Gender differences in T cell regulation and responses to sex hormones

By

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ABSTRACT

Conflicting effects of sex hormones on the immune system could potentially explain the increased susceptibility of females to autoimmune diseases. In this study, I wanted to explore the regulation of the response to sex hormones in T cells from male and female donors. We initially investigated the levels of gene expression for sex hormone receptors and sex hormone metabolising enzymes in $CD45RA^+$ /$CD4^+$ T cells from male and female donors at baseline and after *in vitro* stimulation. I found that expression of 5α-reductase 1, an enzyme which converts testosterone into the more active dihydrotestosterone (DHT), is upregulated both on the mRNA and protein level in T cells from female but not male donors after stimulation. Since androgens are generally thought to have an anti-inflammatory role, this may be a mechanism that regulates the exposure of stimulated T cells to the inhibitory influence of DHT. SLE is a systemic autoimmune disease which mainly affects women. I investigated the regulation of 5α-reductase in peripheral blood mononuclear cells from patients with active or inactive SLE in comparison to normal controls. I did not observe any significant differences in 5α-reductase 1 expression in T cells at the baseline or after stimulation between SLE patients and healthy controls. However, I did find a significantly higher expression of 5α-reductase in B cells from SLE patients compared to healthy controls, suggesting that SLE B cells are increasing their exposure to DHT. In a third section of my experimental work I investigated the effects of a range of physiological concentrations of testosterone and DHT on T cell stimulation and cytokine production. *In vitro* treatment of T cells from female donors with low doses of testosterone resulted in a significant increase of the proportion of IL-2-producing $CD4^+$ T cells. Intriguingly, this effect was neither seen at higher testosterone concentrations nor with DHT, suggesting that it may not be mediated through the androgen receptor. As testosterone, but not DHT can be converted to 17β-oestradiol by aromatase, we tested whether tamoxifen, a competitive antagonist of the oestrogen receptor could block the increase in IL-2 production induced by low doses of testosterone in T cells. These experiments confirmed that low doses of testosterone could lead to oestrogen dependent upregulation of IL-2 production in T cells. In conclusion, we have observed both long-term effects of the hormone environment the T cells have been generated in as well as immediate effects of sex hormones on T cell responses. Attempts have been made to therapeutically target the sex hormone system in patients with autoimmune diseases. For the efficient development of these therapies we need to improve our understanding of the regulatory effects of androgens and oestrogens on the human immune system.

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ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
APC	Antigen Presenting Cells
AR	Androgen Receptor
ARE	Androgen Response Element
BILAG	British Isles Lupus Assessment Group index
DHEA	Dehydroepiandrosterone
DHT	α-Dihydrotestosterone
dsDNA	Double-stranded DNA
EAE	Experimental Autoimmune encephalomyelitis
ER	Oestrogen receptor
ERE	Oestrogen Receptor Element
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin-releasing hormone
HPA-axis	Hypothalamus-Pituitary-Adrenal axis
HPG-axis	Hypothalamus-Pituitary-Gonadal axis
IFNs	Interferons
LH	Luteinizing hormone
MHC	Major Histocompatibility Complex
MS	Multiple Sclerosis
NA-IC	Nucleic Acid-Immune Complex
PPAR	Peroxisome Proliferator-Activated Receptor
RA	Rheumatoid Arthritis
SHBG	Sex Hormone Binding Globulin
SLE	Systemic Lupus Erythematosus
TCR	T-Cell Receptor
TGF	Transforming Growth Factor
TLR	Toll-Like Receptor
TNF	Tumour Necrosis Factor

1 INTRODUCTION

1.1 The immune system

1.1.1 Introduction

The main role of the immune system is to protect the body from infections caused by harmful foreign pathogens including bacteria, viruses and fungi. It can be divided into two arms; the innate and the adaptive (also known as acquired) system. The innate system is usually the first to encounter any newly invading pathogens. Both systems involve cell-mediated responses, with cells directly removing pathogens by phagocytosis or cytotoxicity, and humoral responses, which use soluble immune mediators such as antibodies, cytokines and the complement system. The innate immune system rapidly responds to infection by firstly recognising the presence of an infection and then secondly removing pathogens. Importantly, the response needs to be tightly controlled and down-regulated after the infection has been cleared to prevent the initiation of inappropriate inflammatory responses, which could lead to tissue and organ damage.

Cellular components of the innate immune system include macrophages, neutrophils, natural killer (NK) cells, mast cells, eosinophils and basophils. Phagocytes such as macrophages and their precursors monocytes ingest pathogens by a controlled process called phagocytosis. Phagocytes recognise pathogens through pathogen-associated molecular patterns (PAMPs), which are a group of molecular structures found on pathogens and are recognised by pathogen recognition receptors (PRRs). PRRs include a range of receptors that recognise

different PAMPs on a diverse range of pathogens. For example, macrophages use toll-like receptors (TLRs) to recognise cell wall components of Gram-positive bacteria through binding to TLR2, whereas TLR4 binds lipopolysaccharide (LPS) found in the cell wall of Gram-negative bacteria. Activation of plasma proteins known as complement mediates the recognition of pathogens by coating the surface of bacteria with complement fragments in a process known as opsonisation. They are then recognised by complement receptors on phagocytes, which subsequently result in the phagocytosis of the bacteria. The process of opsonisation is greatly increased by the presence of specific antibodies, a classical example for the interaction of innate and adaptive immunity (Mantovani 1975). Activation of phagocytes results in the production of other immune mediators such as cytokines and chemokines, which are responsible for the recruitment and activation of other immune cells such as dendritic cells, natural killer cells, B cells and T cells, and therefore initiating the involvement of the adaptive immune system.

While the innate immune system responds rapidly, often within minutes, it only recognises a limited number of structures on the surface of pathogens. It recognises groups of pathogens but has a limited specificity for individual species. This is in contrast to the adaptive response, which is slower in its response to new infectious agents, but can subsequently recognize a virtually unlimited number of different structures known as antigens. Furthermore, the adaptive immune system will generate immunological memory by employing specific cells that recognise pathogens previously encountered by the immune system in order to initiate a more rapid response to the infection upon re-exposure. Antigen presenting cells (APC) provide a key link between the innate and adaptive immune system. APCs such as macrophages, dendritic cells and B cells are able to process pathogens

(internalised through phagocytosis or receptor-mediated endocytosis), resulting in the production of short peptides generated from the pathogen are then displayed on the cell surface associated with cell surface glycoproteins called major histocompatibility complex proteins (MHC). These antigenic peptides are then recognised by specific T cells and lead to the differentiation, proliferation and effector function of effector and memory T cells. Activated B cells recognize antigens through the B cell receptor independently of antigen presentation and are able to differentiate into memory B cells or plasma cells, which produce antibodies specific to the encountered antigen. Effective antibody production also involves specific interaction processes between antigen specific T and B cells, in which T helper cells are able to activate B cells through CD40/CD40L interactions.

1.1.2 T cells in adaptive immunity

T lymphocytes (T cells) are antigen specific cells that play a vital role in developing the adaptive immune response. Upon stimulation and differentiation, T cells can produce a wide range of cytokines; specifically kill virus infected cells; provide memory, and contribute to self-tolerance. Immature T cells migrate from the bone marrow to the thymus and after development in the thymus; mature naive (not previously encountered antigen) T cells that express either $CD4^+$ or $CD8^+$ are released into the periphery. $CD4^+$ T helper cells are involved in mediating immune responses either by cytokine production or cognate interaction with other immune cells such as B cells once antigen has been recognised. $CD8^+$ cytotoxic T cells are involved in removal of virally infected and malignant cells. Further $CD4^+$ T cell lineages identified, the first being the $CD45RO^+$ subset (Akbar *et al.* 1988) Later, Mossman and Coffman described the Th1 and Th2 subsets based on the differential cytokine production (Mosmann *et al.* 1986). Differentiation into Th1 cells is driven by cytokines IL-12 and IFN-γ

(also produced by Th1 cells), and transcription factors STAT1, STAT4 and T-bet. Transcription factors STAT6 and GATA-3, and cytokine IL-4 (produced by Th2 cells, and also by mast cells, eosinophils and basophils) promote the development of Th2 cells. Th1 cells are important in defence against intracellular pathogens such as viruses, intracellular bacteria and tumour antigens and are involved in delayed type hypersensitivity reactions. They activate macrophages to increase their phagocytic activity and upregulate expression of MHC class II and other proteins involved in antigen presentation by these cells. In contrast to Th1 cells, Th2 cells are predominately involved in removal of extracellular based pathogens and allergic immune responses via the production of IL-4, IL-5 and IL-13.

In recent years, many more $CD4^+$ T cells subsets have been identified (Palmer and Weaver 2010), which have been characterised by the transcription factors needed for development of these specific T cell lineages and also the signature cytokines each subset produces. T regulatory cells (Treg) differentiation is driven by the transcription factor Foxp3 and cytokine TGF-β. Transcription factors RORγt, RORα and STAT3 alongside cytokines; TGF-β; IL-6, and IL-23 promote the development of Th17 cells, which produce IL-17, IL-21 and IL-22.

