conditions. Moreover, although the intensity of the CD33 signal on the surface of M1 macrophages remained similar following treatment with estrogen, in contrast TREM2 staining showed a more intense signal following estrogen stimulation of M1 macrophages cultured in 11mM or 30mM glucose.

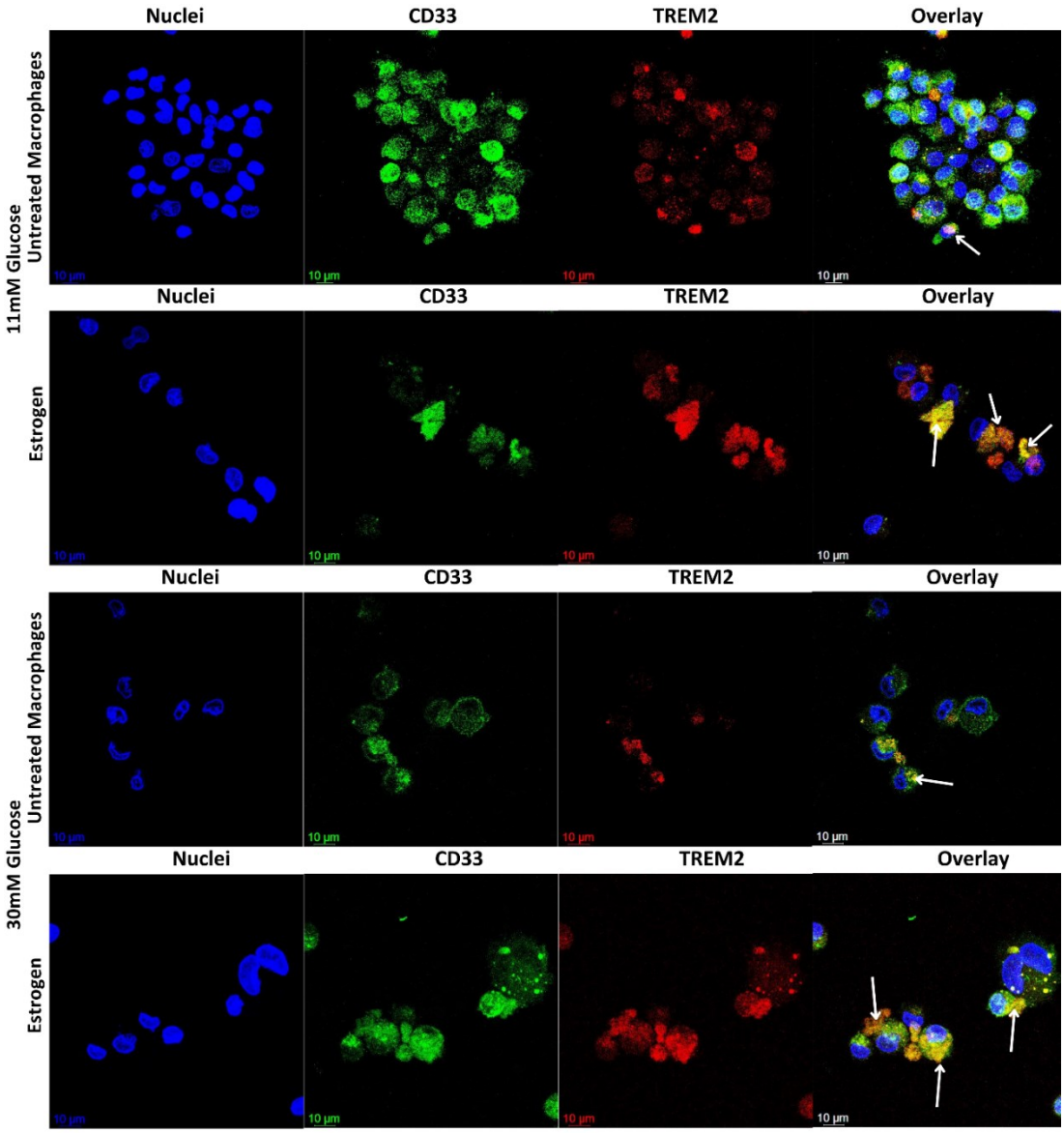

Figure 6.13 Co-localisation of CD33 and TREM2 on M1 Macrophages.
Confocal Images illustrating co-localization of CD33 (green) and TREM2 (red) on the membranes of M1 macrophages under different glucose concentrations (11 mM and 30 mM) and with or without estrogen treatment. The nuclei are stained with DAPI (blue). The overlay images display the co-localization of CD33 and TREM2, appearing in yellow/orange color, indicated by white arrowheads. The images are organised in rows, with each row representing a different condition.; magnification 100. Scale bar = 10 µm.

6.5 Discussion

The link between CD33 and phagocytosis suggests that modulating this receptor could be a potential therapeutic target for impaired bacterial clearance in DFUs. Findings in this chapter demonstrate that knocking out the CD33 gene leads to enhanced phagocytic function and increased inflammatory responses in macrophages under both euglycemic and hyperglycaemic conditions. CD33 knockout significantly boosted the phagocytosis of MRSA and *P. aeruginosa* by monocyte-derived M1 macrophages across all tested glucose concentrations. The absence of CD33 appears to heighten the ability of macrophages to clear bacterial pathogens, which is particularly relevant for DFUs, where impaired phagocytosis contributes to DFIs (Bessman and Sapico, 1992; Aitcheson et al., 2021; Rodríguez-Rodríguez et al., 2022). This aligns with previous studies implicating CD33 as an immunoinhibitory receptor involved in regulating macrophage phagocytosis (Bhattacherjee et al., 2019; Bhattacherjee et al., 2021; Butler et al., 2021; Wißfeld et al., 2021). Several studies show CD33, especially the CD33M isoform, acts as an inhibitory receptor suppressing microglial phagocytosis (Bhattacherjee et al., 2019; Bhattacherjee et al., 2021; Butler et al., 2021), while the CD33m variant increases phagocytosis and may have a protective role in Alzheimer's disease (Butler et al., 2021). Importantly, Wißfeld et al. (2021) found that CD33 knockout enhances the phagocytosis in THP1 macrophages.

