ANDROGENS INHIBIT PHAGOCYTOSIS BY MACROPHAGES VIA THE ANDROGEN RECEPTOR *IN VITRO*

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List of abbreviations

Ab	Antibiotic
AKT	Protein kinase B
APC	Antigen presenting cell
AR	Androgen receptor
ARE	Androgen response element
B alone	Bacteria alone control
BSA	Bovine serum albumin
cAMP	Cyclic adenylyl cyclase
CD11c	Cluster of differentiation 11c
CD14	Cluster of differentiation 14
CYP	Cyproterone acetate
C3a	Complement 3a
C3b	Complement 3b
C5a	Complement 5a
DBD	DNA-binding domain
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone-sulphate
DHT	Dihydrotestosterone
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*

ECM	Extracellular matrix
EDTA	Ethylenediamine tetra-acetic acid
EGF	Epidermal growth factor
ER	Estrogen receptor
FAK	Focal adhesion kinase
FBS	Foetal bovine serum
FcɣR	Fc gamma receptor
FGF	Fibroblast growth factor
FIN	Finasteride
FLU	Flutamide
HSPs	Heat shock proteins
IFN-β	Interferon-*beta*
IFN-ɣ	Interferon-*gamma*
IgG	Immunoglobulin type G
IL-1	Interleukin-1
IL-1R	Interleukin-1 receptor
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-12	Interleukin-12
IL-17	Interleukin-17
LBD	Ligand binding domain
LPS	Lipopolysaccharide

M	Molar
MAPKs	Mitogen-activated protein kinases
MFI	Median fluorescence intensity
mRNA	Messenger ribonucleic acid
MRSA	Methicillin resistant *Staphylococcus aureus*
M1	Macrophage type 1
M2	Macrophage type 2
NC	Negative control
NFκB	Nuclear factor kappa B
NK	Natural killer cell
NTD	N-terminal domain
P. aeruginosa	*Pseudomonas aeruginosa*
PAMPs	Pathogen associated molecular patterns
PBP2a	Penicillin binding protein 2a
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PI3K	Phosphoinositide-3 kinase
PMA	Phorbol myristate acetate
PRR	Pathogen recognition receptor
Rho	Rho GTPase
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute

S. aureus	*Staphylococcus aureus*
SEM	Scanning electron microscopy
STAT	Signal transducer and activator of transcription
T	Testosterone
T_h1	T helper 1 cell
TIR	Toll-interleukin receptor
TLR	Toll like receptor
TLR-4	Toll like receptor 4
TNF	Tumor necrosis factor
TNF-α	Tumor Necrosis Factor-alpha
TGF-β	Transforming growth factor-beta
3β-HSD	3*beta*-hydroxysteroid dehydrogenase
4-DIONE	4-Androstenedione
5α-R	5-*alpha* reductase
5-DIOL	5-Androstenediol
17β-HSD	17*beta*-hydroxysteroid dehydrogenase

Abstract

The cost to healthcare services for the treatment of chronic wounds exceeds $3 billion per year (Menke *et al*, 2007). Chronic wounds are frequently colonised by nosocomial opportunistic pathogens such as Methicillin-resistant *Staphylococcus aureus* (MRSA). Endogenous androgens are known to contribute to delayed healing whilst in contrast, estrogen accelerates healing. Healing in the elderly is impaired, particularly in elderly males due to the concomitant decline in estrogen levels yet largely unchanged levels of circulating androgens. This is predominantly characterised by a prolonged inflammatory phase during wound repair.

In this study a host-pathogen interaction assay was used to investigate the effect of the two main endogenous androgens, testosterone (T) and dihydrotestosterone (DHT), on the clearance of MRSA by macrophages derived from U937 monocytes. Concentrations of T and DHT were chosen to model adult supraphysiological levels following exogenous supplementation (1×10^{-6} M), physiological levels (1×10^{-7} M, 1×10^{-8} M) and androgen deprivation (1×10^{-9} M). The involvement of androgen receptor (AR) activation in bacterial clearance was explored using AR pathway inhibitors.

Supraphysiological and physiological levels of androgens typical of that in adulthood significantly ($P<0.05$; $n=24$) inhibited phagocytosis of MRSA in a concentration-dependent manner compared to the untreated negative control. Scanning electron microscopy (SEM) confirmed androgens inhibit bacterial internalisation by U937 macrophages. AR antagonism reversed the effects of T and DHT, significantly ($P<0.05$) enhancing phagocytosis to levels observed in the untreated negative control.

These findings suggest androgens inhibit the resolution of typical wound bacteria by impairing macrophage-mediated phagocytosis, a phenomenon that can be reversed via direct AR antagonism. More importantly, the 5alpha-reductase inhibitor finasteride significantly ($P<0.05$; $n=24$) increased the clearance of MRSA, suggesting

the inhibition of phagocytosis by T is mediated principally through initial conversion of T to DHT, followed by AR activation by DHT. Novel wound dressings that provide local blockade of the AR or inhibition of 5alpha-reductase may be potential therapeutic treatments to promote the bacterial clearance of colonised wounds in elderly males.

1.0 Introduction

1.1 Acute Inflammation

Inflammation involves the activation of pro-inflammatory cytokine cascades to promote the migration of immune cells to the site of trauma, with the aim to remove pathogens and debris and promote wound healing. The innate immune system is activated immediately following wounding, initiating a local inflammatory response where local mast cells undergo degranulation (Koh and DiPietro, 2011). This in turn promotes pro-inflammatory cytokine release. Furthermore, it is well documented that wounds are commonly hypoxic which in turn stimulates the recruitment of further leukocytes to the site of injury (Sen, 2009). As neutrophils are the most abundant white cell type in circulation, they are the first inflammatory cells to be recruited to the wound. Neutrophils initiate a positive feedback loop by their release of pro-inflammatory cytokines, promoting monocyte recruitment and differentiation into tissue macrophages (Koh and DiPietro, 2011).

Macrophages have the critical pro-inflammatory function of phagocytosis in order to engulf and destroy invading pathogens or debris. Pattern recognition receptors (PRR) on the macrophage cell surface recognise and bind to pathogen–associated molecular patterns (PAMPs) on the target pathogen membrane (Aderem, 2003). For example, scavenger receptors can recognise surface components such as lipopolysaccharide (LPS) on the bacterial membrane (Underhill and Ozinsky, 2002). Particle internalisation involves transformation of the actin cytoskeleton inside macrophages to allow extensions of its plasma membrane called lamellipodia to surround and engulf the pathogen. Protein kinase C and phosphoinositide kinase expressed by macrophages aids lamellipodial formations at the site of attachment (Aderem, 2003). This in turn activates transcription of genes involved in antimicrobial mechanisms such as toxic peptide production that assists in the killing and digestion of internalised pathogens (Aderem, 2003). The inflammatory response is self-limiting and once the infection has cleared, inflammation resolves as acute wound healing progresses (Aderem, 2003).

Unlike acute inflammation, chronic inflammation is not beneficial to the host and ultimately leads to tissue degeneration. Chronic inflammation is characterise as persistent low grade inflammation and can be a result of the following two factors: (i) persistent infection leading to unregulated activation of pro-inflammatory cytokines and signalling pathways such as Nuclear factor kappa B (NFκB) and (ii) dysregulation of anti-inflammatory cytokines aimed to resolve inflammation such as Interleukin-17 (IL-17) (Franceschi and Campisi, 2014).

1.2 Macrophage Biology

Macrophages are established during embryogenesis and take on multiple phenotypical functions in response to a diverse environment during wound healing. They are predominantly responsible for host defence, but also carry out functions such as inflammation control, growth factor production, cell proliferation and tissue restoration (Snyder *et al*, 2016).

Macrophages act independently of the adaptive immune system with remarkable plasticity (Kono and Rock, 2008). Their main function is to clear the interstitial environment of extraneous cellular material (Mosser and Edwards, 2008) through the mechanism of phagocytosis, which was discovered over 100 years ago by Nobel Prize winner Élie Metchnikoff (Nathan, 2008). Macrophages detect danger signals through their toll-like receptors (TLRs) and interleukin-1 receptor (IL-1R), that signal through the adaptor molecule called myeloid differentiation primary-response gene 88 (Mosser and Edwards, 2008).

Additional metabolic functions of macrophages include clearance of approximately 2×10^{11} erythrocytes daily to recycle haemoglobin. Macrophages also play a role in other aspects of immunity such as the adaptive branch, where they act as antigen presenting cells (APC) to help resolve inflammation (Duque and Descoteaux, 2014).

Macrophages are subject to change in their phenotype in response to different stimulants. The macrophage type 1 (M1) subtype promotes pro-inflammatory cytokine release. In comparison, macrophage type 2 (M2) subtype acts in an anti-

inflammatory manner to promote inflammation resolution and tissue repair (Mills, 2012).

1.3 Classically Activated Macrophages

Interferon-gamma (IFN-ɣ) is the most predominant cytokine to activate a classical M1 macrophage (Figure 1). This initiates the release of pro-inflammatory cytokines from the macrophage resulting in increased superoxide anions, oxygen and nitrogen to enhance mycobacterial or tumoricidal capacities during phagocytosis (Dale *et al*, 2008). Macrophages can be activated by IFN-ɣ alone as some TLR ligands such as LPS can also activate Toll-interleukin receptor (TIR) domain-containing adaptor protein, resulting in Interleukin beta (IFNβ) production as an alternative to TNF-α (Yamamoto *et al*, 2003). Downstream genetic transcription pathways initiated during activation include signal transducer and activator of transcription (STAT) molecules following IFN-ɣ receptor ligation, nuclear factor-κB (NFκB) and mitogen-activated protein kinases (MAPKs), which are triggered in response to TLR or TNF receptor ligation (O'Shea and Murray, 2008). In contrast, M2 macrophages are stimulated by interlukin-4 (IL-4) to promote wound repair (Sica and Mantovani, 2012).

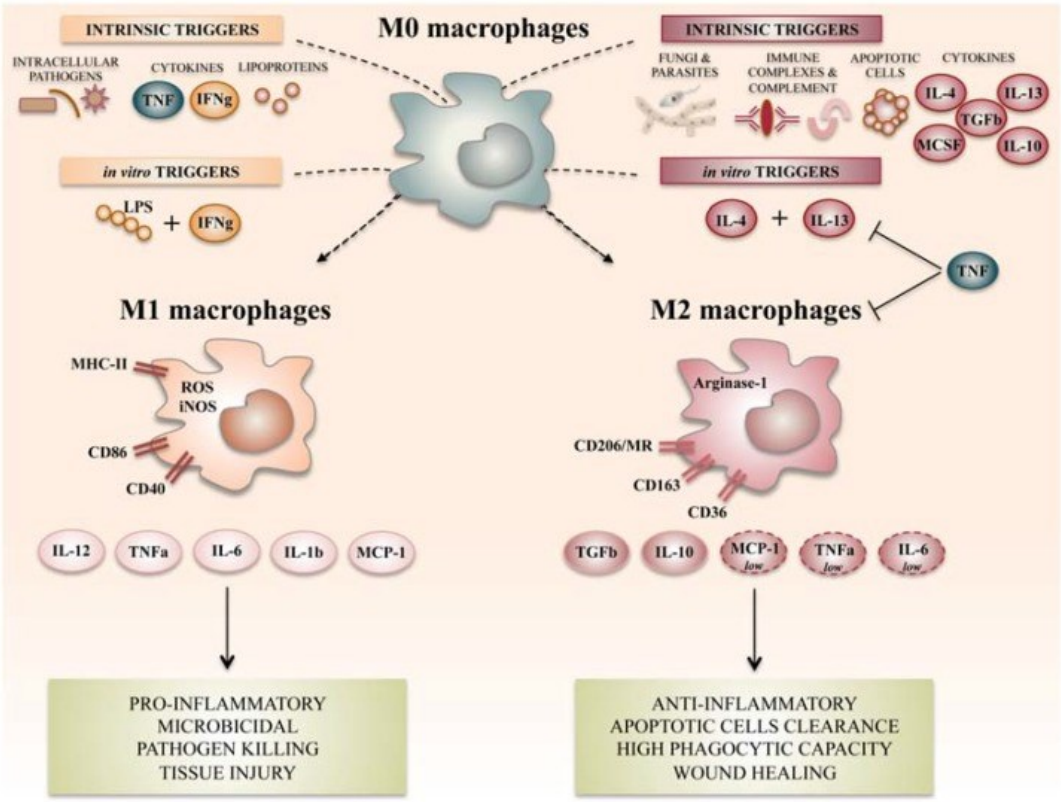


Figure 1: Activation of a Classical macrophage (M1). M0 macrophages are activated by stimuli such as IFN-ɣ and intracellular pathogens. Downstream pathways are initiated through receptor activation including CD40 and CD86. As a result, proinflammatory cytokines are secreted such as IL-6 and TNF-α to promote pathogen elimination (Atri *et al*, 2018).

1.4 Acute Wound Healing

Following injury, restoration of the skin is crucial for infection prevention during cutaneous wound healing. It includes 4 key stages; coagulation, inflammation, proliferation and remodelling. The end goal of the wound healing process is the formation of the mature scar tissue at the site of injury (Lorenz and Longaker, 2008).

The first phase is coagulation which begins immediately after trauma. This phase is characterised by vascular contraction, platelet aggregation and the formation of a thrombus due to activation of the coagulation and complement cascades. This reduces immediate blood loss by acting as a haemostatic plug (Lorenz and Longaker, 2008). The surrounding wound tissue along with the newly formed thrombus release

pro-inflammatory cytokines, growth factors and potent chemoattractants such as transforming growth factor (TGF)-β, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and epidermal growth factor (EGF) that promote the progression of acute wound repair (Guo and DiPietro, 2010).

The inflammatory phase is characterised by inflammatory cell recruitment (chemotaxis) to the wound site (Campos *et al*, 2008). Anaphylatoxins C3a and C5a, activated via the complement cascade, attract primary immune cells directly from the blood stream (Lorenz and Longaker, 2008).

The primary role of inflammation during wound healing is the clearance of pathogens or cellular debris (Guo and DiPietro, 2010). Neutrophil populations peak 24-36 hours after injury whereas monocytes peak at around 7 days post-wounding (Lorenz and Longaker, 2008). Macrophages carry out phagocytosis and release cytokines that promote further recruitment and activation of leukocytes. Upon clearance of apoptotic cells, macrophage populations undergo a phenotypic change to a reparative state that stimulates initiation of the proliferation phase (Mosser and Edwards, 2008).

The proliferation phase begins around 96 hours post injury and is characterised by re-epithelization, angiogenesis and extracellular matrix formation (Guo and DiPietro, 2010 & Lorenz and Longaker, 2008). This phase involves formation of extracellular matrix (ECM) by fibroblasts, angiogenesis by endothelial cells and re-epithelialisation by keratinocytes (Guo and DiPietro, 2010; Maquart and Monboisse, 2014; Li *et al* 2007).