Unlike B lymphocytes, T cells cannot recognise native antigen; thus for T cell stimulation the antigen must be presented to the T cell via the major histocompatibility complex (MHC) on antigen presenting cells (APC) such as B cells, dendritic cells and macrophages (Bretscher 1999). Two signals are required for T cell activation: the first signal comes from the interaction of the T cell receptor (TCR) on the T cell with the MHC associated antigen. The

second signal comes from interaction between co-stimulatory molecules CD80 and CD86 on APC and CD28 on the T cell surface. An alternative ligand expressed by the T cells is CTLA-4, however this mediates an inhibitory signal. This second signal derived from CD80/ CD86 interaction with CD28 is important in T cell self tolerance since antigen recognition in the absence of co-stimulation inactivates naïve T cells, inducing a state of anergy (Sharpe and Freeman 2002). It stimulates the production of IL-2, which is needed in clonal expansion after T cell activation. Salomon and colleagues demonstrated the importance of CD80/CD86 interaction with CD28 in self tolerance and prevention of autoimmune responses through the demonstration of significant reduction in regulatory $CD4^+/CD25^+$ T cells in CD28 knockout mice resulting in exacerbated diabetes in non-obese diabetic (NOD) mice compared to wild type mice (Salomon *et al.* 2000). CTLA-4 (also known as CD152) has also been shown to be important in maintenance of self tolerance in peripheral effector T cells. The fatal condition of lymphoproliferative disease has been observed in CTLA-4 knockout mice and results in significantly elevated levels of T cell stimulation (Ise *et al.* 2010). CTLA-4 is part of the CD28 family and has shown to act as part of a negative feedback system in which T cell activation leads to the binding of B7 and CTLA-4. CTLA-4 delivers an inhibitory signal to the cell, reducing reducing cell proliferation, IL-2 production and T cell mediated immune responses. CTLA-4 has a vital role in preventing self-reactive effector T cells (that have escaped negative selection) from clonal expansion, being expressed by both effector T cells and regulatory T cells.

1.1.3 Autoimmunity

Autoimmunity is defined as the reaction of the adaptive immune system to self-antigen due to loss of immunological tolerance. Autoimmune diseases arise from the organ/tissue damage

caused by autoreactive T or B cells through both cell-mediated and humoral immune responses resulting in array of clinical manifestations. The immune system has various check points in place during lymphocyte development to prevent the maturation of autoreactive lymphocytes. The first stage of inducing tolerance is known as central tolerance, in which immature B and T lymphocytes undergo negative selection to eliminate autoreactive lymphocytes. In the bone marrow, immature B cells expressing IgM antigen receptors that strongly cross-link multivalent self antigens such as MHC molecules are subsequently induced to undergo programmed cell death, apoptosis. These self-reactive cells can be saved from cell death by undergoing receptor editing, in which the light chain of the receptor is rearranged resulting in a new receptor specificity being expressed, thus removing self-reactivity (Hardy and Hayakawa 2001; Tsuiji 2006).

T cell development in the thymus also involves negative selection of self-reactive cells. Firstly, immature T cells receive survival signals if their T-cell receptor demonstrates compatibility with self-MHC molecules on thymic cortical epithelial cells; this is known as positive selection. However, if the TCR expressed by the immature T cell interacts strongly with self peptides associated with self-MHC expressed by antigen presenting dendritic cells in the medulla, then these self-reactive T cells are not able to enter the periphery but are instead removed via apoptosis. This process is known as negative selection.

Once mature lymphocytes have left the central lymphoid organs, autoreactive lymphocytes are held in check by peripheral tolerance mechanisms. Mature self-reactive T cells recognising self antigen can escape the tolerance checkpoints during the early developmental stages as the self antigen they recognise may not be present in the central lymphoid organs,

and therefore continued in the development process without deletion. Mechanisms which prevent the potential destruction caused by autoreactive lymphocytes include anergy (Rocha and von Boehmer 1991), in which self reacting T cells become functionally unresponsive due to absence of co-stimulation by APCs, thus resulting in insufficient cell activation and can be removed by apoptosis. In the absence of added stimuli, termed "danger signals" (Matzinger 2002), proinflammatory mediators such as cytokines are not produced and co-stimulatory molecules on activated APCs are not expressed, therefore adaptive immune activation is suppressed. Another mechanism used in peripheral tolerance is through the use of Tregs that suppress effector T cells by anti-inflammatory cytokines such as IL-10 and TGF-β and expression of CTLA-4. Self-reactive effector T cells can also be differentiated into induced Tregs in the presence of TGF-β (Bluestone and Abbas 2003).

Autoimmunity can occur when mechanisms involved in inducing tolerance are defected or absent. Autoimmune diseases can either be organ-specific or systemic, in which multiple organs are targeted. Organ specific diseases such as multiple sclerosis (MS), type 1 diabetes mellitus and Hashimoto's thyroiditis arise via inflammation mediated tissue/organ damage in the CNS, pancreas and thyroid respectively. Systemic diseases include rheumatoid arthritis (RA), which primarily involves inflammation and destruction of joints, and systemic lupus erythematosus (SLE), in which organs such as the skin, kidneys, lungs, are targeted by autoantibodies and resulting immune complexes. Many autoimmune diseases are characterised by the presence of autoantibodies, for example autoantibodies specific for the myelin sheath in MS (Genain *et al.* 1999); anti-citrullinated protein antibodies (ACPA) and rheumatoid factor (RF) in rheumatoid arthritis (RA) (de Vries-Bouwstra *et al.* 2008); and autoantibodies specific for double stranded DNA in SLE (Swaak and Smeenk 1985). A

significant discovery was the important involvement of T cells in the pathogenesis of various autoimmune conditions.

1.1.4 The role of T cells in autoimmunity

T cells for a long time have been viewed as the main instigators in autoimmune diseases due to their importance in maintaining self tolerance. The $CD4^+$ T cell lineage in particular has been identified as a mediator of autoimmunity (Luger *et al.* 2008; Palmer and Weaver 2010). Many findings support this notion, for example the accumulation of activated $CD4^+$ T cells at sites of inflammation and the association of autoimmune diseases with HLA types (Goronzy and Weyand 1993; Viallard 2001). The involvement of T cells in the development of autoimmune disease has been demonstrated by the ability of self-reactive T cells to confer disease when adoptively transferred from a diseased animal to a healthy one. For example, the transfer of autoreactive $CD4^+$ T cells from mice with experimental autoimmune encephalomyelitis into healthy mice induces disease (Luger *et al.* 2008). Autoreactive $CD8^+$ T cells have been implicated in autoimmune diseases such as type I diabetes, as pathogenic self-reactive $CD8^+$ T cells destroyed insulin-producing beta cells in the pancreas of NOD mice (Nagata *et al.* 1994). A significant finding, which implicated $CD4^+$ T cells in autoimmune diseases, was the observation that susceptibility is associated with MHC genotype, as most diseases have been linked strongly to MHC class II alleles. For example, the MHC II allele subunit human leukocyte antigen-DR (HLA-DR4) is associated with RA (Goronzy and Weyand 1993; Walser-Kuntz *et al.* 1995). As $CD4^+$ T cells recognise antigen associated with MHC class II, whereas $CD8^+$ T cells recognise antigen peptides associated with MHC class I, therefore abnormalities within MHC class II associated genes

sparked the growing interest of $CD4^+$ T cells in autoimmunity. In addition to the identification of MHC genes in controlling susceptibility to autoimmune disease, single nucleotide variants (SNP) in gene loci encoding proteins involved in regulation of T cell activation such as protein kinase C (PKC)-theta, PTPN22 and CTLA-4 are also associated with autoimmune diseases (Barton *et al.* 2008; Fernandez-Blanco *et al.* 2004; Menard 2011; Rieck 2007). While genetic risk factors for autoimmune diseases have been identified most autoimmune conditions are regarded as complex diseases involving both genetic and environmental factors (such as smoking) (Padyukov *et al.* 2004). These factors ultimately result in aberrant immune activation and the production of various immune mediators responsible for the development of destructive immune responses. For example in RA, cytokines contribute to the clinical manifestations seen such as joint synovial tissue inflammation and bone destruction (McInnes and Schett 2007). In MS, myelin specific $CD4^+$ T cells that have crossed the blood-brain barrier are reactivated, leading to inflammation and destruction of the myelin sheath surrounding nerves (demyelination) via the production of cytokines and recruitment of other inflammatory cells (Langrish *et al.* 2005).

Autoimmune diseases such as RA and MS have been thought for a long time to be driven by T-helper cell type 1 (Th1). IL-12 is required for the differentiation into Th1 cells and has been thought to play a key role in the pathogenesis of Th1 driven autoimmune diseases (Leonard *et al.* 1995; Matthys *et al.* 1998). However, the role of the Th1 subset in the pathogenesis of autoimmunity has been questioned due to the discovery of the Th17 subset (Harrington *et al.* 2005), which has been shown to be important in the development of autoimmune conditions. Th17 cells have been characterised by the production of cytokine IL-17. Th17 differentiation depends particularly on the presence of cytokines such as IL-23,

TGF-β, and IL-6. IL-23 has recently been shown to be part of the IL-12 family as it shares the p40 subunit with IL-12 (Oppmann *et al.* 2000). For the cytokine IL-23 the p19 subunit dimerizes with the p40 subunit, whereas for IL-12 the p35 subunit dimerizes with p40. Before this observation was made, the lower susceptibility to autoimmunity observed in p40 deficient mice had been attributed to the role of IL-12 in the promotion of Th1 differentiation. Now studies have shown that both p40 and p19 promote autoimmune diseases in mouse models, but p35 was not necessary for the induction of autoimmunity (Gran *et al.* 2002). Such findings indicate the requirement of IL-23 but not IL-12 for disease development (Cua *et al.* 2003; Langrish *et al.* 2005). The mouse models experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA) have shown that in mice Th17 cells and the presence of IL-23, which is required for differentiation and proliferation of Th17 cells, are responsible for the progression of autoimmunity due to the production of pro-inflammatory cytokines such as IL-6, IL-1 and TNF-α by Th17 cells (Nakae *et al.* 2003; Serada *et al.* 2008). In contrast, mice deficient in IFN-γ, IFN-γR, IL-12Rβ2, and the IL-12p35 chains had an increased susceptibility to developing EAE and CIA, thus suggesting a protective role for IL-12 and IFN-γ mediated responses (Ferber *et al.* 1996; Willenborg *et al.* 1996; Zhang *et al.* 2003).