Furthermore, CD33 knockout induced higher expression of TNF-α in M1 macrophages under hyperglycaemic conditions compared to wildtype CD33+ M1 macrophages. Previous studies have shown that downregulating CD33 on monocytes and macrophages can directly induce TNF-α secretion (Lajaunias et al., 2005; Gonzalez et al., 2012; Guzmán-Beltrán et al., 2022). The increased TNF-α production observed in CD33KO macrophages, particularly under hyperglycaemia, suggests that elevated TNF-α levels could lead to downregulated CD33 expression. The mechanism appears to involve ROS generation induced by high glucose, which disrupts the CD33-sialic acid interaction and downregulates CD33 expression (Lajaunias et al., 2005; Gonzalez et al., 2012). Another study by Shamsasenjan et al.

(2009) also suggested that IL-6 downregulates CD33 expression in myeloma cells (Shamsasenjan et al., 2009). High pro-inflammatory cytokine levels have been widely reported in diabetic environments and attributed to multiple factors. Xu et al. (2013) found that cell necrosis contributes to this phenomenon. Moreover, Ayala et al. (2019) suggests that dysregulation of signalling pathways like TLR4-LPS, PI3K/AKT, and MAPK under hyperglycaemic conditions contributes to an imbalanced inflammatory response, characterised by increased secretion of pro-inflammatory cytokines and impaired release of anti-inflammatory mediators (Ayala et al., 2019). Similarly, S.-M. Huang et al. (2019) propose that high glucose environments promote M1 macrophage polarization and activation of the TNF-α/TIMP1 pathway, which may impair wound healing by inhibiting keratinocyte migration (S.-M. Huang et al., 2019).

In the present study, higher TNF-α secretion was observed in CD33 knockout macrophages under diabetic conditions, with hyperglycaemia directly inducing CD33 downregulation (Chapter 3). The lower TNF-α secretion observed in wildtype CD33+ macrophages cultured under hyperglycaemic conditions compared to CD33 knockout cells suggests a putative role of CD33 in mediating inflammation during DFU infections. However, further research is needed to elucidate the precise relationship between CD33 and TNF-α in diabetic environments.

Intriguingly, flow cytometry and ELISA analyses revealed that CD33 knockout resulted in reduced expression of TREM2 and ApoE in M1 macrophages. The TREM2/ApoE signalling pathway is known to modulate inflammatory responses, including phagocytosis in macrophages (Yeh et al., 2016; Shi and Holtzman, 2018). These findings suggest a potential crosstalk or regulatory role of CD33 in modulating TREM2/ApoE levels during macrophage-mediated phagocytosis. However, the literature presents conflicting reports on the relationship between CD33 and TREM2. The findings of this thesis are supported by work conducted by Chan et al. (2015) who reported that CD33 suppression decreases TREM2 expression in monocytes. When Chan et al. (2015) experimentally blocked CD33 signalling using an anti-CD33 antibody in primary monocytes from study subjects, they observed a consistent decrease in TREM2 surface expression compared to

monocytes treated with a non-specific isotype control antibody. Conversely, Tan et al. (2023) reported that CD33 knockout significantly increased TREM2 expression in murine microglia, while Griciuc et al. (2019) found that CD33 deletion in mice did not directly affect TREM2 expression in microglia. These contrasting results highlight the complex and context-dependent nature of the interactions between CD33 and TREM2 during innate immune responses. Nonetheless, multiple studies have reported opposing roles for CD33 and TREM2 in modulating macrophage responses, underscoring the intricate interplay between these receptors (Griciuc et al., 2013; Chan et al., 2015; Griciuc et al., 2019). Our findings suggest that CD33 suppression of TREM2/ApoE signalling that might extrapolate to impaired bacterial clearance and elevated inflammation in DFU patients.

Building upon the enhanced phagocytic capacity and heightened inflammatory state observed in CD33 knockout macrophages, the potential immunomodulatory effects of estrogen on the phagocytic functions of CD33KO macrophages were investigated in this chapter. Notably, estrogen stimulation enhanced the phagocytic clearance of MRSA and *P. aeruginosa* by classically-activated CD33KO M1 macrophages cultured in a range of glucose environments. These findings suggest that estrogen can exert beneficial immunomodulatory effects, boosting the phagocytic ability of macrophages to clear bacterial pathogens, even in the absence of CD33. In addition, estrogen stimulation reduced TNF-α secretion by CD33KO macrophages. This indicates that estrogen may counteract the heightened inflammatory state induced by CD33 deficiency under hyperglycaemic conditions, potentially exerting an anti-inflammatory effect by dampening the excessive TNF-α production typically associated with DFUs. This anti-inflammatory property of estrogen aligns with previous reports highlighting its immunomodulatory roles in various inflammatory conditions (Villa et al., 2015; Villa et al., 2016; Pepe et al., 2017; Dama et al., 2021). Villa et al. (2015) demonstrated that estrogen acts through ERα to suppress inflammatory responses in two type of macrophage cell lines, RAW 264.7 and J774A.1, by promoting the resolution of inflammation and shifting macrophages towards an anti-inflammatory phenotype. This shift involves suppressing pro-inflammatory signalling pathways and promoting the production of

anti-inflammatory mediators. Subsequent work by Villa et al. (2016) showed estrogen mediates microglial activation and polarization, promoting a neuroprotective phenotype.