Finally, after immune cell populations decline the remodelling phase is characterised by collagen remodelling and vascular maturation/ regression of newly formed capillaries, allowing vascular density to return to normal within the wound. This phase can last over a period of months to years and results in a mature scar (Guo and DiPietro, 2010).

1.5 Impaired Wound Healing

Wound healing becomes impaired with increasing age, leading to prolonged healing and excessive inflammation (Gilliver *et al,* 2007). Chronic wounds are characterised by failure of the skin to regain its anatomic and functional integrity after one month of initial trauma (Clinton and Carter, 2015). Non-healing wounds include diabetic foot ulcers, venous ulcers and arterial ulcers (Harding *et al*, 2002). Non-healing wounds affect about 3 to 6 million people in the United States, with the elderly (+ 65 years) accounting for 85% of cases (Menke *et al*, 2007). Furthermore, chronic wounds give rise to vast health care expenditures, with total costs estimated at more than $3 billion per year (Menke *et al*, 2007). In the UK, chronic wounds cost the NHS around £1 Billion annually, £17 million of which is associated with patients with diabetic foot ulceration (Harding *et al*, 2002). With an aging population and type 2 diabetes on the rise, the costs to healthcare providers is expected to rise substantially. As a consequence, there is an urgency to better understand the pathology of chronic wounds and develop novel therapies.

1.6 Biofilms in Wounds

A biofilm is a structured ecosystem of bacteria surrounded by an exo-polysaccharide layer that aids the survival and adherence of bacteria to the wound bed (Clinton and Carter, 2015). Wounds that have biofilms are the most complicated to treat due to their polymicrobial nature, meaning traditional antibiotics combinations fail to succeed in biofilm clearance (Clinton and Carter, 2015). Biofilms affect 60% of chronic wounds compared to 10% of acute wounds, making wound infection one of the largest factors that prolong the healing of chronic wounds (James *et al*, 2008).

There is increased transfer of antimicrobial genes within biofilms compared to normal bacterial colonisation; such genes can be transferred through those of the same and different species. This dramatically increases the virulence of the biofilm (James *et al*, 2008). Furthermore, the unique metabolism within the biofilm also allows for antimicrobial tolerance, meaning the biofilm can regenerate itself post antibiotic therapy at a stronger tolerability level (Fauvart *et al*, 2011). Once a biofilm is established, it is hard to eliminate and leads to a higher rate of persistent and

reoccurring chronic wounds. Pathogens and debris from a biofilm can elevate pro-inflammatory cytokine levels such as interleukin-1 (IL-1) and TNF-α, resulting in a prolonged inflammatory phase and tissue destruction (Guo and DiPietro, 2010).

1.7 MRSA and Wound Infection

Methicillin resistant *Staphylococcus aureus (*MRSA) is an opportunistic pathogen that asymptomatically colonises humans (Sisirak *et al*, 2010). MRSA is the most common cause for nosocomial infection worldwide (National Nosocomial Infections Surveillance System 2004). Fast spread of infection is associated with overcrowding in wards, a low nurse: patient ratio and high average patient stays. Post-surgical site infections account for 16% of nosocomial infections and up to 40% in developing countries, making them the third most common cause of nosocomial infection spread (Sisirak *et al*, 2010). MRSA is one of many bacterial species associated with biofilm formation, specifically in wound infections and implanted-devices (Côrtes *et al* 2015).

1.8 Global Burden of Antimicrobial Resistance

The 1940s saw the introduction of the 'wonder drug' penicillin in conjunction with chemotherapy to reduce the burden of *Staphylococcus aureus (S.aureus)* infections. It was not long until resistant strains were isolated in the 1950s, caused by the enzyme penicillinase that degrades the antibiotic prior to its delivery to target tissue (Sisirak *et al*, 2010). Methicillin was a second line antibiotic to be introduced but resistance soon developed due to bacterial acquisition of the penicillin binding protein 2a (PBP2a). MRSA soon became resistant to all other antibiotics including vancomycin, with vancomycin resistant isolates found in the USA in 2002. Today, such resistance patterns make MRSA one of the hardest pathogens to treat, with high rates of morbidity and mortality in patients (Sisirak *et al*, 2010). This of course increases the pressure to find alternatives to antibiotics or to expand our knowledge of wound healing and infection to identify strategies to reduce the prevalence of MRSA infections and the spread of antimicrobial resistance.

1.9 Age-Related Impaired Wound Healing

There is increasing evidence suggesting aging has a negative impact on wound healing processes. Studies have found aging to have damaging implications on the inflammatory phase of wound healing, such as prolonged inflammation and increased protease activity (Gilliver *et al*, 2007). Other studies observed delayed re-epithelization and angiogenesis in aged mice compared to young, demonstrating negative effects on the latter stages of wound healing such as the proliferative phase (Swift *et al*, 2001). Thus, aging poses threats to the host in terms of increased risk of wound infection.

Further studies have measured the phagocytic abilities of macrophages in a model of wound healing using latex beads and sheep erythrocytes (Swift *et al*, 2001). They found 27% fewer macrophages from aged mice were able to carry out efficient phagocytosis and those that were phagocytic consumed 37% fewer latex beads compared to the young mice control. When replicated using erythrocytes, 22% fewer macrophages from aged mice were phagocytic. A 43% decrease in the number of erythrocytes ingested per macrophage was observed. Furthermore, this study found there was no significant difference in the volume of receptors on the macrophage surface, suggesting the decline in phagocytic ability is not due to a decline in receptor sites, but a decline in macrophage capabilities through other mechanisms (Swift *et al*, 2001)**.** This study confirmed functional changes in phagocytic cells such as macrophages during aging, thereby prolonging wound healing and increasing host susceptibility to wound infection. With an aging population, these findings highlight the need to expand our knowledge of mechanisms driving age-related impaired healing.

1.10 Sex Differences in Wound Healing

It has been documented that elderly females heal more quickly compared to elderly males, which sparks speculation that sex hormones such as androgens may have negative influence on wound healing processes (Ashcroft *et al*, 1999). Speculation first came about when healing of thick incisional wounds was accelerated in castrated mice compared to non-castrated, aged matched controls suggesting male

hormones such as testosterone increase inflammatory profiles and delay healing independent to aging (Ashcroft and Mills, 2002).

Ashcroft & Mills found a positive correlation between testosterone (T) and wound size in elderly humans (Ashcroft and Mills, 2002). This was in agreement with murine studies that showed a significant delay in murine wound repair, characterised by an enhanced inflammatory response and reduced matrix deposition associated with higher levels of Testosterone (T). In contrast, castrated murine subjects had a lower inflammatory profile and increased collagen disposition with evidence of accelerated wound repair (Ashcroft and Mills, 2002). Collectively, the findings demonstrated androgens are negative regulators of the healing process by increasing the inflammatory response.

In females, estrgoen acts as an anti-inflammtory agent that dampens ones inflammatory profile thereby accelerating the wound repair process (Ashcroft and Mills, 2002). During aging in both males and females humans, estrogen levels decline significantly but testosterone levels are relatively sustained in males. The combined effect of reduced estogen but sustained testsoterone is associated with a pronounced inflammtory profile in elderly human males (Ashcroft and Mills, 2002). This is in concordance with increased prevalance of chronic wounds in elderly males (Wicke *et al* 2009).

1.11 Biosynthesis of Testosterone and Dihydrotestosterone

Testosterone has 3 modes of action in peripheral tissues (Figure 2): conversion into Dihydrotestosterone (5–10%), direct action of T through the androgen receptor (AR) in target tissues or aromatization to estrogen (0.2%) with subsequent activity through the estrogen receptor (ER).

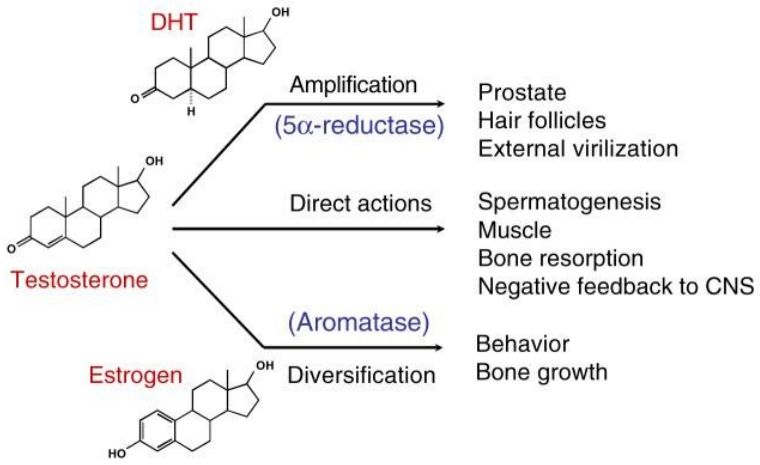


Figure 1: Three modes of action of Testosterone; conversion to Dihydrotestosterone, direct action on target tissues via androgen receptor (AR) and conversion to estrogen (Wenqing *et al*, 2007).

Dehydroepiandrosterone (DHEA) and dehydroepiandrosterone-sulphate (DHEAS) are endogenous steroid hormones released into the blood stream from the adrenal cortex in response to adrenocorticotropin secretion from the anterior pituitary gland (Gilliver *et al* 2003). It is important to note, DHEAS levels in adult males are 100-500 times higher than that of testosterone, proving vast opportunity for conversation into sex steroid hormones (Figure 3) such as active androgens (Labrie *et al*, 2000). DHEAS is converted to DHEA via the steroid sulphatase enzyme secreted from various cell types including macrophages (Gilliver and Wu and Ashcroft, 2003).

The enzyme 3beta-hydroxysteroid dehydrogenase (3β-HSD) converts DHEA to androstenedione (4-DIONE), which is secreted by the adrenal glands in vast quantities. The enzyme 17beta-hydroxysteroid dehydrogenase (17β-HSD) is responsible for converting 4-DIONE to testosterone. The enzyme 5-alpha reductase (5α-R) can additionally convert testosterone to its potent metabolite dihydrotestosterone (DHT). Additionally, a second route involves 17β-HSD in converting DHEA to androstenediol (5-DIOL) and the enzyme 3β-HSD finalising the conversion to testosterone (Gilliver *et al*, 2003).

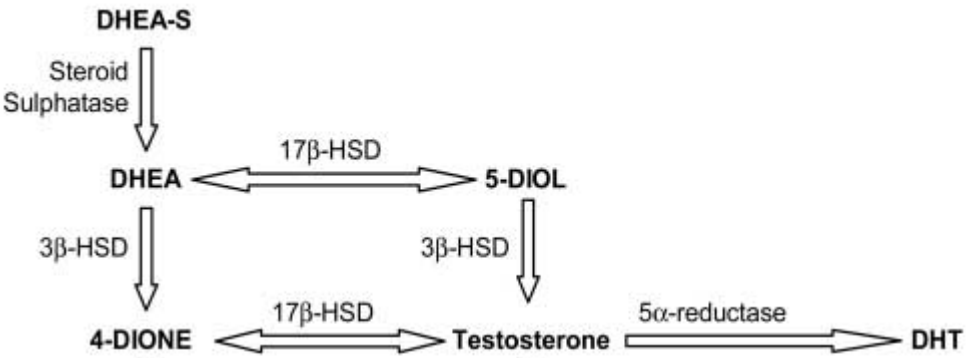


Figure 2: Pathways involved in the biosynthesis of active androgens from dehydroepiandrosterone-sulphate (DHEA-S) (Gilliver *et al,* 2003).

High activity of enzymes in peripheral tissues such as skin allows T and DHT to be localised (Figure 4), resulting in subsequent exposure of androgens to inflammatory cells such as macrophages during wound healing (Gilliver et al, 2003).

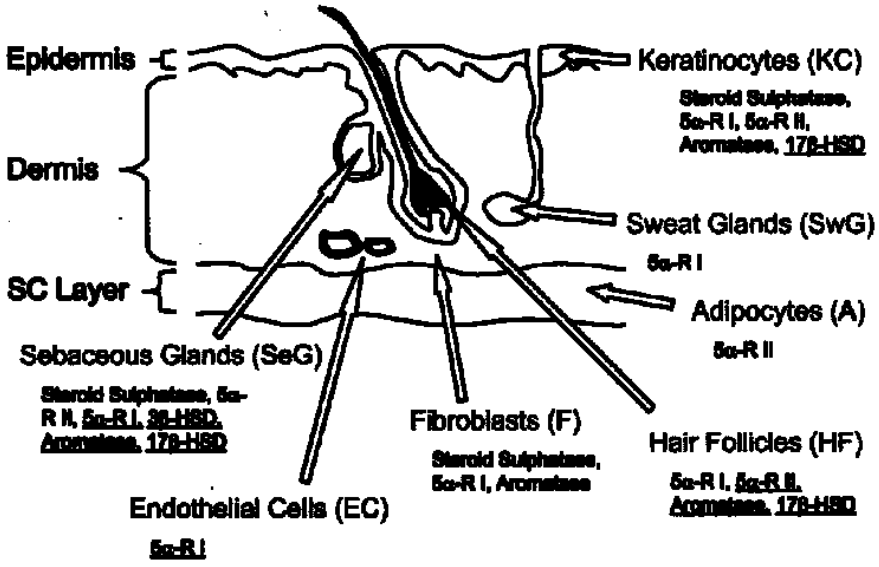


Figure 3: Location of steroid enzymes in the skin. Underlined enzymes represent high enzyme activity (Gilliver *et al,* 2003).

1.12 Androgen Receptor

The 90kb AR is a member of the nuclear receptor family that functions as an intracellular mediator of gene transcription. The structural arrangement of the AR (Figure 5) contains 3 domains; the N-terminal domain (NTD), DNA-binding domain (DBD), and ligand binding domain (LBD). Active androgens such as T and DHT are the most predominant natural steroidal agonists of the AR. These substrates bind to the LBD region that undergoes conformational changes to activate intracellular signalling, resulting in a biological response. The AR is present in many tissues including the skin and immune cells such as macrophages (Ashcroft and Mills, 2002). Such observations suggest androgen signalling in local tissues may be implicated in the pathophysiology of some androgen-dependent skin disorders (Chen *et al*, 1998).