Due to the important role of the Treg subset; either originated from the thymus known as natural Treg or induced in the periphery by transforming growth factor β (TGF-β) known as iTreg, in the regulation of effector T cell function by inducing tolerance and having the ability to suppress T cell activation and proliferation, various studies have been carried out to investigate if Treg activity is suppressed in autoimmune conditions (Haufe 2011; Korn 2007; Yan 2008). However, it has been demonstrated multiple times that there are elevated numbers

of $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs in the synovial fluid of RA patients (Haufe 2011; Mottonen 2005; Ruprecht 2005), and this is further supported by the enhanced demethylation status of the FOXP3 locus within synovial fluid derived $FOXP3^+$ Tregs (Janson 2011). Therefore, Tregs themselves might not be defected but the pro-inflammatory environment could suppress Treg responses as various in vitro studies have shown Tregs isolated from inflamed RA joints are functional. This theory has been investigated in an animal model for SLE (Parietti *et al.* 2008) and in SLE patients (Yan 2008), in which impaired Treg functions were demonstrated in the presence of pro-inflammatory cytokines IL-6 and IFN-α produced by APCs. Tregs have also been shown to be responsive to TNF (Chen 2008; Chen 2010), which is an interesting finding as TNF plays a pivotal role in the development of RA. However, the effects of TNF on Treg responses have conflicting outcomes as mouse models of inflammation have shown TNF to mediate the expansion of the Treg population (Chen *et al.* 2007). In contrast, human in vitro studies have shown TNF is able to suppress Treg proliferation, and in inflamed RA joints there is an inverse correlation between the numbers of Treg cells and the levels of TNF (Herrath 2011). Anti-TNF therapy has shown to compensate for the defect natural Treg population in RA by increasing the population of a Treg subset characterised with a $CD4^+$ $CD25^{high}$ $CD62L^-$ $FOXP3^+$ phenotype, which reduced the production of pro-inflammatory cytokines through a TGF-β dependent mechanism (Ehrenstein 2004; Nadkarni *et al.* 2007).

1.2 Systemic Lupus Erythematosus (SLE)

1.2.1 Introduction

Systemic Lupus Erythematosus (SLE) is an autoimmune disease involving the breakdown of tolerance to nuclear self antigens, leading to the production of autoantibodies and finally resulting in systemic inflammation and tissue damage. There are 5 million people worldwide suffering from SLE and the most common symptoms include extreme fatigue, joint pain, weakened muscles, rashes and hair loss. There is a significant gender bias in SLE with females more likely developing the disease with a 9:1 female to male ratio (Washburn *et al.* 1965; Whitacre 2001). There is also a prominent association of SLE with ethnic background, occurring more often in women from an Afro-Caribbean or Asian background then Caucasian women. SLE has an estimated prevalence of 28 per 100,000 in the general adult UK population, while in Afro-Caribbean women prevalence is around 206 per 100,000. Flares of SLE usually arise during childbearing years (usually between the ages of 15-55yrs) and continue on a cycle of flares and remission for many years often leading to multiple organ damage. The clinical manifestations of SLE are variable. However, approximately 90% of patients present with joint pain, and 75% present with skin rashes. A very well recognised clinical feature of SLE is the appearance of a rash described as butterfly-shaped spanning the face.

1.2.2 Pathogenesis of SLE

The presence of high levels of autoantibodies, in particular anti dsDNA (double-stranded DNA) and anti-nuclear antibodies, is a common biomarker for the development of SLE (Swaak and Smeenk 1985). Many organs are affected in SLE by increased inflammation

which may lead to sometimes life threatening complications such as cardiovascular disease and lupus nephritis (Clynes *et al.* 1998). Also, SLE patients are also likely to develop additional autoimmune conditions such as Sjogren's syndrome, thyroid disease and Hughes syndrome (anti-phospholipid syndrome).

A wide range of autoantibodies against ubiquitous cellular constituents are detected in SLE patients. The main antigens targeted are intracellular nucleosome particles: the nucleosome subunits of chromatin, the spliceosome, and a small cytoplasmic ribunucleoprotein complex containing two proteins known as Ro and La (Arbuckle 2003). These autoantigens become extracellular when exposed on dead and dying cells and are released from injured tissues. The resulting generation of immune complexes comprising autoantibodies and these autoantigens occurs continuously due to the wide availability of the antigens (figure 1.1). Immune complexes may become deposited in the walls of small blood vessels in the renal glomerulus, the glomerular basement membrane, joints, and other organs (Brentjens *et al.* 1975; Churg *et al.* 1980; Schmiedeke *et al.* 1989). This leads to the activation of phagocytic cells through their Fc receptors, resulting in tissue damage and further release of nucleoprotein complexes which initiates further generation of immune complexes. During this process, autoreactive T cells become activated and provide important help to B cells, but can also be directly pathogenic, forming part of the cellular infiltrates found in the skin and kidney and blood vessels. The resultant inflammation in affected tissues may ultimately be sufficient to induce patient mortality.

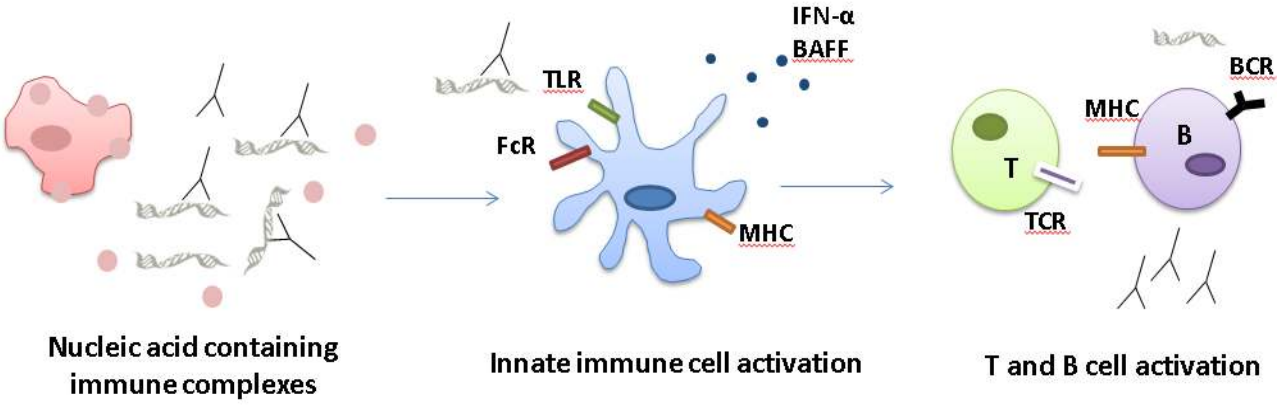

Figure 1.1 Formation of immune complexes through ineffective apoptotic cell clearance and activation of immune responses.
Nucleic acid autoantigens become extracellular when exposed on dead and dying cells and are released from injured tissues. The resulting generation of nucleic acid containing-immune complexes comprising of autoantibodies and autoantigens occurs continuously due to the wide availability of the antigens. This leads to the activation of phagocytic cells through their Fc receptors and TLRs (toll-like receptors), resulting in the production of pro-inflammatory mediators and further release of nucleoprotein complexes, which initiates further generation of immune complexes. During this process, autoreactive T cells become activated and provide important help to autoreactive B cells thus further initiating the production of autoantibodies against nucleic acid.

1.2.3 Treatment of SLE

Treatment options for SLE include non-steroidal anti-inflammatory drugs, antimalarial agents, glucocorticoids, and immunosuppressive agents. The choice of therapy is primarily determined by disease severity and the function of the organs involved. Non-steroidal anti-inflammatory medications are often used in the treatment of mild symptoms to reduce inflammation and dampen joint and muscle pain.

Hydroxychloroquine has previously been used in the treatment for malaria but has shown to reduce SLE symptoms such as fatigue, muscle and joint pain, and skin rashes. Corticosteroids are widely used to reduce inflammation in cases of SLE; however they can lead to many unwanted and serious side effects. Corticosteroids are commonly used in combination with

immunosuppressants such as azathioprine and cyclophosphamide to improve symptoms and limit organ damage in severe cases of SLE. In addition, rituximab, which was originally developed as a cancer drug, has been used off-label and in clinical trials for the treatment of SLE (Merrill 2010). Rituximab is a monoclonal antibody, which depletes CD20-expressing B cells. Since large scale clinical trials showed disappointing results it has not yet been approved for routine therapy.

A further example of a drug used to target B cells is Belimumab, a human monoclonal antibody raised against a cytokine called B cell activating factor (BAFF) also known as BLyS. BAFF is important in many stages of B cell development particularly in the regulating the selection of naïve B cell repertoire and the survival of mature B cells (Benson 2008). Low levels of BAFF induce the negative selection of early autoreactive B cells, while higher levels facilitate their progression into the mature B cell compartment (Lesley *et al.* 2004). BAFF serum levels positively correlate with disease activity and over expression of BAFF in mice led to the induction of SLE in a T cell independent manner. Clinical trials involving the treatment of SLE patients with Belimumab have shown to decrease disease flares and showed to improve serological activity (Furie 2011; Jacobi 2010; Stohl *et al.* 2012). Although the effects were modest, it has now received NICE approval and can be used to treat SLE patients.