Pepe et al. (2017) published a detailed list of genes differentially expressed in peritoneal macrophages in response to estrogen in female mice that are known to regulate wound healing, cell proliferation and immune responses by promoting an anti-inflammatory and pro-resolving macrophage phenotype (Pepe et al., 2017). Dama et al. (2021) also highlighted the role of estrogen in mitigating inflammation in cardiovascular diseases, partly through its modulation of inflammatory signalling pathways (Dama et al., 2021). While these studies focused on different inflammatory conditions and cell types, they collectively support the notion that estrogen possesses broad immunomodulatory properties, particularly in suppressing excessive or chronic inflammation.

The effect of estrogen on TREM2-ApoE expression in CD33KO M1 macrophages was subsequently investigated. Under euglycemic condition, estrogen stimulation of CD33KO cells largely restored both TREM2 and ApoE expression levels, although not to the same extent as in wildtype CD33+ cells. More widely, the findings give an insight into the combined detrimental impacts of hyperglycaemia and the age-related decline in estrogen on the CD33-TREM2/ApoE axis and subsequent impaired immune responses. In contrast, high levels of estrogen found during youth and possibly estrogen supplementation in the elderly are likely to provide protective and restorative effects on the CD33/TREM2-ApoE axis respectively.

Interestingly, even under hyperglycaemic conditions, estrogen treatment effectively reversed the effects of CD33 knockout, restoring TREM2 and APoE levels in CD33KO cells to those observed in CD33+ cells. This might be due to a hyperglycaemia-induced increase in the sensitivity of estrogen receptors or their co-regulators (Zeng et al., 2016), leading to heightened responsiveness to estrogen and subsequent TREM2/ApoE gene expression. However we cannot exclude the possibility that oxidative stress or post-translational modifications caused by hyperglycaemia may possibly contribute to ligand-independent activation of ERs or other transcription factors involved in TREM2/ApoE regulation (Zeng et al., 2016; Gregorio et al., 2021),

bypassing the need for direct estrogen binding. Hyperglycaemia-induced modifications could also modulate the interactions between ERs and their co-regulators, thereby enhancing the transcriptional activity of estrogen-responsive pathways (Chakrabarti and Davidge, 2009; Gregorio et al., 2021). Additionally, metabolic changes under hyperglycaemic conditions may stabilize or protect key transcriptional regulators or co-factors involved in TREM2 regulation (Ihionkhan et al., 2002; Tu et al., 2013; Alemany, 2021), prolonging transcriptional activity and responsiveness to estrogen.

Lastly, the influence of estrogen on the expression of both mCD33 and sCD33 was elucidated. This investigation was driven by the possibility of transcriptional crosstalk mechanism, whereby estrogen might regulate CD33 levels in macrophages, which in turn mediates TREM2/ApoE expression. Interestingly, as seen in Chapter 3, we observed that hyperglycaemia reduced CD33 levels in CD33+ M1 macrophages, which aligns with published data showing decreased CD33 levels in primary human monocytes (Gonzalez et al., 2012). However, estrogen did not significantly alter CD33 expression in M1 macrophages, which suggests that while estrogen and CD33 can modulate TREM2 and ApoE expression, estrogen does not exert a direct regulatory effect on CD33 expression itself.

The novel findings presented in this chapter have multiple clinical implications. Firstly, they suggest that estrogen may have therapeutic potential in modulating the impaired phagocytosis and dysregulated inflammation associated with DFUs. By enhancing the phagocytic clearance of bacterial pathogens and dampening the excessive inflammatory responses observed in DFUs, estrogen could potentially be used as a therapy to promote wound repair and prevent chronic infections in DFUs. Secondly, the interplay between estrogen and the CD33-TREM2/ApoE signalling pathways highlights the potential for more targeted therapeutic approaches that could focus on manipulating TREM2-ApoE and/or CD33 signalling to promote phagocytosis and wound healing in DFUs.

Interestingly, CD33 levels are known to decrease in diabetic patients (Gonzalez et al., 2012), raising the question of whether this is part of the pathology or just a compensatory response. Counterintuitively, increasing local CD33 expression in

wound macrophages could be a potential therapeutic approach. While an increase in CD33 is expected to reduce phagocytosis (Griciuc et al., 2013; Bhattacherjee et al., 2019), the subsequent increase in TREM2/ApoE expression might be more than sufficient to compensate for this initial negative effect, leading to enhanced phagocytosis overall. Furthermore, given an increase in both CD33 and TREM2/ApoE levels are known to reduce inflammation (Gonzalez et al., 2012; Zhong et al., 2017), concomitantly increasing their local expression would dampen excessive hyperglycaemia-induced macrophage release of pro-inflammatory mediators such as TNF-α. Furthermore, increasing local CD33 expression would also align (rather than oppose) with the beneficial effects of local estrogen supplementation or ER-α activation, with a combined therapy expected to lead to upregulation of TREM2/ApoE levels in M1 macrophages and subsequent dampening of inflammation and promotion of bacterial clearance by M1 macrophages.