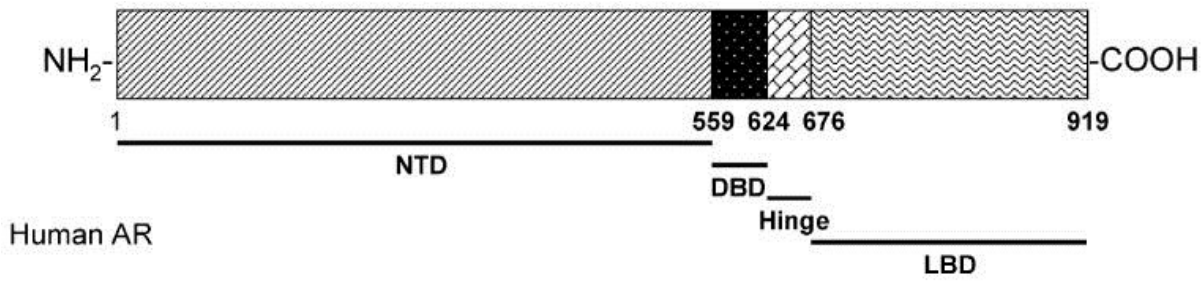


Figure 4**:** Structural arrangement of the human androgen receptor. NTD: N-terminal domain, DBD: DNA-binding domain, LBD: Ligand binding domain (Wenqing, Juhyun and James, 2007).

1.13 Androgen Signalling

Androgen receptors are located in the cell cytoplasm, complexed to heat shock proteins (HSP) (hsp 90, hsp 70, and hsp 56) during their inactive state (Gilliver, Wu and Ashcroft, 2003). DHT has higher affinity for the AR and maintains a more stable DHT-AR complex compared to that of T, making DHT more potent (Chen *et al*, 1998). Following binding of T/DHT to the AR, a phosphorylation reaction occurs resulting in dissociation of HSP and activation of the receptor (Gilliver *et al,* 2003). There are 2

alternate pathways that can occur post-AR activation: genomic signalling and non-genomic signalling (Gilliver *et al*, 2003).

- Genomic signalling:

Following heat shock protein dissociation, the ligand bound receptor dimerizes, and binds to a specific deoxyribonucleic acid (DNA) sequence along the androgen response element (ARE). Alternatively the dimer can interact with cofactors which assist with transcription regulation (Wilkenfeld *et al*, 2018).

- Non- genomic signalling:

Androgens can act as G-coupled-protein receptors on the membrane surface. Upon activation, a series of kinase cascades such as phosphoinositide-3 kinase (PI3K), Protein kinase B (AKT), and MAPK can activate the transcription of various genes in the nucleus (Wilkenfeld *et al,* 2018). Collectively, transcription and translation of AR dependant proteins can give rise to many cell physiological changes such as intracellular calcium influx, upregulation of growth factor receptors and actin skeleton changes (Wilkenfeld *et al,* 2018).

1.14 Pathophysiology of T and DHT

Testosterone is the principle male steroid hormone, predominantly made via Leydig cells in the testis. Testosterone is vital for male development of bone mass, muscle mass and spermatogenesis (Wenqing *et al,* 2007). Males have approximately ten times higher serum testosterone levels compared to females (Gilliver *et al,* 2003). Unlike estrogen, the hormone remains significantly bioactive throughout life however declines gradually with age (Ashcroft and Mills, 2002).

DHT is mainly synthesized in peripheral organs such as the skin, where type one and two 5α-reductase is abundant, lying resident in the cytoplasm of a variety cell types such as sebaceous glands, epidermis cells, sweat glands and hair follicles (Ashcroft and Mills, 2002).

Testosterone levels are reported to decline slightly after the age of 40 at a rate of approximately 0.4%/year in human males (Chen *et al*, 1998). This may be explained

by a decline in testicular function. However DHT, which has a 10-fold higher affinity for the AR and exists in the circulation at around 10% of testosterone levels, has been found to increase up to 3.5%/year relative to testosterone, although total androgen levels decline overall (Chen *et al*, 1998). This phenomenon may be explained in terms of increased 5α-reductase activity, and decreased catabolism of DHT (Feldman *et al*, 2002).

1.15 Effects of Androgens on Inflammatory Response

Studies have shown castration of male mice results in a dampened inflammatory response characterised by a reduction in TNF-α expression compared to intact control subjects (Gilliver *et al*, 2006). Castration but not 5α-reductase inhibition reduced TNF-α positive cell numbers at day 2 following murine post-wounding (Gilliver *et al*, 2006). This evidence suggests testosterone can enhance levels of TNF-α, an important pro-inflammatory mediator, independent of DHT (Ashcroft and Mills, 2002).

IL-6 is a key regulator of the inflammatory phase during wound healing. A significant decline in mRNA IL-6 expression was detected at day 2 post-wounding in murine model wound repair (Gilliver *et al*, 2006). Further investigations has shown testosterone reverses the effects of castration, significantly increasing IL-6 levels (Gilliver *et al*, 2006). These data supports evidence that testosterone alters wound cytokine levels and delays wound healing (Gilliver *et al*, 2006).

Previous studies have shown gender gives rise to differences in the immune system. Increased production of several pro-inflammatory cytokines, including Interleukin-12 (IL-12) and IL-1β, by LPS-stimulated monocytes was observed in *ex-vivo* monocytes from males compared to females following incubation with physiological concentrations of testosterone (Krummen *et al*, 2010). These data suggest that male gender, in addition to testosterone levels, may prolong the inflammatory phase of wound repair (Posma *et al*, 2004). Testosterone also decreases Toll like receptor 4 (TLR-4) expression in primary macrophages and macrophage cell lines, thereby impairing macrophage function in pathogen recognition (Rettew *et al*, 2008).

In vivo and *in vitro* experiments conducted in the late 1990s by Mondal and Rai showed that DHT suppresses phagocytosis in lizard splenic macrophages in a dose and time dependant manner (Mondal and Rai, 1999).

These data support growing evidence that sex steroid hormones are responsible for the differing inflammatory responses between genders. However, such phenomenon warrants further exploration using human cells/tissues and physiological concentrations of androgens to identify true biological effects and underlying mechanisms of action.

1.16 Androgen Inhibitors

Anti-androgens such as AR antagonists and 5α-reductase inhibitors are used to treat a variety of androgen dependent conditions from acne to prostate cancer (Wenqing *et al,* 2007). Two common AR antagonists are cyproterone acetate (CYP) and flutamide (FLU). The 5α-reductase inhibitor finasteride (FIN) is also used to block the conversion of T to DHT (Zhao *et al*, 2017). These various inhibitors are often used within experiments to investigate androgen-mediated responses *in vitro*.

In vitro experiments using CYP in a yeast model have reversed the effects of DHT treatment, increasing phagocytic activity of macrophages (Mondal and Rai, 1999). These data suggest DHT acts through the AR to bring about negative effects on macrophage phagocytosis. However, this response has not been explored in human macrophages with physiological concentrations of DHT to date.

Studies have shown that androgen treatment on guineapig splenic macrophages decreases the expression of Fc-gamma receptors (FcγR) 1&2, thereby decreasing their ability to recognise Immunoglobulin type G (IgG) sensitised complexes (Gomez *et al*, 2000). The authors showed that CYP reversed the findings, confirming the effect was mediated through the AR. However, CYP has not been explored in the context of human wound healing and the clearance of bacteria pathogens such as MRSA.

Flutamide is an acetanilide-derived agent first approved by the FDA in 1989 for locally confined metastatic prostate cancer (Haendler and Cleve, 2012). Flutamide is

a competitive antagonist of the AR which upon binding blocks androgen activity (Sarrabay *et al*, 2015). Flutamide is one of the desired anti-androgens for pharmacological use due to its high specificity for the AR and reduced cross reactivity with other ligands. Flutamide is commonly prescribed for prostate cancer since it is metabolised to 2-hydroxyflutamide *in vivo*, which has higher affinity and potency compared to flutamide itself (Wenqing *et al,* 2007).

In vivo wound healing experiments have shown that flutamide accelerates healing by direct dampening of the inflammatory response, characterised by reduced macrophage TNF-α messenger ribonucleic acid (mRNA) expression and NF-κB activation (Ashcroft and Mills, 2002).

Although flutamide mimics the effects of castration on wound healing, reducing inflammatory and accelerating wound healing, its effect on infection clearance in wounds has not been investigated (Ashcroft and Mills, 2002).

Finasteride blocks the enzyme 5α-reductase that converts T to DHT (Zhao *et al*, 2017), with murine wound healing studies showing it can reduce *in vivo* DHT levels threefold (Gilliver *et al*, 2006).

In vivo investigations of post-operative prostate wound healing has shown finasteride accelerates healing and directly dampens the inflammatory response, characterised by reduced TNF-α expression by M1 macrophages, especially within the first week (Zhao *et al*, 2017). Urine analysis showed IL-6, IL-12 and TNF-α were reduced during finasteride treatment compared to controls. Similarly, decreased TNF-α secretion was observed in AR knockout mice, suggesting the AR is responsible for DHT-induced TNF-α secretion by M1 macrophages. Flow cytometry revealed finasteride reduces the number of M1 macrophages in the wound area compared to controls. To conclude, these data suggest DHT acts through the AR in macrophages to mediate an increased inflammatory profile characterised by pronounced TNF-α, a phenomenon that can be reversed through exposure to finasteride.

A murine study by Gilliver *et al* (2006) shown 5α-reductase inhibition via MK-434 treatment resulted in a significantly decreased wound area and a considerable

decrease in inflammatory cells per unit area, mimicking the effects of castration. These findings confirm that DHT impairs murine wound healing, a phenomenon that can be reversed through castration or blockage of DHT formation.

1.17 Anti-Androgens as Therapeutic Strategies for Bacterial Clearance

To date anti-androgens have not been exploited as potential therapeutic strategies to enhance host immune responses and promote the clearance of bacteria. A deeper understanding of the mechanisms underlying androgen-mediated inhibition of phagocytosis could potentially lead to new therapeutic strategies for bacterial infections that promote host responses of the innate immune system, thereby reducing the over-reliance on traditional, first-line treatments such as antibiotics. Therapeutic success with anti-androgen therapy in the context of wound infections could ultimately decrease global mortality rates and annual healthcare expenditure on chronic wounds. Such treatments could help fight the emergence of antimicrobial resistance by reducing the need for widespread antibiotic usage and minimising the prevalence of problematic hospital- and community-acquired infections such as MRSA.

2.0 Aim and objectives

The aim of this study was to investigate and manipulate the effects of androgens on the clearance of MRSA by human macrophages.

The fundamental objectives of the research were:

- To generate human-derived macrophages from U937 monocytes.
- To model both androgen deprivation and supraphysiological/physiological ranges of T and DHT in an *in vitro* phagocytosis assay using U937 macrophages.
- To measure the rate of clearance of MRSA by U937 macrophages exposed to differing concentrations of androgens.
- To explore the pathways/mechanisms by which androgens/anti-androgens mediate MRSA clearance by U937 macrophages using androgen receptor

antagonists and 5α-reductase inhibition. To visualise androgen-mediated effects on phagocytosis by scanning electron microscopy (SEM).

3.0 Ethical Declarations

No ethical implications were identified during this research. No aspect of the work involved the use of participants, animals or primary human tissues. The study used a commercially available U937 monocyte cell line throughout.

4.0 Materials List

Bovine Serum Albumin (Sigma-Aldrich, Dorset)

Cyproterone acetate (Sigma-Aldrich, Dorset)

Dihydrotestosterone (Sigma-Aldrich, Dorset)

DMSO (Sigma-Aldrich, Dorset)

Finasteride (Sigma-Aldrich, Dorset)

FITC-conjugated anti-human CD11c antibody (Clone Bu15, Biolegend UK)

Flutamide (Sigma-Aldrich, Dorset)

Foetal Bovine Serum (FBS) (Sigma-Aldrich, Dorset)

Glutaraldehyde (Sigma-Aldrich, Dorset)

Gold (Polaron, SC7640)

Human monocyte cell line U937 (Health Protection Agency Culture Collections, Salisbury)

Interferon-gamma (IFN-γ) (Sigma-Aldrich, Dorset)

Lipopolysaccharide (LPS) from Escherichia coli (Sigma-Aldrich, Dorset)

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

Nutrient Agar (Oxoid, Basingstoke)

Nutrient Broth (Oxoid, Basingstoke)

Paraformaldehyde (Sigma, Dorset)

Penicillin – streptomycin (Sigma-Aldrich, Dorset)

Phorbol 12-myristate 13-acetate (PMA) (Applichem, Darmstadt)

Phosphate Buffered Saline (PBS) (Fisher Scientific, Loughborough)

RPMI-1640 Media (Lonza, Slough) supplemented with 10% Foetal Bovine Serum (Sigma-Aldrich, Dorset) and 2% Penicillin-Streptomycin (Lonza, Slough)

Saline (Sigma-Aldrich, Dorset)

Silicon wafers (Sigma-Aldrich, Dorset)

Testosterone (T) (Sigma-Aldrich, Dorset)

Trypsin EDTA (Lonza, Slough)

0.4% Trypan Blue (Lonza, Slough)

5.0 Methods

5.1 Bacterial Culture

Methicillin Resistant *Staphylococcus aureus* strain 11 (MRSA11) were used in this project. One colony of MRSA (Hospital isolates, Manchester, UK) was re-suspended into nutrient broth medium (Oxoid, Basingstoke) and cultured for 24 hours in an orbital shaker at 37˚C. Broths were centrifuged twice for 10 minutes at 3500 RPM and the pellet was washed using saline (Sigma-Aldrich, Dorset). One in ten serial dilutions of the NEAT broth were prepared using 9mL of saline. Serial dilutions 10^{-5}, 10^{-6} and 10^{-7} were cultured overnight on nutrient agar plates (Oxoid, Basingstoke) at 37˚C prior to performing colony counts to determine the bacterial density (colony forming units/mL (CFU/mL) of each broth. Each broth was diluted in saline to a concentration of 2×10^4 CFU/mL.

5.2 Cell Culture

Immortal U937 human monocytes (0.5 x 10^6 cells/mL) (Health Protection Agency Culture Collections, Salisbury) were cultured (37°C with 5% CO_2) under aseptic conditions using RPMI 1640 media (Lonza, Slough) supplemented with 10% Foetal Bovine Serum (FBS) (Sigma-Aldrich, Dorset) and 2% Penicillin-streptomycin (Sigma-Aldrich, Dorset). Cells were re-suspended at 0.5 x 10^6 cells/mL in fresh media every 48 hours.

Cell counting was performed during sub-culturing using a *Biorad* TC10 automated cell counter and trypan blue (Lonza, Slough) exclusion method (equal volume of 0.4% sterile filtered trypan blue and cell suspension) whereby dead cells absorb the blue dye and are excluded from the cell count. Cell viability was maintained above 90% for all experiments in line with previous studies (Krzykowska-Petitjean *et al*, 2012).

5.3 U937 Monocyte Differentiation Assay

U937 monocytes at a concentration of 1 x 10^6 cells/mL were treated with 50 ng/mL Phorbol 12-myristate 13-acetate (PMA) (Applichem, Darmstadt) for 72 hours at 37˚C and 5% CO_2. This induced the differentiation of U937 monocytes to macrophage-like cells as described previously (Sproston *et al*, 2018).