1.2.4 Genetic basis of SLE

The events responsible for induction of SLE are not yet understood; however it is thought that certain genetic traits along with a yet unknown environmental trigger are responsible for the initiation of the disease. Most genetic polymorphisms associated with development of SLE been found in immune-response related genes (Graham 2008; Sanchez 2011).

Considerable evidence suggests that a key driving factor is an impairment or delay in the process of apoptotic clearance (Manderson *et al.* 2004; Marinez Valle *et al.* 2008). Persistent apoptotic cells may undergo necrotic cell death leading to abnormal innate immune responses associated with resultant danger signals, and accessibility to modified autoantigens resulting in the production of autoantibodies. These abnormal immune responses can result in irreversible tissue damage manifested in the clinical features of SLE.

The increased susceptibility of developing SLE among siblings and first degree relatives of SLE patients demonstrates the link between genetic factors and the occurrence of SLE. There is a strong association between gene polymorphisms in HLA class II and SLE, in which certain HLA class II genes have been associated with autoantibodies such as anti-Sm (small nuclear ribonuclear protein) and anti-DNA antibodies. Also, inherited deficiencies in HLA genes class III encoding for components of the complement system (C2 and C4) have also confer increased risk of developing SLE (Howard *et al.* 1986; Senaldi *et al.* 1988).

These abnormalities alongside defects in nucleic acid breakdown by DNase (caused by defects in the *Dnase1* gene) results in an increased exposure of self antigens and nucleic acids and a resulting immune response against antigens, which would not normally be detected by the immune system (Shin *et al.* 2004). Autoantibodies produced are able to form immune-complexes which are recognised by innate inflammatory receptors such as Fc receptors (FcR) and TLRs on or in innate immune cells thus resulting in activation of the innate immune system (Katayama *et al.* 1983).

Polymorphisms in genes encoding components of the innate immune system such as TLRs and TNF are also associated with SLE (Demirci *et al.* 2007; Graham 2008). There is a significant correlation between type 1 IFN levels and disease severity in SLE (Rönnblom *et al.* 2006), and polymorphisms in type 1 IFN have been associated with SLE (Baechler *et al.* 2003). Plasmacytoid dendritic (pDC) cells are a major producer of type 1 IFNs and are activated by nucleic acid-containing immune complexes (Båve *et al.* 2003). Type I IFNs are also able to induce genes involved in the TLR signalling pathway thus amplifying the inflammatory response (Baccala *et al.* 2007).

1.2.5 Defects of innate immunity in SLE

Abnormalities in the innate immune system have been shown to play a key role in the induction of the disease before any clinical features of SLE are visible. Targeting of self organs/tissue by innate immune cells may stem from the absence of safe and efficient clearance of apoptotic cell debris and nucleic acid, which can be opsonised by autoreactive IgM resulting in nucleic acid-containing immune complexes (NA-IC). Every day, vast numbers of cells undergo programmed cell death known as apoptosis. Therefore, it is extremely important that apoptotic cells and their content are effectively removed by phagocytosis without inflammation occurring. This involves a sequence of pathways taking place to prevent secondary necrosis of the cell and leakage of toxic cell components. In order to initiate phagocytosis of the apoptotic cells, apoptotic cells alter their cell membrane by exposing cytosolic phosphatidylserine on the surface and thereby signalling to phagocytes such as macrophages, monocytes and dendritic cells that the cell is undergoing apoptosis. Antibodies and the complement system are also essential for the safe removal of apoptotic cells by opsonising cells which initiates phagocytosis. DNases also assist this process by

breaking down nucleic acid released by apoptotic cells. Defects of these clearance mechanisms such as polymorphisms in C1q and DNase1 lead to diseases sharing features with SLE (Senaldi *et al.* 1988). Clearance abnormalities results in activation of innate receptors such as FcR, TLRs (inside and on the cell surface) on innate cells for instance macrophages, monocytes, dendritic and B cells. Signalling through internal TLRs can also occur when nucleic acid translocates to within the cell by the formation of endosomes, in which unprocessed TLRs are cleaved and interact with internalised nucleic acid. This in turn results in the activation of transcription factor nuclear factor kappa-light chain enhancer of activated B cells (NF-$_K$B) and leads to the production of proinflammatory cytokines such as type one IFNs (IFN-α and IFN-β), TNF, IL-6, IL-1 and IL-17. These inflammatory mediators then activate the adaptive immune system, in which self reactive and long lived T and B effector cells further amplify the proinflammatory immune response resulting in organ and tissue damage (Yasutomo 2003). This vicious cycle continues as autoreactive effector T cells help activate B cells to produce autoantibodies towards double stranded DNA accumulated through abnormal clearance mechanisms thus initiating more proinflammatory innate responses and the cycle repeats itself.

Recently it has also been reported that IFN-α can increase NETosis in neutrophils, which involves dying neutrophils extruding their DNA known as neutrophil extracellular traps (NETs) pDCs are then activated by NA-IC formed from NETs which leads to further production of IFN-α (Garcia-Romo 2011; Lande 2011).

1.2.6 The role of B cells in SLE

In SLE, B cells play a key role in the pathogenesis of the disease as a result of their maturation to pathogenic, autoantibody-secreting plasma cells, and their ability to act as APCs for autoreactive T cells, providing co-stimulation and initiating T cell activation and through production pro-inflammatory cytokines such as TNF and IL-6 In healthy individuals there is a population of low affinity IgM class autoantibodies responsible for mediating the safe clearance of apoptotic cell debris and nucleic acid, however, in SLE this B cell tolerance is disturbed meaning harmless self antigens promote sequential proinflammatory responses leading to irreversible systemic tissue and organ damage which are responsible for the clinical manifestations of SLE. In SLE, the B cell receptor (BCR) on the surface of autoreactive B cells is also able to bind to nucleic acid, which leads to activation and differentiation, and importantly the upregulation of transmembrane activator and calcium modulator ligand interactor (TACI), which binds to important B cell survival factors BAFF and April. BAFF produced by myeloid dendritic cells can also activate survival signals within self reacting B cells (Leadbetter 2002).

The breakdown of B cell tolerance may also be due to defects in the B cell developmental process, which results in the increased production of anti-double stranded DNA antibodies by long-lived autoreactive effector B cells, memory B cells and plasma cells. During B cell maturation immature B cells leave the bone marrow into the circulation, in which they are transported to secondary lymphoid organs, where they go through various check points to prevent maturation of autoreactive B cells, however defects in these checkpoints have been observed in SLE (Yurasov *et al.* 2005).

1.2.7 T cell defects in SLE

T cells play an important role in SLE pathogenesis by contributing to B cell differentiation, as evidenced by the observation that autoantibodies detected in SLE are class-switched and have therefore undergone somatic hypermutation in germinal centres with the help of cognate T cells (Odendahl *et al.* 2000). In addition, T cells directly infiltrate affected organs such as the renal parenchyma, causing tissue damage via direct cytotoxicity and recruitment and activation of macrophages (Kuroiwa and Lee 1998). Antigen-presenting myeloid dendritic cells and B cells with MHC associated with self antigen expressed on their surface are able to activate self-reactive T cells. There is a genetic association between risk alleles within the MHC region and SLE, which may contribute to the accumulation of self-reactive effector T cells. There are potentially genetic defects within the T cell development process as there are various check points in place to prevent the survival of self reactive T cells; these include negative selection within the thymus involving cell death of self reactive cells, receptor editing, induction of anergy to prevent T cell activation, and finally regulatory cells such as Tregs responsible for mediating effector T cell responses (von Boehmer and Melchers 2010). There are significant amount of risk alleles within the MHC region associated with SLE and could be an explanation for the loss of T cell tolerance and the accumulation of self reactive effector T cells.

There are also intrinsic defects within T cells from SLE patients that have been demonstrated; T cells produce very low levels of IL-2 after antigenic and mitogenic activation and are less responsive to IL-2 in comparison to healthy individuals. This may be due to an altered TCR complex that has the CD3 ζ subunit abnormally replaced by the homologous FcRγ chain (Liossis *et al.* 1998). Upon activation, signalling therefore occurs through spleen tyrosine

kinase (SYK) instead of ζ-chain-associated protein kinase 70 (*ZAP70*) resulting in an increase in intracellular calcium concentration. This then can lead to an imbalance in transcription factor activation in which cAMP response element binding protein (CREMB) is favoured over cAMP response element modulator (CREM) thus resulting in a decrease in IL-2 synthesis (Juang 2005). T cells from SLE peripheral blood also have higher CD40 ligand expression, which is important in activating B cells that express CD40 receptor. T cells may therefore have an increased capacity for activating self-reactive B cells, leading to enhanced proliferation and differentiation to autoantibody-producing plasma cells.

Another key finding associated with abnormal T cell responses in SLE, is the presence of defects in CTLA-4 in SLE T cells (Fernandez-Blanco *et al.* 2004). As previously mentioned, CTLA-4 plays a vital role in the regulation of T cell responses in an inhibitory manner; including suppression of prolonged immunological synapse formation, and inhibition of activation induced downstream signalling through the high concentrations of CTLA-4 present in the membrane lipid microdomains that are associated with TCR (Ise *et al.* 2010). CTL4-4 is also key component in regulatory responses by a large proportion of Tregs. CTLA-4 expression has shown to be significantly higher in SLE T cells in both $FOXP3^+$ and $FOXP3^-$ T cells compared to healthy individuals (Jury *et al.* 2010), however it's regulatory role in T cell signalling after subsequent TCR activation has shown to be deficient in SLE T cells, which was due to the absence of CTLA-4 in lipid microdomains, thus reducing CTLA-4 interaction with TCR (Jury *et al.* 2010).