Overall, this chapter highlights the complex interplay between estrogen, CD33 and TREM2/ApoE signalling in regulating phagocytosis and inflammation under euglycemic and hyperglycaemic conditions. Further research is needed to confirm the precise mechanisms involved in order to determine the most effective therapeutic strategies, which may involve a single target or a combination of approaches involving local estrogen supplementation/ ER-α activation, stimulation of TREM2/ApoE expression, and/or induction of CD33 to achieve an overall beneficial outcome.

Chapter 7: General Discussion and Future Work

7.1 General Discussion

DFUs present a significant challenge in healthcare, as they often become infected with a range of wound-associated pathogens including *S aureus* and *P aeruginosa* (Rybtke et al., 2015; Ishwarya and Neelusree, 2019; Akash et al., 2020; Macdonald et al., 2021). The susceptibility to wound infections is heightened in diabetic patients, particularly the elderly, due to delayed wound healing and impaired immune function (Joshi et al., 1999; Cooper et al., 2015; Hodgson et al., 2015; Swoboda and Held, 2022). Serious infections can increase the risk of partial or complete amputation, especially in elderly diabetic patients (Pecoraro et al., 1990; Rajagopalan, 2005). Treating serious bacterial infections in diabetic patients can often require aggressive medication, including the systemic use of last-resort antibiotics (Nagendra et al., 2022).

In the well-synchronised healing of acute wounds, macrophages play a crucial role, initiating the inflammatory response and subsequently promoting tissue regeneration through the controlled release of cytokines and growth factors (Werner and Grose, 2003; Barrientos et al., 2008). However, chronic hyperglycaemia associated with diabetes disrupts this process, leading to multiple adverse effects on macrophage function (Barman and Koh, 2020; Al Sadoun, 2022). To investigate these effects, an *in vitro* model was established using U937 monocytes cultured under high glucose concentrations to mimic the hyperglycaemia associated with the development of diabetes. Previous research has employed glucose concentrations ranging from 15 to 40 mM to simulate diabetic hyperglycaemia in cell culture (Simone et al., 2008; Kim et al., 2010; Torres-Castro et al., 2016; Grosick et al., 2018; Pavlou et al., 2018; Zhu et al., 2020).

Similar to published studies (Martin-Manso et al., 2008; Torres-Castro et al., 2016; Magatti et al., 2017), this project utilised U937 monocytes that were differentiated into M0-like macrophages and then polarized into the classically-activated proinflammatory M1-like phenotype, for subsequent use in investigations of M1 macrophage function under simulated hyperglycaemia. This *in vitro* methodology provides a well-established model to study the impact of chronic hyperglycaemia

on macrophage function, which is crucial for understanding the disruption of the wound healing processes such as phagocytosis in the context of diabetes.

The differentiation of monocytes into M0 macrophages was accomplished using PMA, a chemical known to stimulate monocyte differentiation by inducing the expression of cell adhesion molecules such as β-2 integrins, as well as activating protein kinase C (PKC) in cells, which triggers a signalling cascade leading to macrophage differentiation (Luscinskas et al., 1994; Pinto et al., 2021). Flow cytometry was used to identify the CD11b surface marker, which is used to confirm the differentiation of U937-derived monocytes into M0-like macrophages (Sintiprungrat et al., 2010; Jundi et al., 2020). M0 macrophages were then polarized into the proinflammatory M1-like phenotype by stimulation with LPS and IFN-γ, which are known to promote polarisation to the M1 phenotype (Nathan et al., 1983; Murray et al., 2014). Flow cytometry was used to confirm the M1 phenotype through the increased expression of the CD197 (CCR7) surface marker, characteristic of the M1 macrophage population (Rios de la Rosa et al., 2017).

Hyperglycaemia was found to promote both the planktonic growth and biofilm formation of MRSA and *P. aeruginosa* (Section 3.4.3), two typical wound pathogens commonly associated with infected DFUs (Rybtke et al., 2015; Nagendra et al., 2022; Mamdoh et al., 2023; Wada et al., 2023). These findings aligned with existing research demonstrating hyperglycaemia promotes bacterial proliferation and biofilm development (Chávez-Reyes et al., 2021; Fernández-Grajera et al., 2022). Glucose is known to upregulate the expression of genes involved in the bacterial production of extracellular polysaccharides, key components of the biofilm matrix, leading to a strengthening of the biofilm structure by increasing secretion of the extracellular matrix (She et al., 2019).

In terms of host cell function, prolonged exposure to high glucose concentrations (more than two weeks) led to reduced viability of both M0 and M1 macrophages (Section 3.4.4.1), corroborating previous studies showing that immune cells, including macrophages, can undergo apoptosis when subjected to hyperglycaemic conditions (Smart and Li, 2007; Dasu et al., 2010b; Morey et al., 2019). Furthermore, hyperglycaemia led to a shift in macrophage polarization towards the

proinflammatory M1 phenotype (Section 3.4.4.2), while altering their cytokine secretion. This results in a sustained inflammatory state, which is detrimental to the wound healing process. Specifically, hyperglycaemic conditions led to an increase in the production of TNF-α by M1 macrophages (Section 3.4.5.1), a pro-inflammatory cytokine known to contribute to excessive inflammation in diabetes (Xu et al., 2013). Conversely, the production of IFN-β, a crucial factor for the resolution of inflammation and the transition to the tissue-repairing M2 phenotype (Vidyarthi et al., 2018), was reduced in M1 macrophages (Section 3.4.5.2). A published comparative study of macrophages derived from wounds of healthy and diabetic individuals has revealed the importance of IFN-β signalling in this context, with impaired IFN-β signalling in diabetic patients resulting in the failure to switch from the M1 to the M2 phenotype (Newby and Mathews, 2017; Wolf et al., 2022). Consequently, an accumulation of proinflammatory M1 macrophages has been reported in the diabetic wounds, which contributes to delayed and functionally-impaired wound healing in diabetic individuals (Kimball et al., 2019). Furthermore, in this project hyperglycaemia was found to decrease NO production by M1 macrophages (Section 3.4.6). Adequate NO levels are essential for the antimicrobial activity and wound healing functions of M1 macrophages (Yoshii and Mizushima, 2017; Pavlou et al., 2018). These alterations in macrophage and immunomodulatory mediator profiles induced by hyperglycaemic stress align with heightened inflammatory responses and impaired wound healing observed in diabetes (Acosta et al., 2008; Graves and Dasu, 2011; Xu et al., 2013; Wolf et al., 2022).