5.4 Flow Cytometry

Following treatment with PMA for 72 hours (section 5.3), the U937 cells were centrifuged at 500 g for 5 minutes before being washed twice with phosphate buffered saline (PBS) (Fisher Scientific, Loughborough). Cells were fixed with 4% paraformaldehyde (Sigma, Dorset) in PBS for 15 minutes at room temperature. The supernatants were discarded and the cells were washed twice with PBS. Cells were then stained with FITC-conjugated anti-human CD11c antibody (Clone Bu15, Biolegend UK), (diluted 1:50 with 10% FBS in PBS) at room temperature for 30 minutes. Cells were washed twice with PBS before resuspension in 500 µL of PBS. CD11c expression was measured with a BD Accuri C6F1 cytometer using BD Accuri C6 software. Data from three experimental replicates were presented as the median

fluorescence intensity (MFI) relative to unstained monocytes (negative control) as described previously (Sproston *et al*, 2018).

5.5 Host-Pathogen (Phagocytosis) Assay

Testosterone (T) / Dihydrotestosterone (DHT) Treatments:

U937 differentiated macrophages were treated (n=24 per treatment) for 24 hours at 37°C in 5% CO_2 with differing concentrations of T/DHT (Sigma-Aldrich, Dorset) in antibiotic free RPMI media to represent supraphysiological concentrations (1 x 10^{-6} M), physiological concentrations (1 x 10^{-7} M and 1 x 10^{-8} M) and androgen deprivation (1 x 10^{-9} M) or an absolute absence of androgens (negative control) as described previously (Gilliver *et al*, 2006).

Androgen Pathway Inhibitor Treatments:

U937 adherent macrophages were treated (n=24 per treatment) with either an AR inhibitor (Cyproterone Acetate or Flutamide) or the 5-alpha (5α-) reductase inhibitor (Finasteride) (Sigma-Aldrich, Dorset) suspended in RPMI media at concentration 1 x 10^{-6} M for 24 hours at 37°C in 5% CO_2. Supernatants were removed and U937 macrophages were then treated with T/DHT (1 x 10^{-6} M) for 24 hours before performing the host-pathogen assay.

Host-pathogen Interaction:

Following treatments with androgens or androgen inhibitors, U937 macrophages were stimulated with 50 µL Interferon-gamma/Lipopolysaccharide (IFN-ɣ/LPS) (100 ng/mL and 1 µg/mL respectively) suspended in antibiotic-free RPMI media for 2 hours at 37˚C in 5% CO_2 as an *in vitro* model of infection/ inflammation.

Supernatants were discarded and the U937 macrophages were incubated with LPS/IFN-ɣ (Sigma-Aldrich, Dorset) and MRSA (10^4 CFU) in a total volume of 100 µL for 3 hours to allow phagocytosis to take place. The supernatants were aspirated and collected. Adherent U937 cells were detached using 450 µL EDTA-Trypsin (Lonza, Slough) for 5 minutes before adding 450 µL of RPMI media to neutralise the trypsin. The content of each well (900 µL) was then added to the collected

supernatant. Bacterial recovery was determined by culturing a 100 µL aliquot of each sample onto duplicate nutrient agar plates and incubating for 24 hours at 37°C in 5% CO_2. The number of MRSA colonies recovered on the agar plates were used to calculate the mean bacterial recovery.

Controls:

Bacterial controls (n=24) consisting of MRSA (10^4 CFU) incubated in 100 µL antibiotic (Ab)-free RPMI media at 37°C and 5% CO_2 were performed within the assay to take into account the rate of bacterial growth in the absence of host (U937 macrophages) interaction. Bovine serum albumin (BSA) (Sigma-Aldrich, Dorset) controls and androgen inhibitor controls were prepared, consisting of 1 x 10^{-6} M BSA/inhibitor suspended in Ab-free RPMI media and incubated for 3 hours at 37°C in 5% CO_2. The negative control consisted of macrophages incubated in the absence of androgens (absolute absence of androgens). The inhibitor controls were used to determine the direct effect of androgen inhibitors on phagocytosis in the absence of T/DHT.

U937 Cell Counts:

Total cell counts were performed before the host-pathogen interaction between U937 macrophages and MRSA. Cell culture media was removed from one replicate well of each treatment/control and U937 macrophages were detached using 50 µL of Trypsin-EDTA for 5 minutes. The trypsin was neutralised with 50 µL of Ab-free media and a automated cell counter *Biorad* was used to determine the number of total and viable U937 cells (average of two readings) using the trypan blue exclusion methodology as described in section 5.2.

5.6 Statistical Analysis

Statistically significant differences ($P<0.05$) in mean bacterial recovery (CFU/mL) between treatments and control groups were determined using SPSS software (Version 24) by performing normality tests and ANOVA followed by Tukey Pairwise comparisons. In all cases $P<0.05$ was considered statistically significant.

5.7 Scanning Electron Microscopy (SEM)

Silicon wafers (Sigma-Aldrich, Dorset) prepared at size 1 cm^2 were sterilised using 70% ethanol for 30 minutes and allowed to dry in a class II cabinet. PMA-treated U937 cells (1 x 10^6/mL) were added to wells containing the silicon wafers and incubated for 72 hours at 37˚C and 5% CO_2. The U937 macrophages generated were treated with T/DHT and stimulated with IFN-ɣ/LPS (100 ng/mL and 1 µg/mL respectively) before being incubated with MRSA for 3 hours as described in section 5.5. Similarly, controls were generated as described in section 5.5 using silicon wafers in control wells. The silicon wafers were removed from each well and placed in 4% glutaraldehyde (Sigma-Aldrich, Dorset) at 4°C overnight to allow sample fixation. Each silicon wafer was washed with increasing concentrations of ethanol (20, 40, 60, 80 and 100% ethanol) for 30 minutes each. Samples were dried overnight in a vacuum-assisted desiccator at room temperature. Samples were coated with gold (Polaron, SC7640) ready for imaging by a Scanning Electron Microscope (SEM; Carl Zeiss Ltd Supra 40VP).

6.0 Results

6.1 Flow Cytometry

Flow cytometry was used to confirm differentiation of U937 monocytes into U937 macrophages through the detection of surface marker CD11c that is specific to macrophage populations. Figure 6 shows low detection of CD11c on monocyte and unstained macrophage populations (negative controls). However, PMA-differentiated cells demonstrated significantly ($P<0.01$) higher levels of CD11c compared to controls [$P<0.01$]. These data confirmed differentiation of U937 monocytes into CD11c-positive (CD11c+) U937 macrophages (Figure 6 & 7).

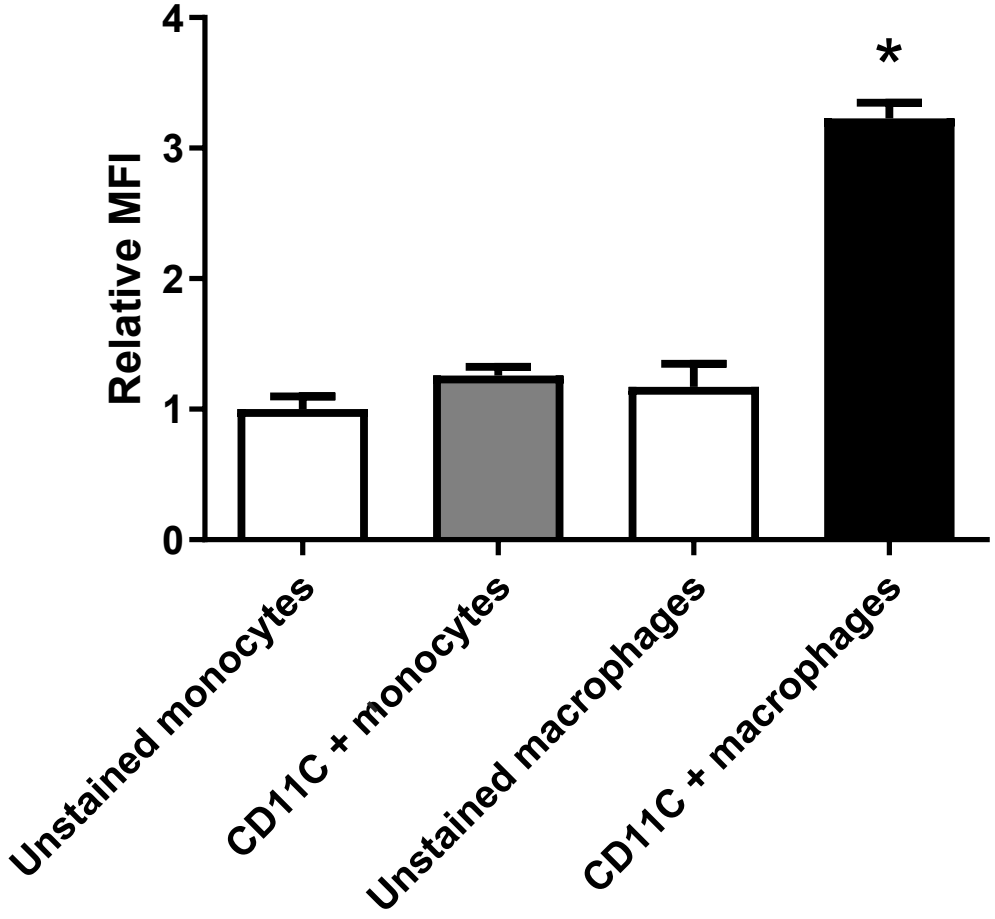


Figure 5: Detection of the surface marker CD11c using flow cytometry to confirm differentiation of U937 monocytes into macrophages. Differentiated cells expressed significantly (n=3; $P<0.01$) higher levels of the CD11c marker compared to monocyte and unstained macrophage controls. The relative Median Florescence Intensity (MFI) for unstained monocytes: 1.00; CD11c positive (cD11c+) monocytes: 1.26; unstained macrophages: 1.17; CD11c-Positive (CD11c+) macrophages: 3.28. Bars represent mean MFI ± standard error of the mean in all cases. * indicates significant difference between CD11c+ macrophages and controls.

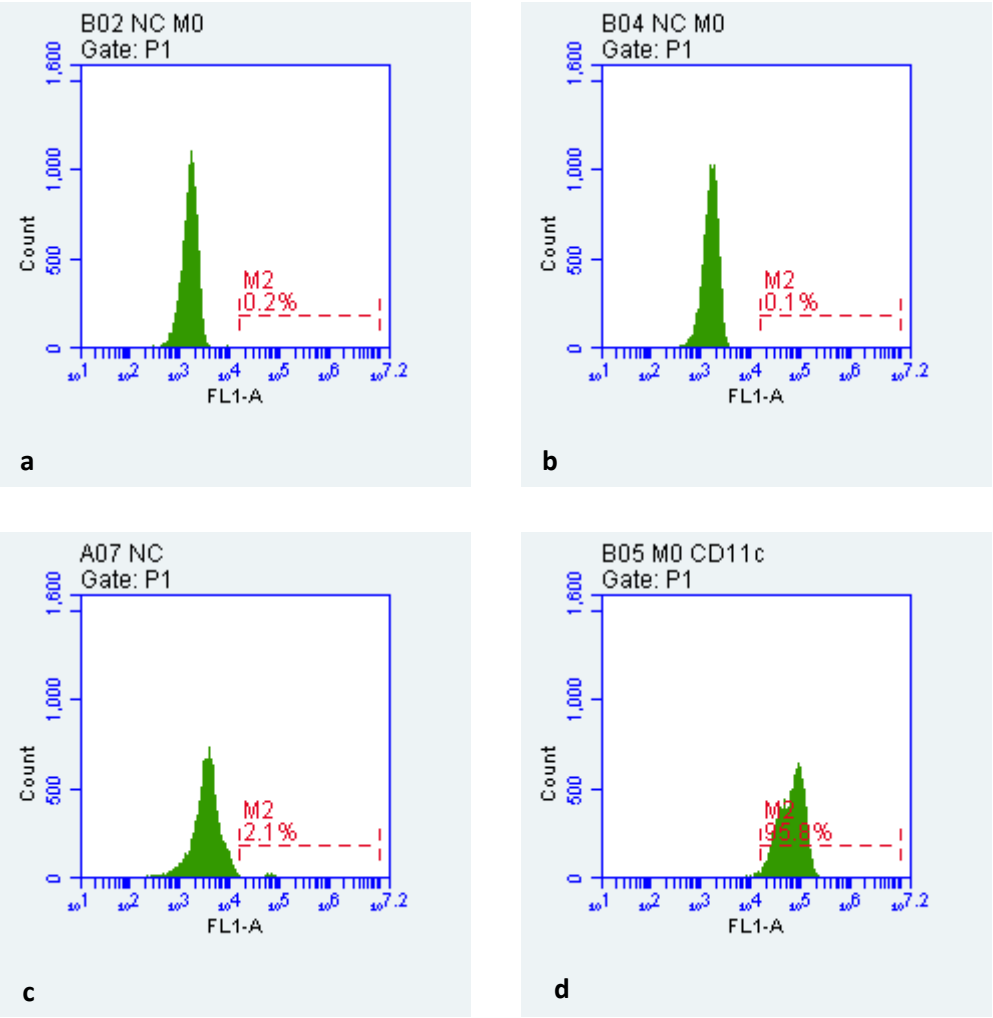


Figure 6**:** Flow cytometry charts confirming monocytes were differentiated into macrophage populations. a) Unstained monocytes. b) Unstained macrophages. c) CD11c+ monocytes. d) CD11c+ macrophages.

6.2 Effect of Testosterone on Phagocytosis of MRSA

A one-way ANOVA identified significant (P=0.01) differences in bacterial recovery per million viable macrophages (CFU/mL) between androgen-treated (T: 1 x 10^{-6} M, 1 x 10^{-7} M, 1 x 10^{-8} M, 1 x 10^{-9} M) and untreated control groups.

Bacterial recovery significantly (P<0.01) reduced in the presence of untreated macrophages (negative control, NC) compared to MRSA recovery in absence of any phagocytes (bacteria alone control), suggesting the U937 macrophages were highly effective at phagocytosis in the absence of androgens.

Tukey pairwise comparison tests showed higher concentrations of testosterone (T: 1 x 10^{-6} M, 1 x 10^{-7} M, 1 x 10^{-8} M) significantly ($P<0.01$) increased bacterial recovery CFU/mL in a dose dependant manner (Figure 8) compared to the untreated negative control, suggesting testosterone concentrations spanning physiological levels found in adult males inhibit phagocytosis. Supraphysiological concentrations of testosterone (1 x 10^{-6} M) resulted in the highest MRSA recovery of 9298 CFU/mL, whilst physiological concentrations of testosterone typical of adult males (1 x 10^{-7} M and 1 x 10^{-8} M) also gave elevated MRSA recoveries of 7583 CFU/mL and 6808 CFU/mL respectively compared to the untreated NC (1864 CFU/mL). In contrast, although the mean MRSA recovery with testosterone deprivation (1 x 10^{-9} M) was elevated (5280 CFU/mL) compared to the untreated NC (absolute absence of androgens), there was no evidence to suggest this concentration of testosterone significantly inhibited phagocytosis ($P=0.08$).