1.3 Gender differences in autoimmunity

1.3.1 Introduction

It is well established that the prevalence of autoimmune conditions in females is significantly higher in than in males (figure 1.2). There is a significant difference in autoimmune susceptibility between genders; the female to male incidence ratio for RA is 3:1, in multiple sclerosis it is 2:1, and more drastic differences are observed in autoimmune conditions such as SLE, Hashimoto's thyroiditis and Sjogren's syndrome, in which the ratio is 9:1 (Whitacre 2001). However, there are a few exceptions to the higher incidence of autoimmunity in females such as Ankylosing Spondylitis, in which males are more susceptible with a male to female ratio of 3:1; studies have shown that mutations in androgen-responsive genes have been linked to the development Ankylosing Spondylitis (Tsui *et al.* 2005).

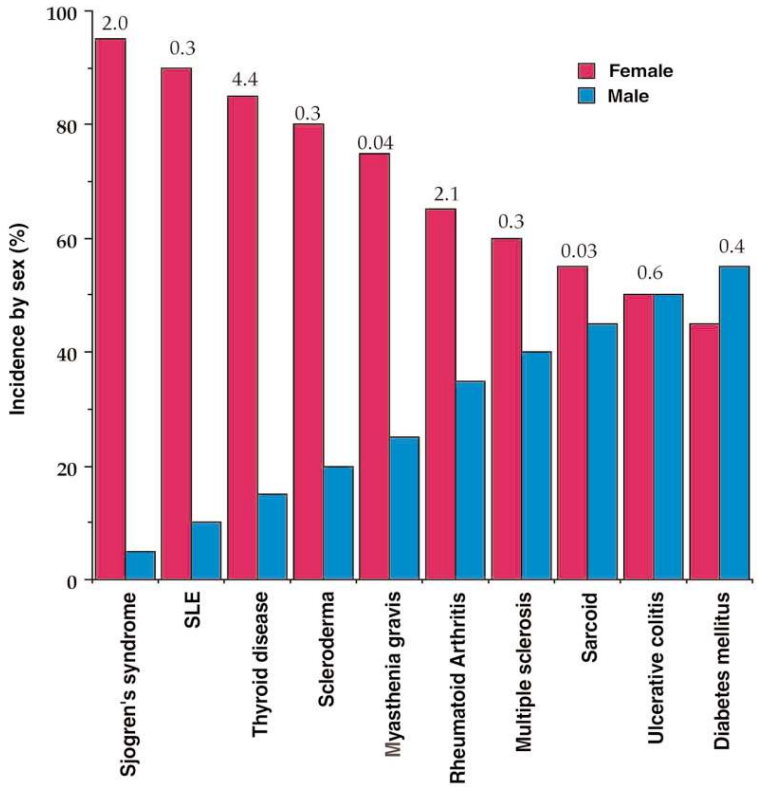

Figure 1.2 The incidences of autoimmune diseases by sex.
This shows the percentage incidence of various autoimmune conditions for both genders and the number of incidences (x 1,000,000) in females above each pink bar. Taken from Whitacre, 'Sex differences in autoimmune disease.' Nature (2001).

There are various factors that could be responsible for this sex difference, one being that females have been demonstrated to having a more robust immune responses, in comparison to their male counterparts, which also has been argued as the reason for significantly longer life spans in women compared to men (Aspinall 2000). It has been reported that women have stronger humoral and cellular immunity, and respond more robustly to infections, vaccinations and trauma by producing higher antibody titres, especially higher levels of serum IgM (Lichtman, Vaughan *et al* 1967) (Michaels and Rogers 1971). Also, females have shown to have a higher $CD4^+$ T cell count (Amadori *et al.* 1995), and predominately Th2

immune responses in contrast to males, who have more severe Th1 immune responses. From a very young age onwards severe bacterial and viral infections are far more frequent in males.

Environmental factors could also potentially explain the gender differences in autoimmunity. A Canadian study showed a rapid and significant increase in the female to male ratio for MS over the past 50 years (Orton *et al.* 2006), thus suggesting the potential role of environmental factors in the increased incidence of MS in females. For an example, there is evidence to suggest that exposure to chemicals and toxins found in cosmetics could increase the susceptibility of autoimmunity in females (Finckh *et al.* 2006; Wang *et al.* 2008). Studies have also investigated the influence of sunlight especially in MS, as low exposure to sunlight has shown to be a risk factor in developing MS (Pugliatti *et al.* 2002). Females have shown to have lower exposure to sunlight compared to males and also males tend to use sunscreen less often compared to their female counterparts (Hall *et al.* 1997). These differences in sunlight exposure between the sexes will thus have an effect on the production of steroid vitamin D, which has shown to have immune-modulating effects (Smolders *et al.* 2008). MS sufferers have shown be vitamin D deficient (Nieves *et al.* 1994), and in mice females supplemented with vitamin D were protected against the development of EAE (Experimental Autoimmune encephalomyelitis) (Spach and Hayes 2005) , which is the mouse model for MS.

Another area that has also been investigated extensively is pregnancy and its effect on autoimmune diseases. Lifestyle changes could also be responsible for the increase in female to male ratio for certain autoimmune diseases. Throughout evolution, women would have been pregnant and breastfeeding throughout their fertile life, and therefore would have been protected against the development of certain autoimmune conditions such as MS and RA,

which have shown to improve during pregnancy due to high oestrogen levels (Latman 1983; Offner and Polanczyk 2006). However, women are now having fewer children and much later in their fertile life, thus this protection from certain autoimmune diseases could be absent. Other then the effect of oestrogen (discussed later on in this chapter), there have been studies investigating microchimerism, which occurs during pregnancy and involves the transfer of haemopoietic cells from the foetus to the mother that then reside there for a short term or persist as either stem cells or lymphocytes. An environmental trigger can then break the tolerance of microchimeric cells towards the recipient and thus lead to the development of autoimmunity in the mother. This has been observed in the autoimmune disease scleroderma as it resembles many features of graft-versus host disease (Artlett 2003; Evans *et al.* 1999).

Trauma is also key area in which a significant sex difference is observed; males recover less efficiently from severe traumatic injuries such as haemorrhage and other physical traumas leading to more trauma-related deaths compared to females (figure 1.3) (Choudhry *et al.* 2007). There is a fine balance in which the immune system operates; it should protect the body from potentially harmful foreign antigens through the mediation of various immune cells and production of inflammatory mediators without these inflammatory processes damaging to tissue and organs. This balance is disturbed through the induction of traumatic injuries, thus leaving the body very vulnerable to severe infections. Firstly, trauma related injuries leads to an amplified proinflammatory response followed a significantly suppressed immune response (known as immune depression) resulting in severe bacterial or viral infections (Choudhry *et al.* 2006). The observation that males are more vulnerable to developing induced subsequent sepsis suggests that they have less robust immune systems compared to females. There is a significant reduction in pro-inflammatory cytokines

produced by macrophages such as IL-1, IL-6 and TNF-α, antigen presentation to T cells, and a decrease in T cell proliferation and production of cytokines such as IL-2 and IFN-γ. In contrast, there is a significant increase in anti-inflammatory cytokine IL-10.

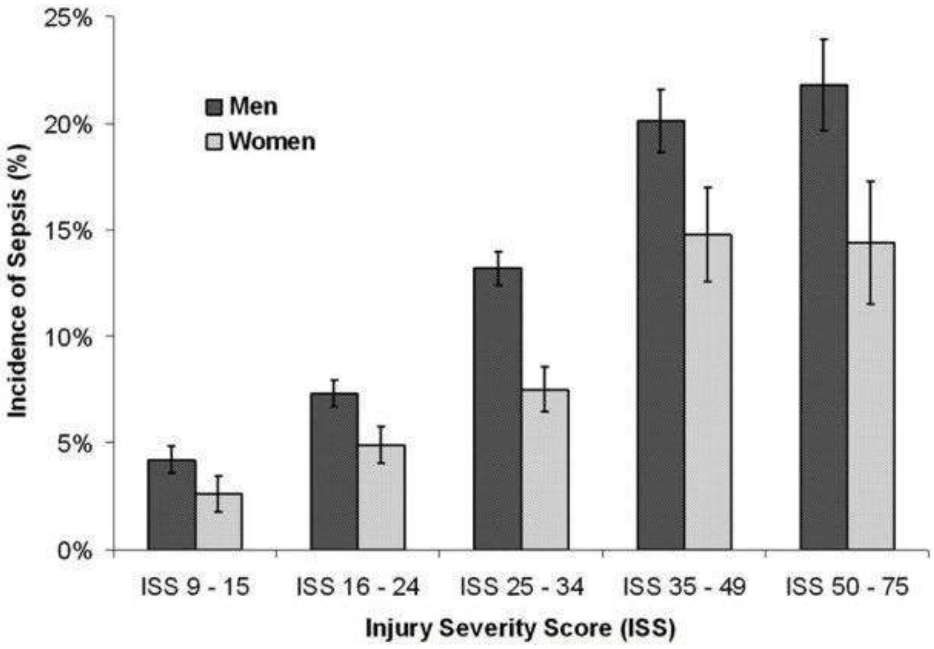

Figure 1.3 The incidence of sepsis in multiple trauma patients between the sexes. This shows the incidence of sepsis in males and females according to the patient injury severity score (ISS). Error bars show 95% confidence intervals. Taken from Wafaisade *et al,* 'Epidemiology and Risk Factors of Sepsis after Multiple Trauma'. Critical Care Medicine (2011)

These changes in immune cell responses however are not observed in females and remain constant during traumatic stress. This advantageous protection against various infections females have acquired is also responsible for disadvantageous increased susceptibility of many autoimmune conditions.