Hyperglycaemia activated NF-κB in M1 macrophages (Section 3.4.7), a transcription factor which induces the inflammatory response and bacterial clearance (Lawrence, 2014). Additionally, hyperglycaemia altered M1 receptor expression, increasing CD14 but decreasing both CD33 and TREM2 (Section 3.4.8), leading to an overall increase in inflammation but compromising phagocytosis (Nareika et al., 2008; Gonzalez et al., 2012). Moreover, hyperglycaemia induced structural changes in the actin cytoskeleton of M1 macrophages (Section 3.4.9), potentially impairing cellular motility and phagocytosis (Möller et al., 2002; Plekhova et al., 2017).

Estrogen plays a key role in regulating wound healing process, and its decline with age is linked to problematic wound healing in the elderly (Ashcroft et al., 1999b; Margolis et al., 2002). While estrogen helps regulate inflammation, the decline in estrogen levels with age can impair the ability of inflammatory cells to effectively clear bacteria from infected wounds (Thomas, 2001; Guo and DiPietro, 2010; Giefing-Kröll et al., 2015).

The potential role of estrogen in regulating macrophage-mediated clearance of bacterial pathogens, particularly in the context of DFUs, has not been fully characterized to date. To elucidate the effect of age-related changes in estrogen levels on the clearance of bacteria by diabetic macrophages, this study investigated the *in vitro* phagocytosis of MRSA and *P. aeruginosa* by glucose-supplemented U937-derived M1 macrophages. Experiments were performed under conditions modelling estrogen deprivation (to mimic the elderly) and estrogen supplementation (to mimic supraphysiological levels). Estrogen supplementation was able to reverse the detrimental effects of hyperglycaemia on macrophage-mediated phagocytosis (Section 4.4.2, 4.4.3 and 4.4.4). Estrogen treatment enhanced the clearance of both planktonic bacteria as well as mature biofilms formed by MRSA and *P. aeruginosa* under all glycaemic conditions tested (Section 4.4.6). Confocal microscopy and SEM further validated these findings (Section 4.4.7), showing increased intracellular fluorescence from GFP-*S. aureus* and mCherry *P. aeruginosa* in estrogen-treated macrophages, as well as pronounced morphological changes such as the formation of longer and more extensive membrane extensions (pseudopodia) in response to bacteria. These findings indicated $1x10^{-7}$M estrogen can to a large extent mitigate the phagocytic impairment caused by sustained hyperglycaemia, a hallmark of poorly controlled diabetes. This builds on previous research showing that estrogen stimulation can increase the phagocytic function of macrophages by enhancing the uptake of amyloid β-peptide and *E. coli* by microglia (Li et al., 2000; Loiola et al., 2019). It is important to note however that microglia, the resident macrophages of the central nervous system, have some functional differences compared to macrophages-derived monocytes. Microglia exhibit distinct responses to stimuli compared to

peripheral macrophages (Ginhoux and Prinz, 2015), so whilst both cell types demonstrate enhanced phagocytic activity in response to estrogen, the precise mechanisms may differ between microglia and peripheral macrophages.

Previous research from the Ashworth group also found that estrogen supplementation increases the phagocytosis of both MRSA and *P aeruginosa* planktonic bacteria (El Mohtadi, 2019). Similarly, Souza et al. (2021) demonstrated a substantial increase in the phagocytic activity of estrogen-treated macrophages against planktonic *S. aureus*. The current study extends these findings by demonstrating that estrogen can enhance the phagocytic clearance of both planktonic and biofilm-forming bacteria by M1 macrophages under sustained hyperglycaemia. Biofilms are known to be more resistant to host immune clearance and antibiotic therapies compared to planktonic bacteria (Jamal et al., 2018). Therefore, the ability of estrogen to improve the elimination of biofilm-associated bacteria under hyperglycaemic conditions is an important novel finding that has profound implications for the management of diabetic wound infections.

Transitioning to the analysis of mechanistic pathways mediating the responses of estrogen in Chapter 5 revealed the beneficial effects of estrogen on the phagocytosis of bacteria by M1 macrophages were mediated solely through ERα, with no evidence of a contribution from ERβ activation. Moreover, estrogen treatment acted through ERα alone to significantly decrease the secretion of the pro-inflammatory cytokine TNF-α by M1 macrophages, irrespective of glycaemic levels (Section 5.4.1). Multiple human and animal studies have previously shown estrogen's key role in modulating TNF-α across various disease contexts . Estrogen has been reported to inhibit TNF-α expression in both murine and human macrophage cell lines (An et al., 1999; Srivastava et al., 1999). The binding of estrogen to ER-α can activate the IGF-1 receptor pathway, which has been shown to downregulate TNF-α expression (Kahlert et al., 2000). This is particularly relevant since the hyperglycaemia associated with diabetes can impair IGF-1 signalling through decreased receptor desensitization (Kajstura et al., 2001). Thus, estrogen supplementation may have the potential to reverse the hyperglycaemia-induced

impairment of IGF-1 signalling, representing a key mechanistic pathway warranting further investigation.