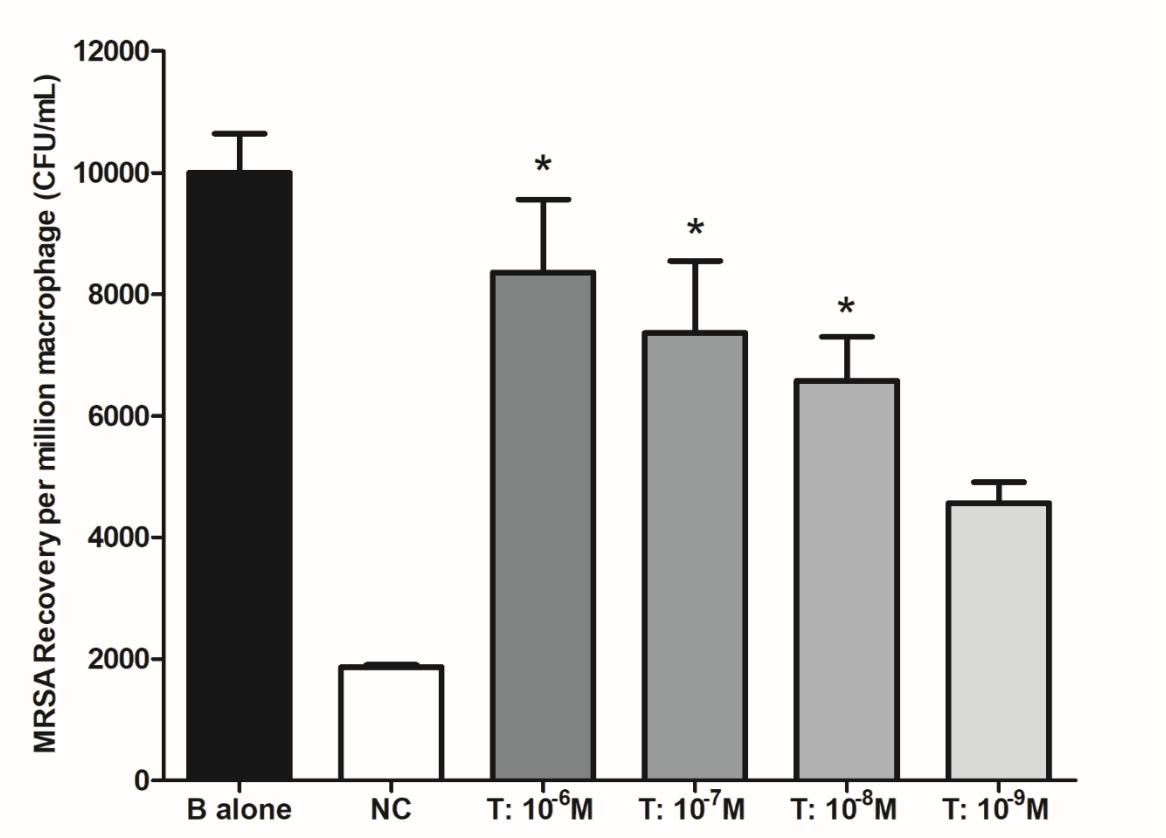


Figure 7: Effect of Testosterone (T) on the phagocytosis of MRSA by U937 macrophages (n=24). MRSA recovery significantly (P<0.01) increased in a dose-dependent manner following exposure of macrophages to testosterone (T: 1 x 10^{-6} M, 1 x 10^{-7} M, 1 x 10^{-8} M). Bacterial control (B alone) 10^4 CFU/mL, Negative control (NC) 1864 CFU/mL, T: 10^{-6} M 9298 CFU/mL, T: 10^{-7} M 7583 CFU/mL, T: 10^{-8} M 6808 CFU/mL, T: 10^{-9} M 5280 CFU/mL. Bars represent mean MRSA recovery per million macrophage (CFU/mL) ± standard error of the mean (SEM). * indicates significant difference (P<0.01) between T treatment and NC.

6.3 Effect of Dihydrotestosterone (DHT) on Phagocytosis of MRSA

A one-way ANOVA identified significant (P=0.01) differences in bacterial recovery per million viable macrophages (CFU/mL) between DHT-treated (DHT: 1 x 10^{-6} M, 1 x 10^{-7} M, 1 x 10^{-8} M, 1 x 10^{-9} M) and untreated control groups.

Again bacterial recovery significantly (P<0.01) reduced in the presence of untreated macrophages (negative control, NC) compared to MRSA recovery in absence of any

phagocytes (bacteria alone control), suggesting the U937 macrophages were highly effective at phagocytosis in the absence of androgens.

Tukey pairwise comparison tests showed DHT treatments significantly ($P<0.03$) increased bacterial recovery CFU/mL in a dose dependant manner (Figure 9), suggesting concentrations of DHT that span physiological concentrations in adult males inhibit phagocytosis. Supraphysiological and physiological concentrations of DHT (1×10^{-6} M, 1×10^{-7} M and 1×10^{-8} M) appeared to almost arrest phagocytosis, with the bacterial recovery (9508 CFU/mL, 8505 CFU/mL and 7510 CFU/mL) approaching that found in the absence of any phagocytes (bacteria alone control). In contrast, DHT concentration 1×10^{-9} M increased the mean MRSA recovery (6429 CFU/mL) compared to the untreated NC, however shown no evidence to suggest this concentration of DHT significantly inhibit phagocytosis ($P=0.2$).

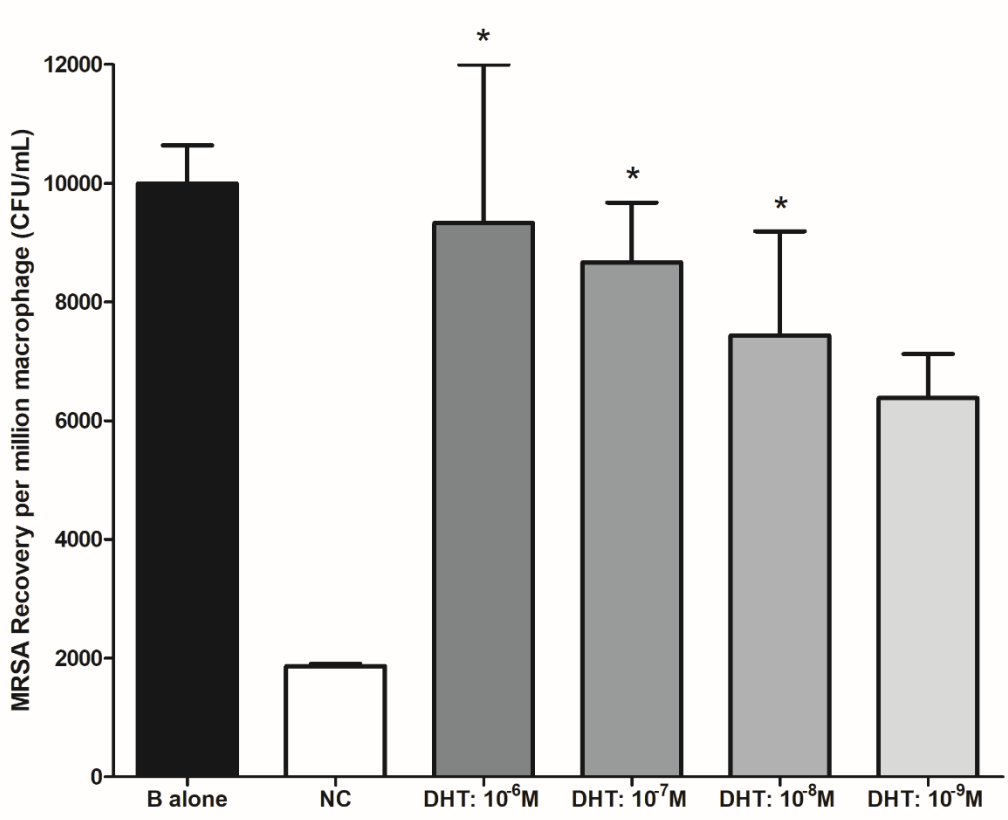


Figure 8: Effect of Dihydrotestosterone (DHT) on the phagocytosis of MRSA by U937 macrophages (n=24). MRSA recovery significantly (P<0.01) increased in a dose-dependent manner following exposure of macrophages to DHT (DHT: 10^{-6} M, 10^{-7} M). Bacterial control (B alone) = 10^4 CFU/mL, Negative control (NC) = 1866 CFU/mL, DHT: 10^{-6} M = 9508 CFU/mL, DHT: 10^{-7} M = 8605 CFU/mL, DHT: 10^{-8} M = 7510 CFU/mL, DHT: 10^{-9} M = 6429 CFU/mL. Bars represent mean MRSA recovery per million macrophage (CFU/mL) ± standard error of the mean (SEM). * indicates significant difference (P<0.01) between DHT treatment and NC.

6.4 Comparison of the Effects of Testosterone (T) and Dihydrotestosterone (DHT) on Phagocytosis of MRSA

Both T and DHT followed the same trend, increasing MRSA recovery compared to the untreated negative control (NC). However, on average DHT increased bacterial recovery by an additional 8% compared to T (Figure 10). The data suggest both T and DHT inhibited phagocytosis in a dose-dependent manner, with supraphysiological concentrations (1 x 10^{-6} M) of T and particularly DHT almost arresting phagocytosis. Collectively, these findings suggest DHT was substantially

more potent at inhibiting phagocytosis than T, with even very low concentrations of DHT leading to MRSA persistence.

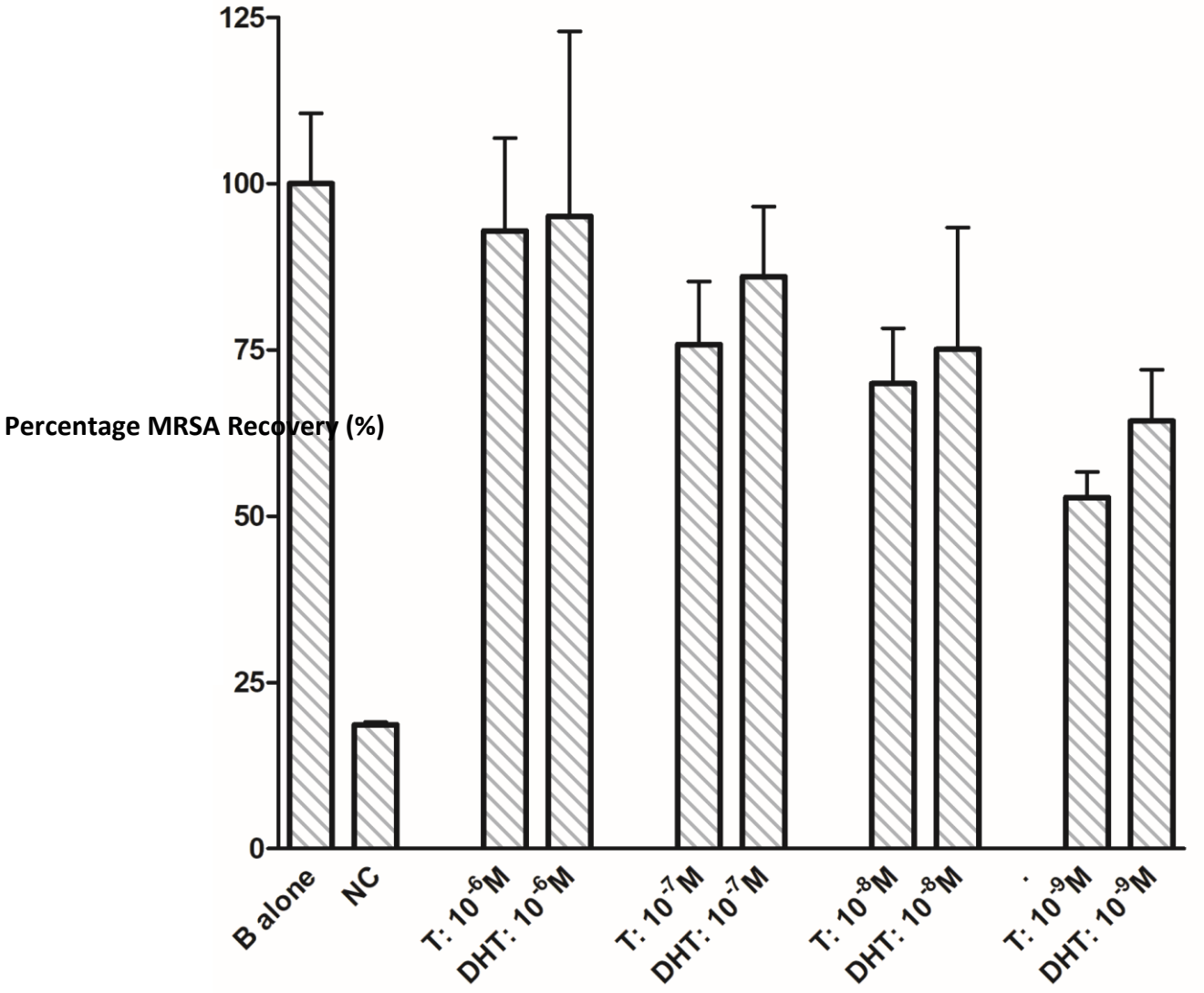


Figure 9: Comparison of the effect of testosterone (T) and dihydrotestosterone (DHT) on the phagocytosis of MRSA. Both T and DHT increased the percentage (%) bacterial recovery compared to the bacterial (B alone) and untreated negative control (NC) groups (n=24) in a dose-dependent manner. Bacterial Control = 100%, Negative Control = 19%, T: 10^{-6} M = 93% v DHT: 10^{-6} M = 95%, T: 10^{-7} M = 76% v DHT: 10^{-7} M = 86%, T: 10^{-8} M = 70% v DHT: 10^{-8} M = 75%, T: 10^{-9} M = 53% v DHT: 10^{-9} M = 64%. Bars represent mean MRSA recovery per million macrophage (CFU/mL) ± standard error of the mean (SEM).

6.5 Androgen Receptor Inhibition

The androgen receptor antagonists, Flutamide (FLU) and Cyproterone acetate (CYP), and the 5-alpha (5α-) reductase inhibitor, Finasteride (FIN), were explored to determine the potential mechanisms through which androgens mediate phagocytosis.