1.3.2 Genetic factors responsible for sex differences in autoimmunity

The obvious answer to the sex difference in autoimmunity may lie in sex linked genes. Various studies have addressed the role of the X chromosome on the immune defects seen in autoimmunity. During early stages of female embryogenesis one X chromosome is randomly silenced leading to its inactivation in order to prevent the double dosage of X chromosome genes. This process results in cellular mosaicism; this means that in females half the cells contain genes originating from the maternal X chromosome and half the cells containing genes from the paternal X chromosome. The X chromosome contains genes that directly or indirectly involved in the immune system, and therefore having two different sets of X chromosome derived genes protects females from deleterious gene-mutations and provides them with an increased immunological diversity (Spolarics 2007). This is clearly demonstrated in X-linked inherited recessive diseases, in which the phenotypes are more prominent in males as they only have one X chromosome. Many of these X-linked diseases significantly disrupt immune responses, these are known as X-linked primary immunodeficiencies (X-linked PIDs). The X chromosome codes for many genes involved in immune processes such as transcription factor forkhead box P3 (*FOXP3*), *CD40L*, *TLR-7* and interleukin-2 receptor-γ (*IL2RG).* Mutations in these genes lead to various loss of function defects within immune function resulting in severe immunedeficiency, thus being responsible for recurring viral and bacterial infections, which are often fatal in male newborns. X-linked chronic granulomatous disease (X-CGD) occurs from mutations in cytochrome b-245 β-subunit (*CYBB* or *NOX2*) (Teahan *et al.* 1987), which is important in superoxide production during phagocytosis; mutations in CYBB leads to deficient bacterial or fungal killing by phagocytes and an increased susceptibility to SLE. Another example of an X-linked PID is X-linked severe combined immunodeficiency (X-SCID) which arises due to a mutation in the *IL-2RG* gene which encodes the IL-2 receptor gamma chain common to receptors for several

interleukin receptors (Noguchi 1993). Mutations in this gene disrupt cytokine signalling, and in the absence of IL-7 and IL-15 signalling, T cell and natural killer cells fail to develop. Additionally there is decreased production of immunoglobulins by B cells (also demonstrated in X-linked CD40L deficiency). One loss of function mutation in particular is so detrimental that males die in *utero*; this condition is known as incontinentia pigmenti, whereas mutations that do not completely inactivate but reduce the activity of the protein lead to a rare congenital disease called anhidrotic ectodermal dysplasia. These conditions arise from mutations in the inhibitor of NF-κB kinase-γ gene (*IKBKG*), which is important in various innate inflammatory responses (Smahi 2000).

Although, inactivation of one X-chromosome in females occurs in order to prevent double dosage of X-linked genes and therefore protects females from lose of function and hypomorphic mutations, sometimes genes can escape silencing through cellular mosaicism, which can be beneficial as they can escape deleterious X-linked mutations and genes involved in immunity can enhance protection against infections in females. Genes found both on the X chromosome and the Y chromosome (XY homologous genes) usually escape inactivation, but can vary in levels of expression and unequal expression in different tissues (Ditton *et al.* 2004).

In females, a process called skewing of X chromosome inactivation occurs when X chromosome inactivation selects for or against alleles on the active X chromosome. This results in over-expression of either the maternal or paternal X-chromosome in certain tissues, which could be detrimental as this could lead to expression of mutant genes, however cells carrying normal X-chromosomes can outgrow cells carrying mutant alleles, thus protecting

against potential diseases (Migeon 2006). However, this so-called immune advantage in females can also be a disadvantage as seen in the increased susceptibility of autoimmune conditions in females, thus a loss in immunological tolerance must have some X-chromosome involvement.

There are three main theories as to how X chromosomes might be responsible for this sex difference in autoimmunity. Firstly, it has been proposed that loss of mosaicism might be associated with loss of tolerance; random X chromosome inactivation results in X chromosome mosaicism in females meaning different cells contain certain genetic material from the maternal side or from the paternal side (Kast 1977; Migeon 2006). However, if there is an imbalance of X-linked gene dosage (more maternal then paternal X-linked genes and vice versa) due to skewing of X chromosome inactivation, this can result in loss of tolerance as X-linked self-antigens may escape presentation in the thymus or other peripheral sites involved in tolerance induction. Self-reactive T cells and B cells may therefore (either specific to maternal or paternal X-linked antigens) escape negative selection depending on the specificity of the antigen presenting cells, thus leading to the breach in tolerance by activating B cells specific to X-linked antigen to produce autoantibodies and the induction of autoimmunity.

The second hypothesis for the induction of autoimmunity on the genetic level is the reactivation of genes on the previously inactivated X-chromosome; this can result in an over dose of X-linked such as genes involved in immune responses thus leading to autoimmunity (Lu 2007). It has been shown SLE T cells have over-expressed CD40L due to reactivation of this gene on the inactive X-chromosome. This results in enhanced IgG production by self reactive B cells, which has been shown to correlate with disease activity (Zhou 2009).

The third hypothesis for the involvement of the X-chromosome in autoimmunity is the haploinsufficiency of X-genes meaning when two copies of a gene are required for a efficiently functioning protein, however only one copy is present (Invernizzi 2005; Invernizzi 2007). This can occur due to complete deletion or absence of part of one of the X chromosomes (Sybert and McCauley 2004).

Another area of interest alongside sex chromosomes in understanding why women are more susceptible to autoimmune conditions is the role of steroid hormones in this sex difference, as drastic changes in hormone levels have been associated to disease progression.

1.4 Steroid sex hormones

1.4.1 Hypothalamic-pituitary-gonadal axis

Another explanation for the increased susceptibility of women to autoimmune conditions may lie in the role of sex-specific steroid hormones. Sex hormones are produced in the gonads; the ovaries produce oestrogens such as oestrone and 17β-oestradiol in females and the testes in males produce testosterone, and this process is regulated by the hypothalamus-pituitary-gonadal axis (HPG-axis) (Luu-The and Labrie 2010). This involves the coordination of the hypothalamus, pituitary gland and gonads working as a single unit. Gonadotropin-releasing hormone (GnRH) is produced and secreted by the hypothalamus, which in turn stimulates the production of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) by the anterior pituitary gland (Silverman and Sternberg 2012). Finally, these hormones act on the gonadal organs (ovaries in females and testes in males) and stimulate the production and secretion of sex hormones (oestrogen and testosterone) i.e. during the menstrual cycle in females. This HPG-axis is controlled by a negative feedback mechanism; oestrogen predominately inhibits LH secretion from the anterior pituitary gland, whereas testosterone and other androgens modulate the process at the hypothalamus level (Rochira *et al.* 2006). Although oestradiol levels in men are lower compared to testosterone levels, oestradiol is much more potent then testosterone, and therefore oestradiol is the main inhibitor of LH and FSH secretion by the anterior pituitary gland. Gonadal organs are also able to produce other regulatory factors such as activins and inhibins, both belonging to the transforming growth factor (TGF-β) superfamily (Bilezikjian *et al.* 2006). Activins and inhibins have conflicting roles in regulating the HPG-axis; activins enhance FSH production and secretion. In contrast, FSH stimulated synthesis of inhibins forms a negative feedback

mechanism, in which inhibins prevent the production and secretion of FSH by the anterior pituitary gland by interacting with specific inhibin receptors (Risbridger *et al.* 2001).

1.4.2 Hypothalamus-Pituitary-Adrenal axis

Androgens and oestrogens are predominately produced by gonads, however alongside the production of cortisol; sex hormones can also be produced by adrenal steroid dehydroepiandrosterone (DHEA) through stimulation of the Hypothalamus-Pituitary-Adrenal-axis (HPA-axis). DHEA and its sulphated precursor DHEA-sulphate (DHEAS) are the most abundant steroid hormones and function as precursors of active androgens and oestrogens.

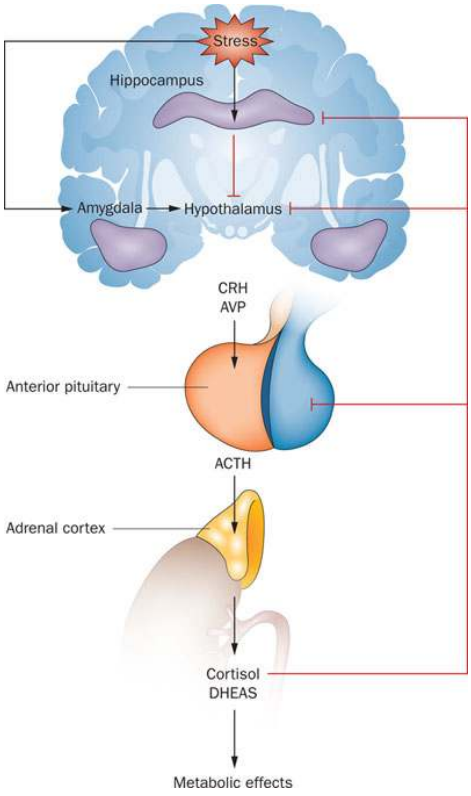

Figure 1.4 The HPA-axis in response to stress signals.
HPA-axis is able to respond to stress signals by stimulating the release of vasopressin and corticotrophin-releasing hormone (CRH) from the hypothalamus, which then results in the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland. ACTH stimulates the production of a glucocorticoid hormone called cortisol, which is able to modulate its own production through a negative feedback mechanism (red lines). Taken from Papadopoulos and Cleare, 'Hypothalamic-pituitary-adrenal axis dysfunction in chronic fatigue syndrome,' Nature (2012).