The mechanistic investigations in this study suggest that the inhibitory effects of estrogen on hyperglycaemia-induced TNF-α secretion by M1 macrophages could be mediated through suppression of the NF-κB signalling pathway. It is worth noting that while multiple studies have investigated the effect of estrogen on LPS-induced NF-κB activation, very few have examined its impact on hyperglycaemia-induced NF-κB signalling, highlighting a gap in current knowledge in the context of hyperglycaemia that is likely to have implications for DFUs and DFIs. The activation of ERα by estrogen led to decreased nuclear translocation of the NF-κB p65 subunit (Section 5.4.2), reversing the hyperactivation of NF-κB signalling observed under hyperglycaemic conditions (Section 3.4.7). This finding is consistent with studies demonstrating that estrogen can inhibit NF-κB signalling in various cell types (Kalaitzidis and Gilmore, 2005), reportedly through coordinated regulation of the NF-κB inhibitor, IκB-Ras2, via modulation of specific microRNAs, including let-7a and miR-125b (Straub, 2007; Murphy et al., 2010). However, it is important to consider that hyperglycaemia is known to activate multiple signalling cascades beyond NF-κB, including MAPK, PI3K/Akt and TLRs pathways, all of which can also regulate cytokine production (Dasu et al., 2008; Jialal and Kaur, 2012; Mirza et al., 2020). Thus, the wider contribution from these other pathways would also need to be evaluated to gain a full understanding of hyperglycaemia-induced production of TNF-α by M1 macrophages. Additionally, estrogen-induced changes in the expression of microRNAs may also potentially affect multiple signalling pathways, not just the NF-κB axis (Pérez-Cremades et al., 2018; McKiernan et al., 2020; Khayati et al., 2023).

Further investigations focused on the influence of estrogen on modulating the expression of key receptor proteins or ligands involved in pathogen recognition and phagocytosis, including CD14, TREM2 and ApoE. Results showed that hyperglycaemia upregulates the expression of both mCD14 and sCD14 in M1 macrophages, whereas estrogen treatment significantly decreased the levels of both mCD14 and sCD14 in M1 macrophages, independent of glycaemic status

(Section 4.4.3.1). These findings suggest downregulation of CD14 might reverse the effects of hyperglycaemia and mediate the estrogen-induced enhancement of biofilm clearance. The estrogen-induced suppression of NF-κB activation is likely to contribute to the downregulation of CD14 given NF-κB has been shown to negatively regulate CD14 expression (Li et al., 2013; da Silva et al., 2017; Jiang et al., 2019). In addition, (Yu et al., 2021) found that the downregulation of mRNA and protein levels of CD14 inhibits the NF-κB pathway by decreasing the total p65 expression level and preventing the phosphorylation of p65 in intestinal epithelial cells. The repression the CD14/ NF-κB pathways arising from this feedback loop aligns with the dual modulatory effects of estrogen confirmed in this study, notably the concurrent inhibition of hyperglycaemia-induced NF-κB activation and decreased CD14 expression.

Results demonstrated that hyperglycaemia had an inhibitory effect on levels of mTREM2 and sTREM2, as well as its ApoE ligand (Section 3.4.8.3), in M1 macrophages. In contrast, estrogen treatment could reverse this effect, significantly increasing levels of mTREM2, sTREM2 and ApoE in M1 macrophages. This finding suggests the TREM2/ApoE pathway might also mediate the beneficial effects of estrogen on biofilm clearance under hyperglycaemic conditions. This is supported by several previous studies highlighting the essential role of TREM2 in regulating phagocytic responses. For example, Yang et al. (2021) reported that TREM2 overexpression played a pivotal role in modulating macrophage responses to *E. coli* infection by enhancing phagocytosis, regulating ROS production, and inhibiting inflammasome-mediated inflammatory responses and cell death pathways, Similarly, N'Diaye et al. (2009) demonstrated that TREM2 signalling is required for the efficient phagocytosis of various bacterial species, including *E. coli*, *P. aeruginosa*, and *F. tularensis*, by professional phagocytic macrophages. Moreover, they showed the process was dependent on the activity of Src kinase, the phosphorylation of the ITAM domain of DAP12 at tyrosine residues, Syk kinase, and the involvement of the small GTPases Rac1 and Cdc42 (N'Diaye et al., 2009). Importantly, previous research by the Ashworth group (El Mohtadi (2019) have observed that estrogen increases the protein levels of the small GTPases Rac1 and

Cdc42. This suggests that estrogen may enhance phagocytosis through the TREM2-mediated activation of these key signalling molecules, thereby involved in the regulation and organisation of actin cytoskeleton essential for the phagocytic process (Swanson and Hoppe, 2004). These findings collectively indicate that estrogen upregulates the TREM2/ApoE immunomodulatory axis, which is disrupted by chronic hyperglycaemic environments. The TREM2/ApoE signalling pathway has been shown to inhibit pro-inflammatory cytokine secretion and NF-κB activation, whilst enhancing phagocytosis and associated signalling cascades, including SYK/PI3K/AKT pathways (Hsieh et al., 2009; Yao et al., 2019; K. Peng et al., 2022).