6.6 Reversal of Testosterone (T)-Mediated Inhibition of Phagocytosis

U937 macrophages were treated with the androgen inhibitors at a concentration of 10^{-6} M prior to exposure to T at concentration of 10^{-6} M (Figure 11). A one-way ANOVA indicated significant differences between treatment groups ($P<0.01$). The bacterial recovery per million viable macrophages (CFU/mL) was significantly ($P<0.01$) lower in inhibitor treated groups (FIN/ FLU/ CYP + T: 10^{-6} M) compared to T treatment group (T: 10^{-6} M), indicating all the inhibitors block the inhibitory effect of T on phagocytosis. All inhibitor treatments showed no statistical significant difference ($P>0.01$) compared to the untreated negative control (NC), confirming the addition of inhibitors effectively reverse the inhibitory effects of T on phagocytosis.

Flutamide was the most effective inhibitor, decreasing bacterial recovery to 1436 CFU/mL, whilst cyproterone acetate decreased MRSA recovery to 3545 CFU/mL. These findings indicate that T acts through the AR to inhibit phagocytosis. The 5α-reductase inhibitor, finasteride (FIN), also decreased MRSA recovery to 3297 CFU/mL, suggesting the inhibition of phagocytosis by T is mediated principally through initial conversion of T to DHT, followed by action of DHT on the AR.

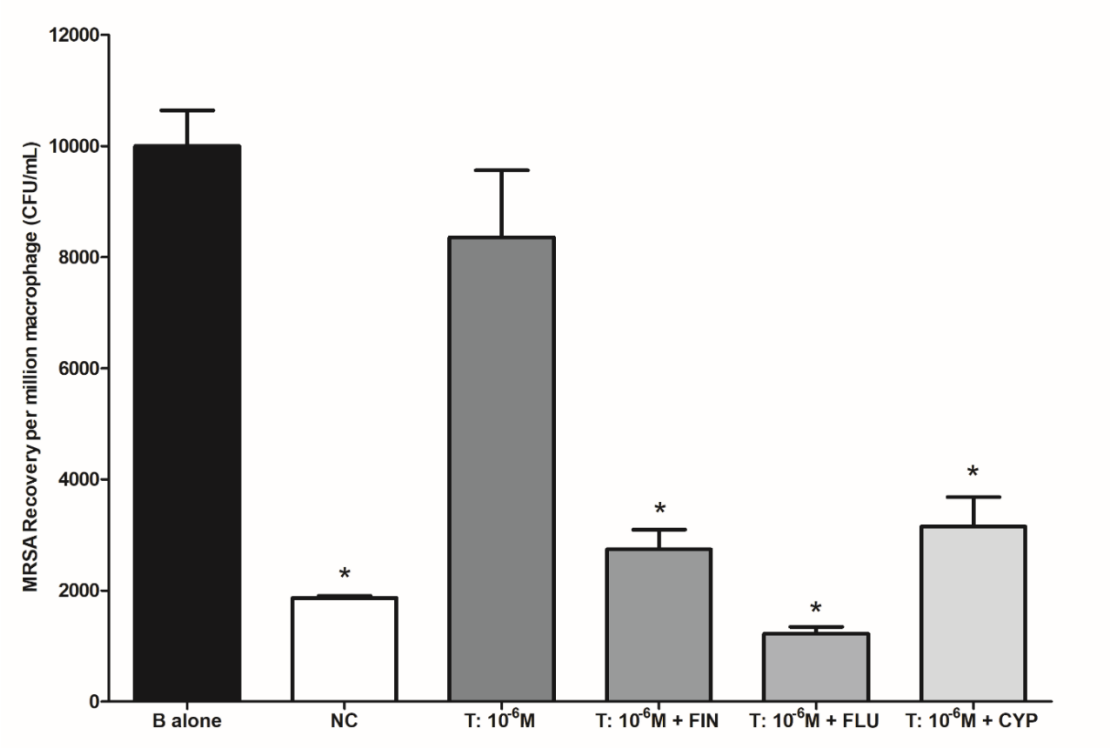


Figure 10: Reversal of testosterone (T)-mediated inhibition of phagocytosis via androgen receptor (AR) antagonism, using 10^{-6} M flutamide (FLU) or cyproterone acetate (CYP), or via inhibition of 5-alpha (5α-) reductase, using 10^{-6} M finasteride (FIN). Exposure of macrophages to androgen inhibitors significantly ($P<0.01$; n=24) blocked the effects of T on MRSA recovery. Bacterial control (B alone) = 10^4, CFU/mL, negative control (NC) = 1864 CFU/mL, T: 10^{-6} M + FIN = 3297 CFU/mL, T: 10^{-6} M + FLU = 1436 CFU/mL, T: 10^{-6} M + CYP = 3545 CFU/mL. Bars represent mean MRSA recovery per million macrophage (CFU/mL) ± standard error of the mean (SEM). * indicates significant differences ($P<0.01$) compared to the T: 10^{-6} M treatment.

6.7 Reversal of Dihydrotestosterone (DHT)-Mediated Inhibition of Phagocytosis

U937 macrophages were treated with the androgen inhibitors at a concentration of 10^{-6} M prior to exposure to DHT at a concentration of 10^{-6} M (Figure 12). A one-way ANOVA indicated significant differences between treatment groups ($P=0.01$). The bacterial recovery per million viable macrophages (CFU/mL) was significantly ($P=0.03$ for FLU; $P=0.01$ for CYP) lower following treatment with AR antagonists (FLU/ CCYP + DHT: 10^{-6} M) compared to the DHT treatment (DHT: 10^{-6} M), suggesting the inhibitory effect of DHT on phagocytosis is mediated through the AR. Flutamide and cyproterone acetate treatments reduced MRSA recovery to 3645 and 3076

CFU/mL respectively, compared to DHT treatment alone (9507 CFU/mL). Treatment with AR antagonists (FLU/CA + DHT: 10^{-6} M) showed no significant differences (P>0.05) compared to the untreated negative control (NC), confirming antagonism of the AR reverses the inhibitory effect of DHT on phagocytosis.

No statistical significance (P>0.05) was found between treatment with the 5α-reductase inhibitor finasteride (FIN + DHT: 10^{-6} M) and treatment with DHT alone (DHT: 10^{-6} M), confirming the enzymatic conversion of T to DHT by 5α-reductase is only relevant following treatment with T (Figure 12) but not with DHT treatment.

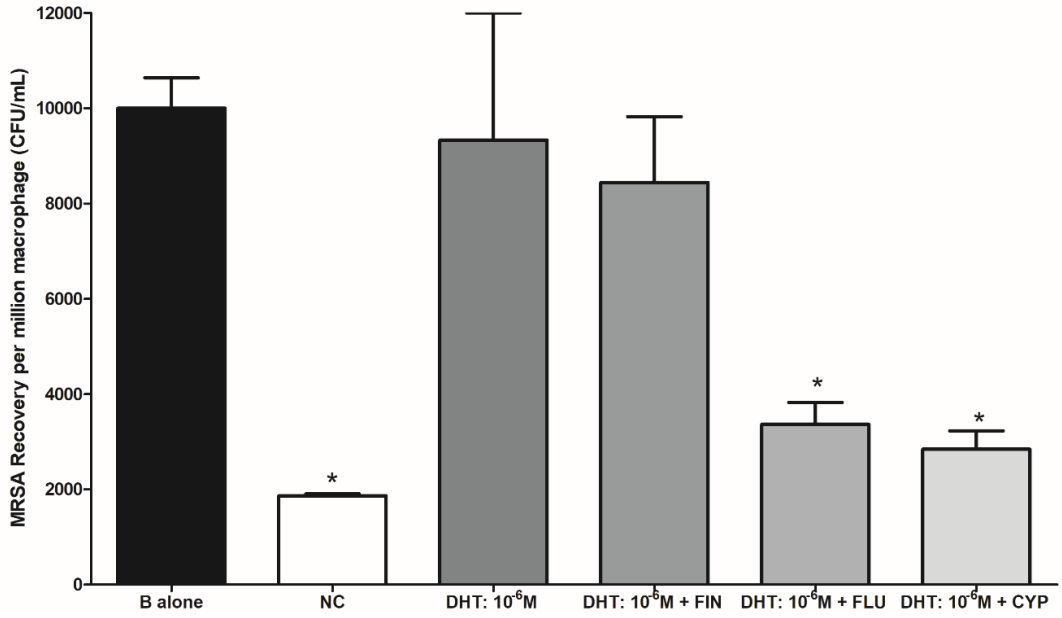


Figure 11: Reversal of dihydrotestosterone (DHT)-mediated inhibition of phagocytosis via androgen receptor (AR) antagonism, using 10^{-6} M flutamide (FLU) or cyproterone acetate (CYP). Exposure of macrophages to androgen receptor antagonists significantly (P<0.01; n=24) blocked the effects of DHT on MRSA recovery. Treatment with the 5-alpha (5α-) reductase inhibitor, finasteride (FIN), had no significant (P>0.05; n=24) effect on DHT-mediated MRSA recovery. Bacterial control (B alone) = 10^4 CFU/mL, negative control (NC) = 1864 CFU/mL, DHT: 10^{-6} M = 9507 CFU/mL, DHT: 10^{-6} M + FIN = 8540 CFU/mL, DHT: 10^{-6} M + FLU = 3645 CFU/mL, DHT: 10^{-6} M + CYP = 3079 CFU/mL. Bars represent mean MRSA recovery per million macrophage (CFU/mL) ± standard error of the mean (SEM). * indicates significant differences (P<0.05) compared to the DHT: 10^{-6} M treatment.

6.8 Experimental Controls

Several experimental controls (n=24 in each control group) were performed within the phagocytosis assays to ensure robustness of the data (Figure 13). The bacterial control (B alone) represents normalised MRSA recovery (10^4 CFU/mL) in absence of androgens (T/DHT) and U937 macrophages. The negative control represents bacterial recovery following incubation with untreated U937 phagocytes (i.e. macrophages with no androgen treatment). The bacterial recovery was reduced by 81% ($P<0.01$) in the negative control compared to the bacterial control, indicating phagocyte function was highly effective in the absence of androgens. The BSA control represents macrophages treated with bovine serum albumin (BSA) instead of androgens to confirm changes in MRSA recovery were due to the specific activity of androgens. The BSA control had no effect on MRSA recovery ($P>0.05$) compared to the negative control, confirming phagocyte function was unaffected by this non-androgen treatment. Inhibitor controls represent treatment with androgen inhibitors (FIN, FLU and CYP) alone (in the absence of androgens). The inhibitor controls had no effect on MRSA recovery compared to the negative control, demonstrating the inhibitors had no direct effect on phagocytosis themselves, in the absence of androgens.

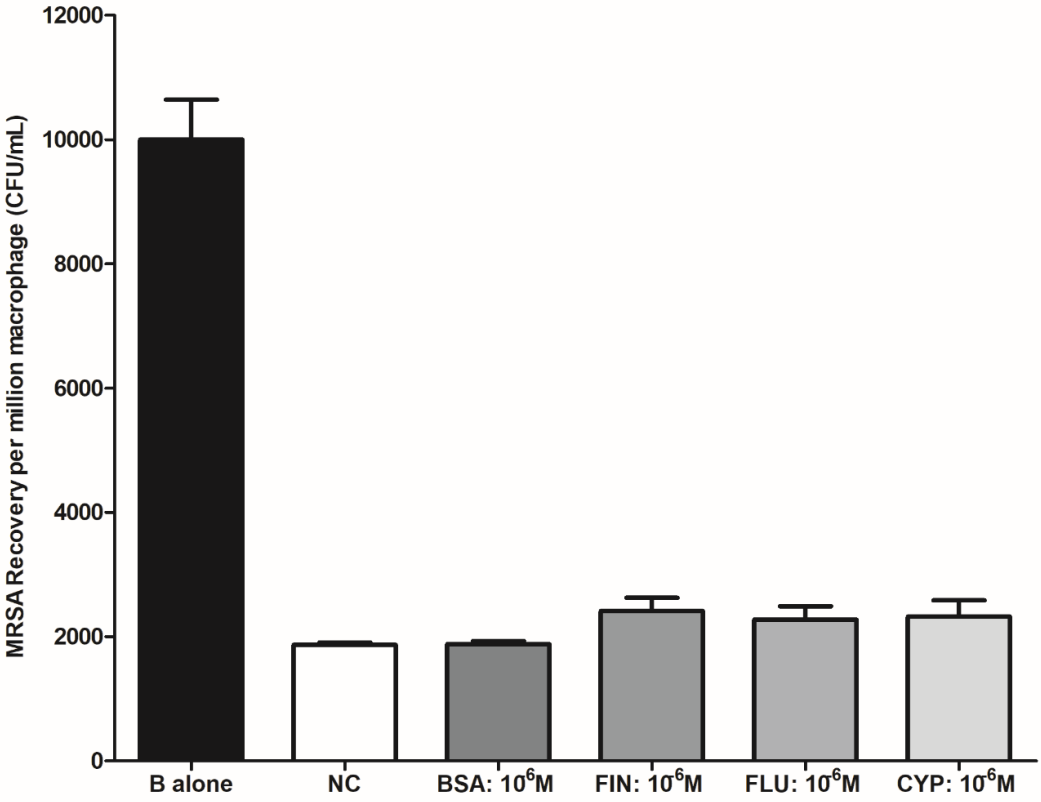
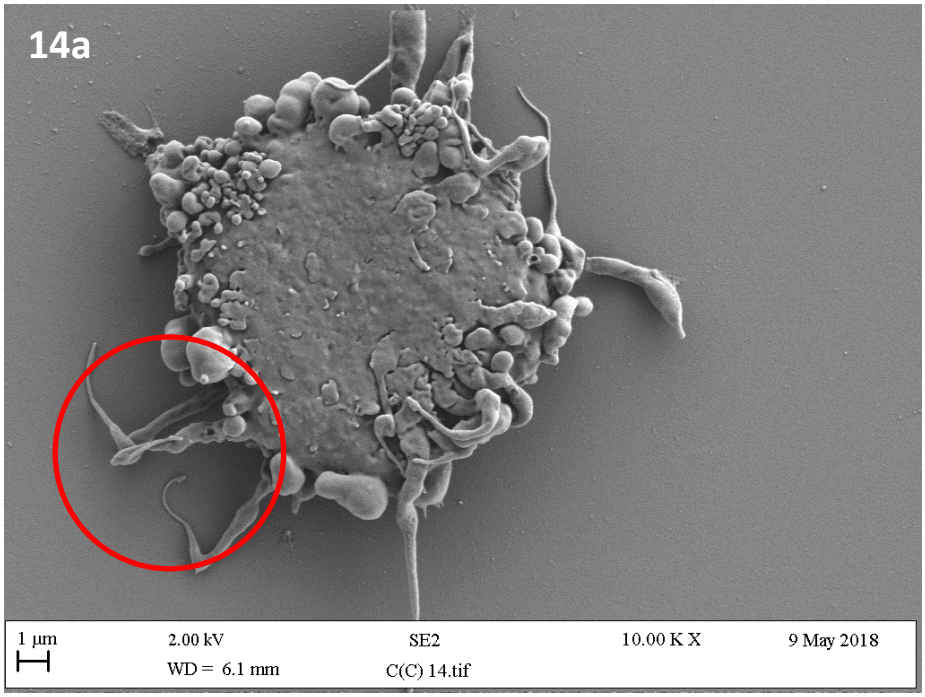


Figure 12: Experimental controls for the phagocytosis assay. MRSA recovery per million viable macrophage (CFU/mL) was unaffected by the non-specific BSA control or inhibitor control treatments compared to the untreated negative control (NC). Bacterial control (B alone): = 10^4 CFU/mL. Negative control (NC): = 1864 CFU/mL. BSA control: = 1886 CFU/mL. Finasteride (FIN) control: = 2734 CFU/mL, Flutamide (FLU) control: = 2510 CFU/mL, Cyproterone acetate (CYP) control: = 2632 CFU/mL. No significant differences were found between the BSA/inhibitor control treatments and the NC. Bars represent mean MRSA recovery per million macrophage (CFU/mL) ± standard error of the mean (SEM).