DHEA is produced in the zona reticularis in the adrenal glands and can then be secreted into peripheral tissue, where it acts in an intracine manner meaning most of the androgens and oestrogens synthesised from DHEA locally in organs such as skin, liver and adipose tissue, can then act on the same tissues that produced them (Labrie *et al.* 2001). This local production of androgens and oestrogens by DHEA metabolism is particularly important in post-menopausal women as the ovaries are no longer producing high levels of oestrogen and virtually all of the androgen being synthesised is through DHEA metabolism. Labrie and

colleagues demonstrated that intra-vaginal DHEA administration to post-menopausal women rapidly diminished vaginal atrophy and improved sexual function without raising levels of androgen and oestrogen in the circulation (Labrie *et al.* 2009).

1.4.3 Steroidogenesis

Cholesterol is the main precursor for many active steroid hormones (figure 1.4). Cholesterol, a C27 compound is converted by CYP11A1 into the first steroid pregnenolone a C21 compound, which is then converted to progesterone. These two compounds are then responsible for the synthesis for three main groups of steroids: C21 compounds known as mineralocorticoids and glucocorticoids, C19 compounds which are classed as androgens, and thirdly the C18 compounds that form oestrogens.

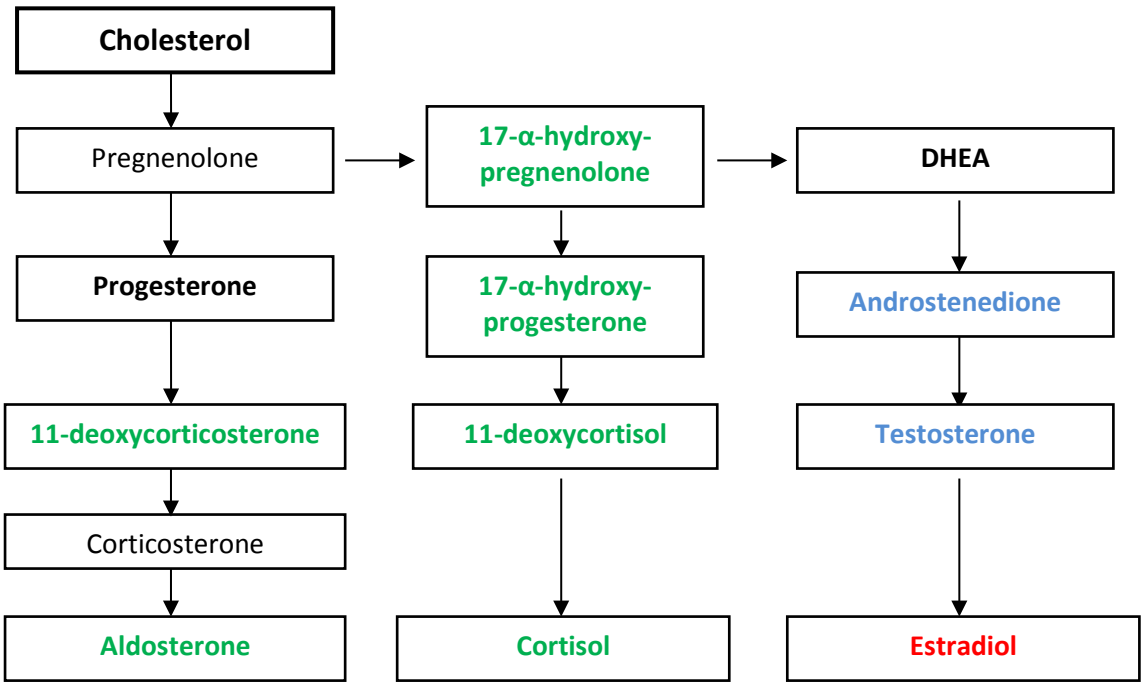

Figure 1.5 Steroid synthesis via cholesterol metabolism.
Steroids highlighted in green are glucocorticoids and mineralocorticoids, in blue are androgens and in red are oestrogens.

Only low levels of the active sex steroids made locally by peripheral tissues are secreted into the circulation, as they are inactivated locally before entering in the circulation, whereas most of the active steroids in circulation are secreted by the gonads. Active sex steroids such as DHT are usually converted into androsterone (ADT) and then made more water soluble by the addition of glucuronide derivatives (ADT-G) (Luu-The and Labrie 2010).

Also, a high concentration of sex hormones (particularly the most biologically active sex hormones); testosterone; DHT; oestradiol are transported in the periphery bound to glycoproteins called sex hormone-binding globulins (SHBG), which are produced in the liver. Sex hormones can also bind to albumin but with a much lower affinity compared to SHBG. Therefore, the levels of bioavailable active sex hormones is determined by the levels

of SHBG in circulation; bound sex hormones are no longer lipophilic, thus are unable to travel through the cell membrane and bind to inactive sex hormone specific nuclear receptors and transcription factors within the cytosol. When measuring the total concentration of sex hormones, both the levels of unbound sex hormones (known as free sex hormones) and SHBG bound sex hormones are taken into account.

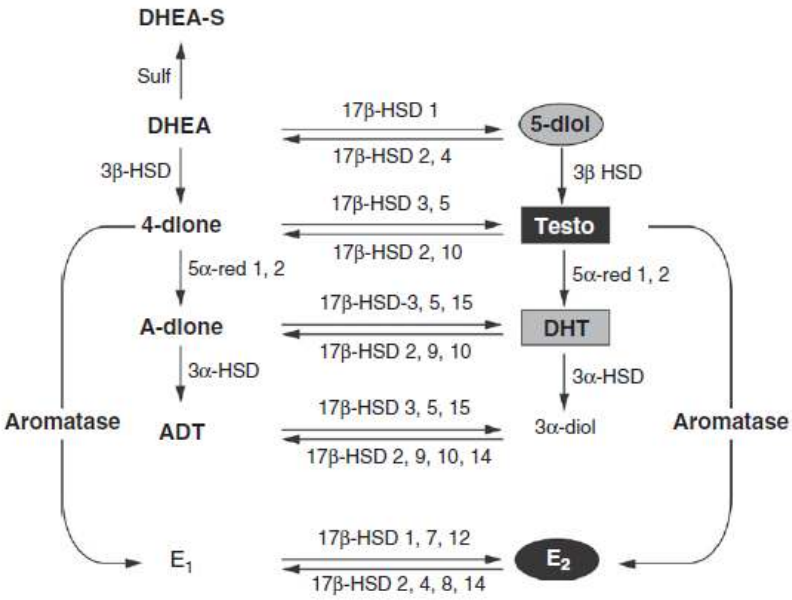

Figure 1.6 Downstream conversions of DHEA/DHEAS to sex hormones.
DHEA/DHEAS is metabolised into biologically active androgens via many metabolic enzymes. Androgens (testosterone and androstenedione) can be converted into oestrogens; oestrone (E1) and oestradiol (E2) by aromatase. Abbreviations: 5α-reductase 1 and 2 (5α-Red 1, 2); 3β- and 3α-hydroxysteroid dehydrogenase (3β/α-HSDs); 17β-hydroxysteroid dehydrogenase (17β-HSDs); androsterone (ADT); 5-androstenediol (5-diol); 4-androstenedione (4-dione); androstanedione (A-dione); dihydrotestosterone (DHT). Taken from Luu and Fernand, 'The intracine sex steroid biosynthesis pathways.' Progress in Brain Research, (2010).

The family of 17β-HSD enzymes are known as the backbone of androgen metabolism (figure 1.5), as they are required for synthesis of various androgens and indirectly in synthesis of

oestrogens. However, the most potent and active androgen DHT, is produced by the reduction of testosterone by the enzyme 5α-reductase and therefore plays a vital role in androgen metabolism.

1.4.4 5α-reductase

5α-reductase, also known as 3-oxo-5α-steroid 4-dehydrogenase, is an important enzyme in mineralocorticoids, glucocorticoids and androgen synthesis. It is responsible for the reduction of progesterone to 5α-dehydroprogesterone (5α-DHP) or 3α-, 5α-tetrahydrodeoxycorticosterone (3α, 5α-THDOC), which then can be converted into other glucocorticoids or mineralocorticoids. In androgen metabolism, 5α-reductase reduces androstenedione to androstanedione, but more importantly it converts testosterone in an NADPH irreversible dependent reaction into DHT, the most potent androgen receptor agonist (figure 1.6); as DHT has a 10 fold higher affinity for the androgen receptor compared to testosterone (Jin and Penning 2001). Testosterone is essential for the formation of male internal sex organs and maintaining sexual function. In contrast, DHT is important in the formation of external male genitalia, prostate and also facial/body hair.