This study also uncovered a potential regulatory role for the immunoinhibitory receptor CD33 in M1 macrophage responses. Knockout of CD33 enhanced TNF-α production and the phagocytic capacity of MRSA and *P. aeruginosa* bacteria by M1 macrophages, particularly under hyperglycaemic conditions (Section 6.4.1 and 6.4.2), indicating that CD33 plays a role in modulating inflammatory responses in M1 macrophages, with its absence leading to heightened inflammation and enhanced bacterial clearance. Further investigations revealed that CD33 knockout led to reduced expression of TREM2 and ApoE in M1 macrophages, suggesting a regulatory role of CD33 in maintaining TREM2 and ApoE levels, which in turn are known to modulate inflammatory responses and phagocytosis. Interestingly, estrogen treatment in CD33 knockout M1 macrophages had a anti-inflammatory effect, enhancing the clearance of bacterial pathogens and reducing TNF-α secretion, across all glycaemic conditions tested. Furthermore, estrogen supplementation upregulated TREM2 and ApoE expression levels in CD33 knockout M1 macrophages. Notably, estrogen treatment did not alter the protein expression of mCD33 or sCD33, suggesting that estrogen's regulation of TREM2 and ApoE is not mediated through altered CD33 levels.

Collectively these findings indicate that estrogen signalling can finely tune an interconnected, glycaemic-dependent CD33/TREM2-ApoE axis. This is consistent with the broader understanding that estrogen can exert its protective effects through multiple pathways and signalling cascades, not limited to a single receptor or axis (Villa et al., 2015; Villa et al., 2016; Pepe et al., 2017; Dama et al., 2021).

Confocal microscopy revealed that under euglycemic conditions, CD33 and TREM2 exhibited moderate co-localization on the surface of M1 macrophages, this co-localization was reduced under hyperglycaemic conditions with more diffuse distribution of the markers. Estrogen treatment visibly restored the co-localization of CD33 and TREM2 at the cell surface of M1 macrophages cultured under both euglycemic and hyperglycaemic conditions. suggesting potential crosstalk between the signalling pathways of these two receptors. These findings build upon the existing research that has implicated CD33 as an immunoinhibitory receptor that negatively regulating the TREM2/ApoE signalling and macrophage phagocytosis (Bhattacherjee et al., 2019; Bhattacherjee et al., 2021; Butler et al., 2021; Wißfeld et al., 2021). The insights gained from the complex interplay between estrogen signalling and the CD33/TREM2-ApoE axis will contribute to a more comprehensive understanding of the mechanisms underlying macrophage dysregulation in diabetic complications.

The interplay between estrogen and the CD33-TREM2/ApoE signalling pathways highlights the potential for targeted therapeutic approaches that could focus on manipulating TREM2-ApoE and/or CD33 signalling to promote phagocytosis and wound healing in DFUs. Interestingly, CD33 levels are known to decrease in diabetic patients (Gonzalez et al., 2012), raising the question of whether this is part of the pathology or just a compensatory response. Counterintuitively, increasing local CD33 expression in wound macrophages could be a potential therapeutic approach. While an increase in CD33 is expected to reduce phagocytosis (Butler et al., 2021), the subsequent increase in TREM2/ApoE expression might be more than sufficient to compensate for this initial negative effect, leading to enhanced phagocytosis overall. Furthermore, given an increase in both CD33 and TREM2/ApoE levels are known to reduce inflammation (Gonzalez et al., 2012; Zhong et al., 2017), concomitantly increasing their local expression would dampen excessive hyperglycaemia-induced macrophage release of pro-inflammatory mediators such as TNF-α. Furthermore, increasing local CD33 expression would also align (rather than oppose) with the beneficial effects of local estrogen supplementation or ER-α activation, with a combined therapy expected to lead to upregulation of

TREM2/ApoE levels in M1 macrophages and subsequent dampening of inflammation and promotion of bacterial clearance by M1 macrophages.

In summary, the findings of this thesis give an insight into the combined detrimental impacts of hyperglycaemia and the age-related decline in estrogen on the CD14/ NF-κB and CD33/TREM2-ApoE axes and subsequent impaired immune responses. Results consistently indicate estrogen acts through ERα to mitigate the detrimental effects of hyperglycaemia on macrophage function by promoting TREM2-ApoE signalling and blocking CD14/ NF-κB signalling to enhance the phagocytic capacity of M1 macrophages and dampen inflammatory cytokine production. Taken together, the investigations lay the foundation for the development of novel, multifaceted therapeutic interventions that can harness the beneficial effects of local estrogen supplementation to resolve DFIs and promote healing in DFU patients. Localised application of estrogen/ ERα agonists to DFUs may be a promising therapeutic strategy for diabetic patients that have developed a DFI. Moreover, the use of TREM2 agonists and localised upregulation of TREM2 expression might provide a more targeted approach for the treatment of patients with a DFI, thereby avoiding the potential negative hyperproliferative effects of estrogen. Alternatively, reducing CD14 expression or blocking the CD14/ NF-κB pathway in tissue macrophages within a DFU might be another targeted approach for the treatment of patients with a DFI. More widely, the findings also highlight the importance of addressing the underlying metabolic dysregulation in diabetic patients, ensuring they comply with prescribed medications (hypoglycaemic agents) to maintain glycaemic control. Reversing the detrimental effects of hyperglycaemia through careful glycaemic control in diabetic patients is an important complementary strategy to improve wound healing and reduce infection susceptibility.

7.2 Future Work

The insights gained from this thesis, elucidating the combined impacts of hyperglycaemia and age-related estrogen decline on macrophage function, phagocytosis and inflammatory signalling pathways, open up several promising avenues for future research aimed at developing novel therapeutic strategies to improve wound healing and infection resolution in diabetic patients.