6.9 Scanning Electron Microscopy (SEM)

SEM was used capture visual evidence of phagocytosis taking place after 3 hours of host-pathogen interaction. Figures 14, 15 and 16 illustrate macrophage activity under the following conditions, i) absence of androgens (untreated negative control), ii) presence of T and iii) presence of DHT.



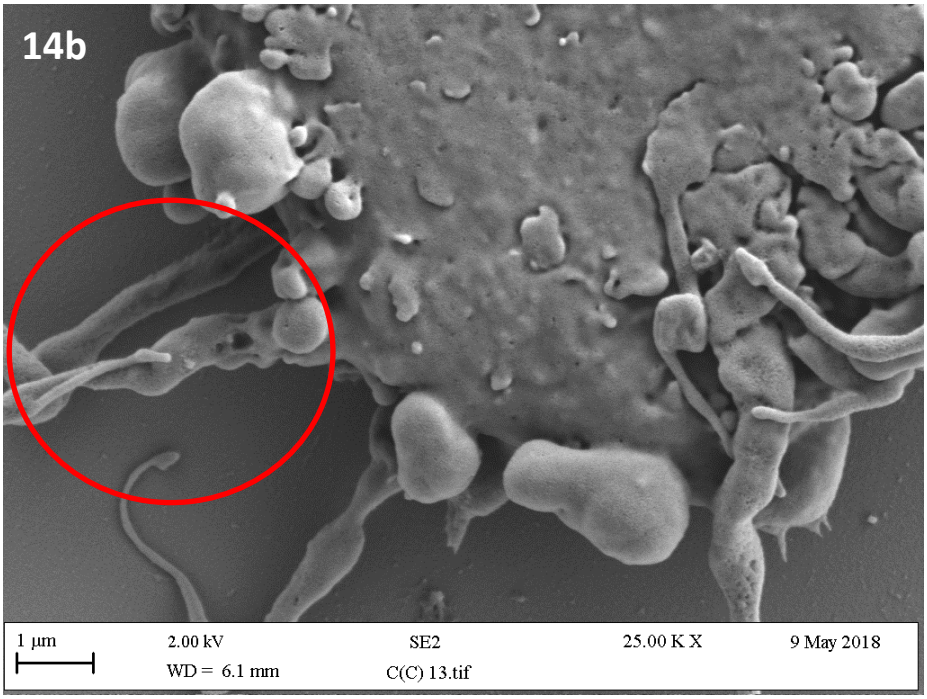
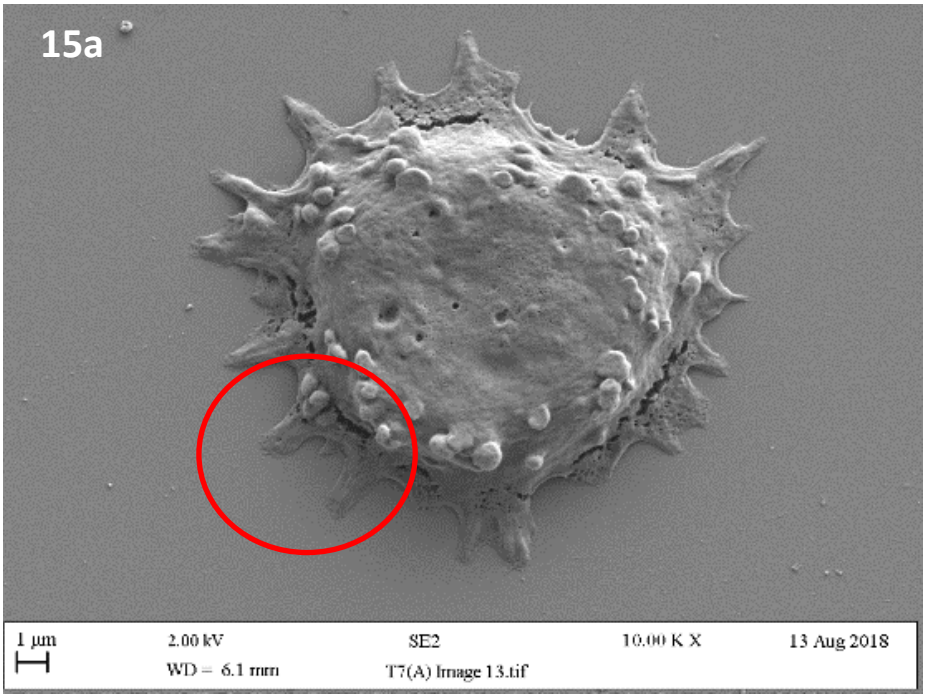


Figure 13a & 14b: SEM image to visualise host-pathogen interaction in the absence of androgens (untreated negative control). Frequent internalisation of MRSA was acompanied by prominent membrane extensions (example shown in red circle) by the U937 macrophages, typical of phagocytosis.



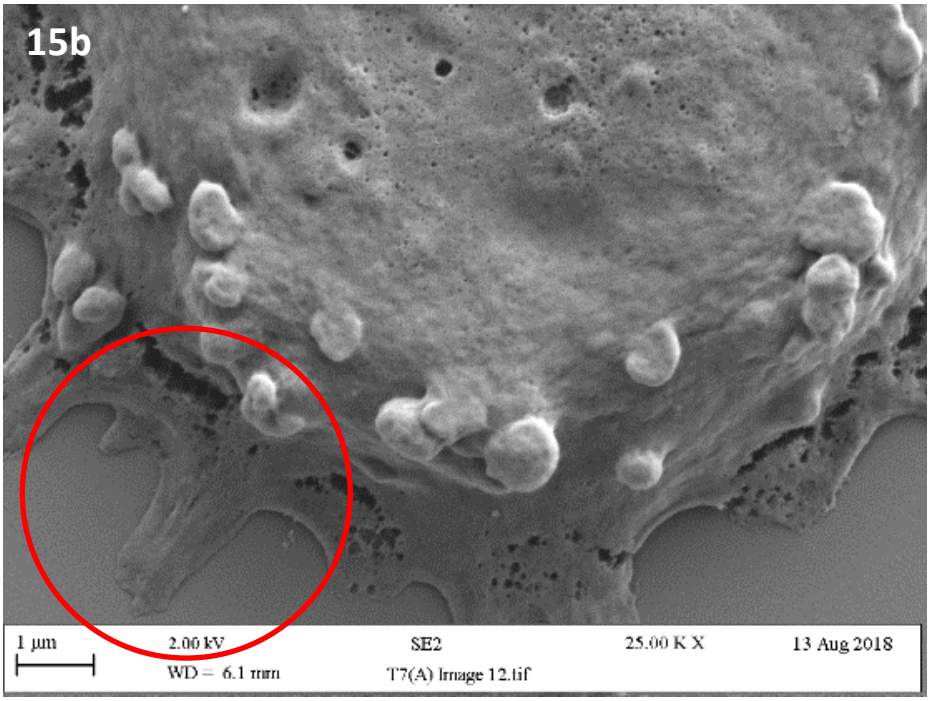
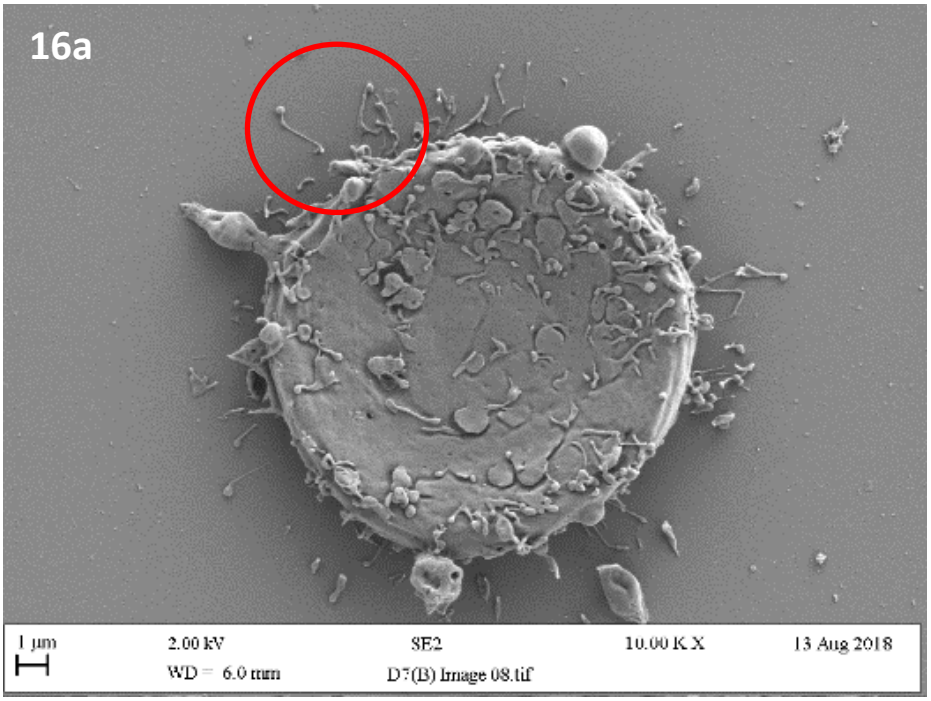


Figure 14a & 15b. SEM image of host-pathogen interaction in the presence of testosterone (T). Internalisation of MRSA was reduced compared to the untreated negative control, with fewer macrophage-bacteria interactions suggesting impaired phagocytosis. Phagocyte morphology was altered compared to the untreated negative control, with macrophages producing fewer and shorter membrane extensions (example shown inside red circle).



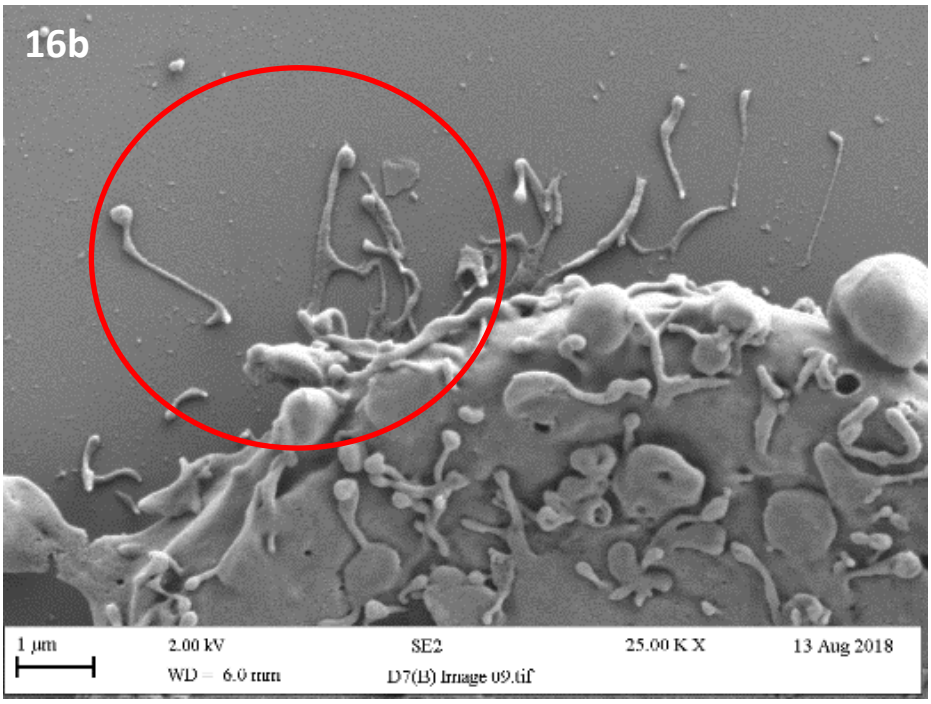


Figure 15a & 16b. SEM image of host-pathogen interaction in the presence of dihydrotestosterone (DHT). Internalisation of MRSA by U937 macrophages was substantially diminished compared to the untreated negative control, with fewer macrophage-bacteria engagements, suggesting impaired phagocytosis. Phagocyte morphology was altered compared to the untreated negative control, with negligible membrane extensions from macrophages (example shown inside red circle).

7.0 Discussion and Future Work

This study investigated for the first time the effects of the two main active androgens (T or DHT) on the clearance of MRSA by human-derived phagocytes. This novel data showed physiological and supra-physiological levels of both T and DHT significantly ($P<0.05$; $n=24$) reduce the clearance of MRSA in a dose dependent manner. Thus, the findings suggest androgens modulate pathways that directly or indirectly inhibit phagocytosis by activated U937 macrophages *in vitro*. DHT was 12% more effective at exhibiting inhibitory effects on phagocytosis, which is consistent with the known biology of DHT being a more potent metabolite of testosterone, formed through the conversion to T to DHT by the enzyme 5α-reductase (Chen *et al*, 1998). More importantly, the 5α-reductase inhibitor finasteride (FIN) significantly ($P<0.05$; $n=24$) increased the clearance of MRSA to levels seen in the untreated negative control, suggesting the inhibition of phagocytosis by T is mediated principally through initial conversion of T to DHT, followed by the action of DHT on the AR. Thus, collectively the findings indicate that DHT (rather than T) is the principle androgen mediating the impairment of phagocyte function. Given T declines more slowly than estrogen during male ageing, resulting in the relative (%) amount of DHT effectively increasing (although absolute levels decline) with age, these data support growing evidence that an imbalance of sex hormones during male ageing contributes to impaired healing processes and increased propensity of elderly males to develop chronic wounds (Ashcroft and Mills, 2002; Gilliver *et al*, 2006; Gilliver *et al,* 2003).