- One key aspect to further investigate if there is any relationship and crosstalk between CD14 and the CD33/TREM2-ApoE axis in regulating M1 macrophage function under diabetic conditions. The results in chapter 5 demonstrated estrogen-mediated enhancement of phagocytosis was dependent on CD14. For instance, examining how CD33 knockout affects the expression and signalling of CD14 would be of interest. Could responses induced by CD33 lead to compensatory changes in CD14-mediated pathways? Conversely, investigating whether CD14 modulation might influence CD33 expression and downstream signalling could help elucidate the intricate balance between these pathways and their overall on bacterial clearance and cytokine production by M1 macrophages.
- Further investigations to elucidate the molecular mechanisms underlying the CD33/TREM2-ApoE axis, and how it is influenced by hyperglycaemic environments, could reveal novel therapeutic targets for enhancing bacterial clearance and resolving inflammation in DFUs. For instance, does CD33 directly influence the transcriptional or post-translational regulation of TREM2 and ApoE, and how does estrogen signalling intersect with this regulatory mechanism? Are there intermediate mediators that regulate the effects of CD33 on TREM2-ApoE, and if so are they modulated by estrogen?
- In addition to interrogating the underlying molecular mechanisms and crosstalk between estrogen/ ERα, CD33, TREM2 and CD14/ NF-κB in more detail, future work could investigate combination strategies involving simultaneous dual/triple manipulation of these pathways to determine

potential treatments that might magnify the beneficial effects on bacterial clearance and/or inflammation.

- Hyperglycaemia induced structural changes in the actin cytoskeleton of M1 macrophages. The phagocytosis induced by TREM2 involves GTPases Rac1 and Cdc42 (N'Diaye et al., 2009). El Mohtadi (2019) showed estrogen increases levels of the small GTPases, Rac1 and Cdc42, in phagocytic M1 macrophages. Thus, further investigations could be performed to elucidate the effect of estrogen on the actin cytoskeleton and key regulators of phagocytosis in relation to TREM2 signalling to reveal novel downstream targets for therapeutic intervention.
- The findings of this study were derived from *in vitro* host-pathogen interaction assays using U937-derived M1 macrophage models. The investigations could be repeated using additional macrophage cell lines or *ex vivo* macrophages isolated from DFU patients. The beneficial effects of estrogen and the involvement of specific pathways or mediators could also be confirmed *in vivo* using a diabetic murine model.
- The clinical relevance of observed relationships could be confirmed by analysing clinical samples from patients with DFUs. Use biological replicates to assess how factors such as age, sex, or diabetes duration might influence the observed effects of estrogen and hyperglycaemia on macrophage function. The expression/activation status of CD14/ NF-κB, CD33, TREM2/ ApoE, and other estrogen-regulated markers could be confirmed and correlated with disease progression, infection severity, glycaemic control and wound healing outcomes.
- Expanding on Technical Replicates: Throughout this thesis, we primarily used technical replicates in our model. While this approach provided high precision in our measurements, it also limits the generalizability of our findings to broader populations. Future work should prioritize the inclusion of biological replicates to address this limitation.

- Future work could include investigating the wider influence of hyperglycaemia and estrogen on other receptors and signalling molecules (e.g. TLRs, MAPK, PI3K/Akt) or inflammatory cells (e.g. neutrophils) involved in immune responses to build a broader picture of the immunomodulatory effects of estrogen under diabetic conditions.

Conclusion

This comprehensive set of investigations has made a substantial contribution to the understanding of the protective role of estrogen in regulating macrophage-mediated clearance of bacterial pathogens and dampening excessive inflammation in the context of diabetic and age-related wound complications. The findings demonstrate that estrogen acts through ERα to mitigate the detrimental effects of hyperglycaemia on macrophage function. Estrogen negates the effect of hyperglycaemia, promoting TREM2-ApoE and blocking CD14/ NF-κB signalling to enhance the phagocytic capacity of M1 macrophages and dampen inflammatory cytokine production.

Since estrogen levels decline rapidly with ageing, the increased propensity for chronic wound infections in the elderly may be directly attributed to impaired macrophage function arising from a loss of protection afforded by estrogen during youth. Collectively, the findings of this thesis suggest the anti-inflammatory effects of estrogen, combined with its ability to promote bacterial clearance, may collectively translate to improved immune function and enhanced wound healing in DFUs. Thus, localised application of estrogen/ ERα agonists to DFUs may be a promising therapeutic strategy for diabetic patients that have developed a DFI. Moreover, the findings suggest the use of TREM2 agonists (like ApoE) and localised upregulation of TREM2 and/or CD33 expression might provide a more targeted approach for the treatment of patients with a DFI, thereby avoiding the potential negative hyperproliferative effects of estrogen on non-target tissues. Alternatively, reducing CD14 expression or blocking the CD14/ NF-κB pathway in tissue macrophages within a DFU might be another targeted approach for the treatment of patients with a DFI. More widely, the findings highlight the importance of

addressing the underlying metabolic dysregulation through the use and careful patient compliance with medications (hypoglycaemic agents) to ensure glycaemic control in diabetic patients. Reversing the detrimental effects of hyperglycaemia through careful glycaemic control in diabetic patients may be a crucial complementary strategy to improve wound healing and reduce infection susceptibility.

In conclusion, the data presented in this thesis indicate that directly or indirectly restoring estrogen signalling could be a game-changing approach to transform the clinical management and treatment of DFIs, not only to enhance bacterial clearance but also to resolve the chronic inflammation associated with diabetic and age-related wound healing.

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