SEM imaging provided qualitative data confirming impaired Internalisation of MRSA following treatment with T and DHT compared to the untreated negative control, with fewer macrophage-bacteria interactions suggesting impaired phagocytosis. Phagocyte morphology was altered following T and particularly DHT treatment compared to the untreated negative control, with macrophages producing fewer membrane extensions towards bacteria. Formation of lamellopodia by phagocytes is a key morphological process required for the engagement and subsequent engulfment of bacteria (Aderem, 2003; Rossi *et al*, 1998). T and particularly DHT

treatments appear to interfere with this critical process and block the internalisation of bacteria, in concordance with a previous study by Mondal and Rai (1999).

The underlying mechanism by which androgens inhibit the phagocytic function of macrophages is poorly defined but previous studies suggest it is mediated via the AR (Gilliver *et al*, 2006; Mondal and Rai 1999). In a murine model of wound healing, the competitive AR antagonist FLU was shown to dampen the inflammatory profile and accelerate healing by decreasing levels of TNF-α mRNA expression and NF-κB activation, reversing the effects observed with T treatment (Ashcroft and Mills, 2002). However, FIN has anti-androgenic effects by targeting the enzyme 5α-reductase, thereby inhibiting the conversion of T to DHT. A study by Gilliver *et al* (2006) found treatment with FIN reversed the effects of T, significantly accelerating murine wound healing by reducing the inflammatory response. Collectively, these findings suggest that the detrimental effects of T on wound healing require the initial conversion of T into DHT, followed by activation of the AR by DHT.

Similarly, CYP is an AR antagonist that has been shown to increase the phagocytosis of yeast cells by non-human splenic macrophages *in vitro,* reversing the effects of DHT (Mondal and Rai 1999). This study has shown the AR antagonists (FLU and CYP) significantly ($P<0.05$; $n=24$) increased the phagocytosis of MRSA in the *in vitro* model of host pathogen interaction following treatment with T or DHT. These data suggest active androgens inhibit phagocytosis by macrophages directly or indirectly through AR activation, in a similar manner to androgen activity found in murine wound healing studies. More importantly, this study investigated whether the conversion of T to DHT is required to inhibit phagocytosis. In concordance with wound healing investigations (Gilliver *et al*, 2006), novel findings from this study showed blocking the conversion of T to DHT with finasteride reversed the inhibition of phagocytosis observed with T. These findings suggest that the inhibition of phagocytosis by T is mediated principally through initial conversion of T to DHT, followed by AR activation by DHT. Future work could measure DHT levels produced by macrophages following incubation with T to confirm this finding. It is important to note, finasteride was unable to reverse the inhibition of phagocytosis following treatment with DHT, which is logical since 5α-reductase inhibition can block the conversion of T to DHT

but has no effect on pre-existing DHT. Inhibition of phagocytosis following DHT treatment was only reversed by antagonism of the AR.

The SEM from this study supports growing evidence that androgens can alter macrophage surface morphology. AR activation may result in decreased transcription of pathogen recognition receptors (PPRs) such as TLR-4 and CD14 on the macrophage surface, thereby decreasing their ability to detect pathogens and carry out phagocytosis (Rettew *et al*, 2008). To investigate this idea further, future work could involve flow cytometry to explore differences in surface receptor expression following treatment of macrophages with active androgens.

Other areas of potential interest arising from the SEM data includes androgen-induced changes in the cytoskeleton of phagocytes. Androgens have been shown to alter the distribution of actin in the cytoskeleton (Papakonstanti *et al*, 2003), which might impair the formation of lamellopodia by macrophages during host-pathogen interactions. Mediators involved in lamellopodial formation, such as focal adhesion kinase (FAK) and Rac1 (Kallergi *et al*, 2007) could be investigated in future work to determine the downstream mechanisms by which androgens may alter host-pathogen interactions. Stimulation of the AR with physiological concentrations of androgens may inhibit phagocytosis through signalling pathways such as the cAMP pathway. Elevation of intracellular cAMP may induce phenotypic changes known to alter cell shape and the disassembly of structures involved in pathogen engulfment such as membrane extensions (Rossi *et al*, 1998). Future work could involve measuring cAMP levels in phagocytes following treatment with androgens.

This research studied the effects of androgens typical of adulthood on phagocytosis using the immortal commercially available U937 monocyte cell line. These assays should be confirmed using *ex vivo* human peripheral macrophages derived from the circulation of age-matched, healthy control male subjects. An *in vivo* murine model of wound healing could also be conducted, similar to that of Gilliver *et al* (2006), to investigate the effect of local DHT application on bacterial clearance within acute wounds inoculated with MRSA.

8.0 Conclusion

It is well established that estrogen has a pivotal role in the acceleration of wound repair whereas androgens exhibit the opposite effect. During male aging, the decline in sex hormone precursors but somewhat maintained levels of androgens in circulation from gonadal production result in a substantial reduction in estrogen compared to T and DHT. This imbalance in sex hormones potentially explains why elderly men heal poorly compared to age-matched females and why there is greater male susceptibility to chronic wounds. Studies have demonstrated the effects of androgens on wound healing, particularly the heightened inflammatory profile. However, the effects of androgens on infection clearance have not been fully explored to date.

This study demonstrated for the first time that endogenous androgens inhibit phagocytosis in a macrophage-MRSA model of host-pathogen interaction through activation of the AR by DHT. SEM imaging confirmed reduced MRSA-macrophage interaction following androgen treatment and this was associated with altered phagocyte morphology involving fewer and shorter lamellopodia. These findings warrant further investigations to determine the precise underlying cellular processes and molecular mechanisms involved in androgen-mediated inhibition of phagocytosis.

9.0 Impact

The novel data in this study suggests local AR antagonism (e.g. using flutamide) or inhibition of 5α-reductase activity could be potential therapeutic strategies to promote the resolution of infected wounds in elderly males, including biofilms associated with chronic wounds and wounds that become colonised by antibiotic-resistant pathogens. Effective treatment of such wounds could save healthcare services over £4 billion worldwide, alongside reducing pressure on medical resources and hospital bed space.

Blocking the conversion of T to DHT locally at the wound site may bring potential benefits in terms of infection clearance without causing systemic side effects such as a reduction in bone and muscle-mass typically observed following androgen deprivation (Toraldo *et al*, 2010). It is well known that 5α-reductase is expressed locally in peripheral tissues such as skin (Gilliver *et al,* 2003). Thus, potential therapies to promote bacterial clearance could include specialised wound dressings or topical creams containing 5α-reductase inhibitors such as finasteride that can deliver the required therapy directly to the wound site.

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11.0 Appendix

Androgens inhibit phagocytosis by macrophages via the androgen receptor

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BACKGROUND

- Post-surgical wound infections caused by pathogens such as methicillin resistant *Staphylococcus aureus* (MRSA) are the third most common cause of nosocomial infection.[1]
- Non-healing wounds predominantly occur in the elderly and frequently become colonised by bacteria.[2, 3]
- Treatments for chronic wounds cost the UK national health service (NHS) over £1 billion annually.[4] An aging population and the emergence of antibiotic-resistant bacteria highlights the urgent need to identify novel therapeutic approaches to treat infected chronic wounds.
- Steroid hormones are key mediators of wound repair, with both human and animal studies demonstrating endogenous estrogens accelerate healing whereas androgens inhibit cutaneous repair. [5, 6]
- Age-related impaired healing in the elderly is associated with declining levels of estrogen in both genders. [5] In contrast, androgens levels are largely sustained in elderly males, supporting emerging data that elderly males heal more slowly than elderly females. [6]
- However, the effect of androgens on the clearance of typical wound bacteria is largely unknown.

AIM

To characterise the effect of endogenous androgens on the phagocytic function of macrophages using an *in vitro* model of host-pathogen interaction.

METHODS

- U937 monocytes were cultured and differentiated into U937 macrophages using PMA (50ng/mL for) 72 hours (Figure 1).

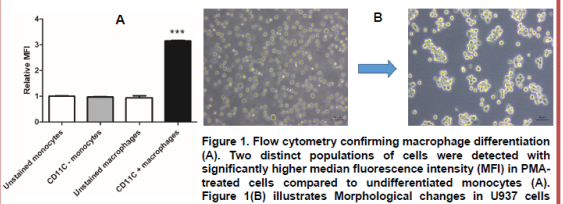

Figure 1. Flow cytometry confirming macrophage differentiation (A). Two distinct populations of cells were detected with significantly higher median fluorescence intensity (MFI) in PMA-treated cells compared to undifferentiated monocytes (A). Figure 1(B) illustrates Morphological changes in U937 cells after treatment with PMA for 72 hours.

- U937 macrophages were treated with (10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} M) testosterone (T) or dihydrotestosterone (DHT), with/without anti-androgens (10^{-6}M) including androgen receptor (AR) antagonists (flutamide, cyproterone acetate) or a 5 alpha (5a)-reductase inhibitor (finasteride) for 24 hours prior to stimulation with LPS and IFN-g for 5 hours.
- Phagocytosis of MRSA strain 11 or GFP-*S. aureus* (10^3 CFU/ml) by the U937 macrophages was then determined by bacterial recovery following 3 hours of host-pathogen interaction. Fluorescent microscopy and scanning electron microscopy (SEM) were used to visualise host-pathogen interactions using GFP- *S. aureus* and MRSA.

CONCLUSION

This study confirms androgens inhibit the clearance of *S. aureus* by macrophages in a dose-dependent manner. The findings suggest androgens act through the androgen receptor (AR), principally via conversion of T into the more potent DHT. The use of a 5-α reductase inhibitor such as finasteride enhances bacterial clearance by preventing T-induced inhibition of phagocytosis. Furthermore, androgen-mediated inhibition of phagocytosis is blocked by androgen receptor (AR) antagonists such as flutamide. Thus, the development of novel wound dressings that provide local AR blockade and/or prevent conversion of T to DHT may be an effective treatment to promote the bacterial clearance of colonised wounds, particularly chronic wounds in elderly males.

1) Sisirak, M et al. Bosnian Journal of Basic Medical Sciences, 2010; 10(1):32-37. 2) James, GA et al. Wound Repair Regeneration, 2008; 16(1):37-44. 3) Menke, NB et al. Clinical Dermatology, 2007; 25(1):19-25. 4). Harding, K et al. British Medical Journal, 2002; 324(7330):160-162. 5) Ashcroft, G. S et al. The American Journal of Pathology, 2007; 155(4): 1137–1146. 6) Gilliver SC et al. Journal of Cell Science, 2006; 119(4):722-732.

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RESULTS

Both T and DHT significantly (P<0.01) inhibited phagocytosis, increasing the recovery of MRSA (CFU/mL) in a dose-dependent manner compared to the negative control (Figure 2 & 3).

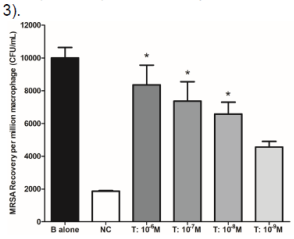

Figure 2. Effect of testosterone (T) on the recovery of MRSA (n=25)

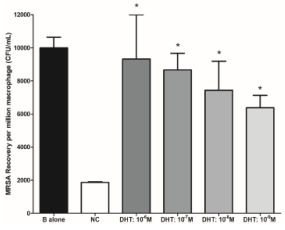

Figure 3. Effect of dihydrotestosterone (DHT) on the recovery of MRSA (n=25)

The addition of anti-androgens significantly (P<0.01) reversed the inhibitory effect of T on phagocytosis (Figure 4) . In contrast, only the AR antagonists, flutamide and cyproterone acetate, significantly (P<0.01) reversed the inhibitory effects of DHT on phagocytosis (Figure 5) . The 5a-reductase inhibitor finasteride showed no statistical effect on DHT-mediated inhibition of phagocytosis but significantly (P<0.01) reversed the inhibitory effect of T on phagocytosis.

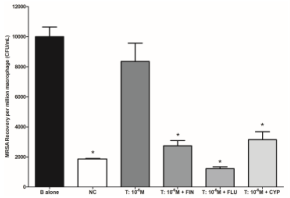

Figure 4. Effect of antagonists Finasteride (FIN), Flutamide (FLU) or Cyproterone Acetate (CA) on T-mediated inhibition of phagocytosis of MRSA (n=25)

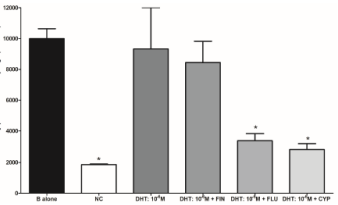

Figure 5. Effect of antagonists Finasteride (FIN), Flutamide (FLU) or Cyproterone Acetate (CA) on DHT-mediated inhibition of phagocytosis of MRSA (n=25)

Fluorescent Microscopy

Fluorescent microscopy confirmed internalization of GFP- *S. aureus* (green) by U937 macrophages (red). Treatment with T/DHT resulted in reduced *S. aureus* internalization compared to untreated cells (Figure 6).

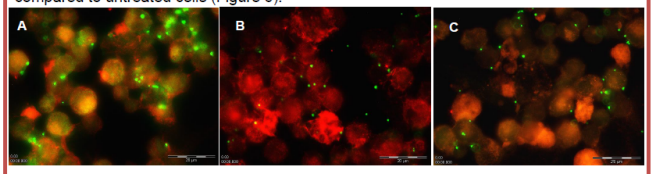

Figure 6. Internalization of GFP-*S. aureus* by a) untreated, b) testosterone (T)- and c) dihydrotestosterone (DHT)- treated U937 macrophages (stained with red-phalloidin) following 3 hours host-pathogen interaction with GFP-*S aureus* (green). Images were captured by epifluorescent microscopy (100X).

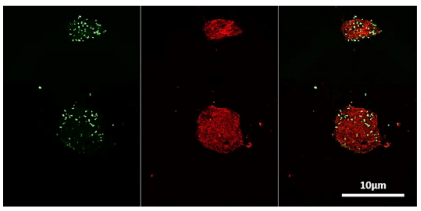

Figure 7. GFP - *S. aureus* localized inside untreated Phalloidin-stained U937 cells after eliminating extracellular bacteria. Images were captured using a confocal microscopy (63X)

Scanning Electron Microscopy

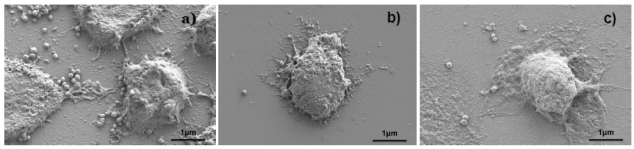

Figure 8: SEM images of a) untreated, b) testosterone (T)-treated (10^{-6}M) and c) dihydrotestosterone (DHT)-treated (10^{-6}M) U937 macrophages following 3 hours host-pathogen interaction with MRSA.