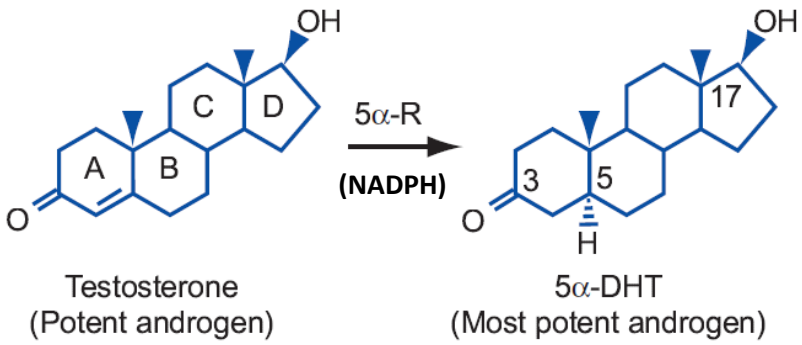

Figure 1.7 Conversion of testosterone by DHT by 5α-reductase.

This shows chemical reaction for the synthesis of DHT through the reduction of testosterone by 5α-reductase in the presence of co-factor NADPH. Adapted from Yin, 'Steroid 5αreductases and 3a-hydroxysteroid dehydrogenases: key enzymes in androgen Metabolism.' Best Practice & Research Clinical Endocrinology and Metabolism, (2001).

5α-reductases are members of the aldo keto-reductase family of enzymes and are located in the microsomal membrane. They act in a NADPH-dependent manner, in which androgens, mineralocorticoids, or progestins with a C3 ketone group and a double bond between C4-C5 are reduced to form 5α-reduced products. 5α-reductases are low abundance proteins with a molecular weight between 28-29kDa (Andersson and Russell 1990). There have been three isoenzymes identified; 5α-reductase 1 (gene SRD5A1), 5α-reductase 2 (gene SRD5A2) and more recently 5α-reductase 3 (gene SRD5A3), however there has been little evidence of androgen metabolism activity by SRD5A3 (Uemura *et al.* 2008). 5α-reductase isoforms are expressed in a tissue specific manner; 5α-reductase 1 is predominately found in the sebaceous glands in skin, hair follicles, and the liver (Berman and Russell 1993; Silver *et al.* 1994), where as 5α-reductase 2 is the main isoform found in the male accessory reproductive structures such as the Wolffian ducts, prostate, the epididymis and the seminal vesicles (Russell and Wilson 1994). Male pseudohermaphroditism is a rare genetic disorder, associated with a mutation in the SRD5A2 gene, which results in the formation of female resembling external genitalia and no prostate formation during embryogenesis (Andersson *et al.* 1991; Wilson *et al.* 1993). It is not until these individuals reach adolescence, where they start to develop characteristic male external genitalia but fail to develop a prostate. There is limited homology between the different isoforms, which is around 47%. These three isoform genes are located on separate chromosomes with the SRD5A1 gene located on chromosome 5 in p15, SRD5A2 gene on chromosome 2 p23 and SRD5A3 on chromosome 4 q12 (Jin and

Penning, 2001). However, the positions of the introns are the same in both SRD5A1 and SRD5A2 genes.

A study showed that human lymphocytes from patients with primary 5α-reductase deficiency have significantly higher levels of cytosine methylated 5α-reductase 2 genes, thus resulting in reduced gene expression (Rodriguez-Dorantes *et al.* 2002). Also, a murine study found that there were differential methylation patterns in the 5α-reductase 1 genes within various organs. They observed an increase in adenine methylation levels in the 5α-reductase 2 gene in the epididymis and in the testis in contrast to the liver (Reyes *et al.* 1997). Adenine methylation is associated with increased gene expression, thus the 5α-reductase 2 gene was highly expressed in the epidydimis and in the testis compared to in the liver. There is no evidence of 5α-reductase 1 genetic deficiency, however mice studies have shown that removal of 5α-reductase 1 in female mice leads to defects in reproduction (Mahendroo and Russell 1999). Also, there is evidence to suggest that 5α-reductase is regulated by levels of androgens present; testosterone given to castrated mice reduced 5α-reductase activity (Wright *et al.* 1999).

1.4.5 Steroid nuclear receptors

Androgens and oestrogens exert their effects through binding of nuclear receptors called androgen and oestrogen receptors respectively, in target cells. Both androgen and oestrogen receptors are members of the nuclear receptor superfamily of transcription factors and are found abundantly in sex hormone responding tissues such as the prostate. There are two isoforms of oestrogen receptors; oestrogen receptor-alpha (ER-α) (the dominate isoform in most tissues) and oestrogen receptor-beta (ER-β), which have been shown to have opposite

effects to ER-α on gene transcription depending on the activating ligand, as well as target DNA regions (Paech *et al.* 1997). ER-β is able to control ER-α activation as it can form heterodimers with ER-α, thus suppressing ER-α transcriptional activity (Lindberg *et al.* 2003). Ligand binding, i.e. when DHT binds to androgen receptor and 17β-oestradiol binds to oestrogen receptors, results in translocation from the cytosol into the nucleus (figure 1.8). This then leads to receptor dimerization with other nuclear receptors, and subsequent binding to specific regions in promoters of target genes (figure 1.8). Receptor activation initiates the recruitment of co-activators and co-repressors, which in turn changes the structure of chromatin surrounding target genes thus initiating transcription. Both testosterone and DHT are able to bind androgen receptors however, DHT has a 10 fold higher affinity for the receptor and therefore lower concentrations of DHT compared to testosterone are needed for androgen receptor activation.

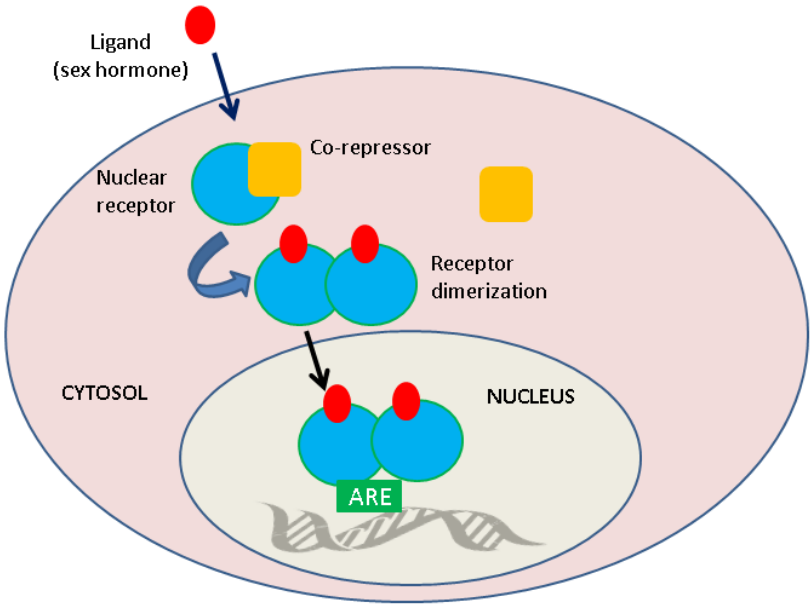

Figure 1.8 Steroid hormone nuclear receptor activation.
Ligand binding to inactive nuclear receptors results in detachment from co-repressors and translocation from the cytosol into the nucleus. This then leads to receptor dimerization with other nuclear receptors, and subsequent binding to specific regions (such as androgen

response elements (ARE) in promoters of target genes. Receptor activation initiates the recruitment of co-activators and co-repressors, which in turn changes the structure of chromatin surrounding target genes thus initiating transcription.

Studies have shown that androgen and oestrogen receptors are regulated through epigenetic modifications and by exposure to androgens and oestrogens (Imamura 2011). In some tissues, AR mRNA expression has been shown to be regulated in a negative feedback manner, in which low androgen levels increase AR expression and in contrast, high androgen levels decrease AR expression (Khetawat *et al.* 2000; Sader *et al.* 2005). Mutations in steroid receptors have shown to have severe impacts on development, as seen in indirect 5α-reductase deficiency, in which a point mutation in the androgen receptor gene (type 1 occurs from a mutation in the 5α-reductase gene), resulting in the absence of the androgen receptor. This leads to defects in male embryonic development, in which formation process of external male genitalia is defected (Wilson *et al.* 1993).

1.4.6 Steroid hormones and the immune system

The HPA-axis is the focal point of the body's stress response system and has a direct effect on immune responses. Glucocorticoid hormone, cortisol, is produced by the anterior adrenal gland through the HPA-axis and is essential in exerting a suppressive effect on the immune system to maintain homeostasis and preventing the development of inflammatory conditions.

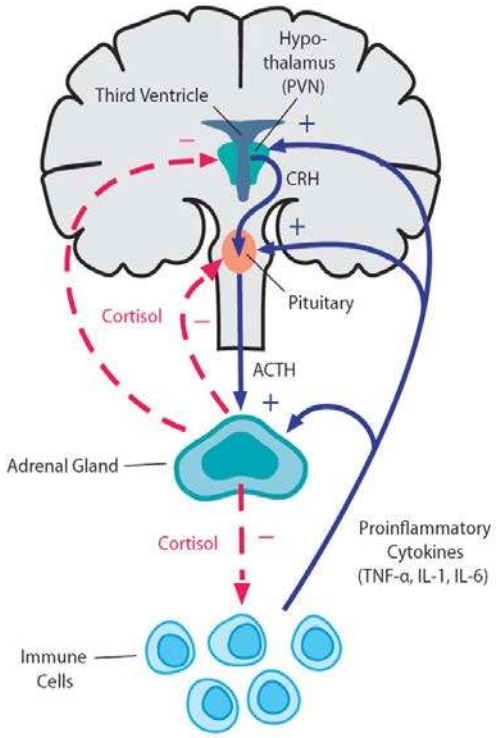

Figure 1.9 Response to pro-inflammatory cytokines by the HPA-axis.
Pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 stimulates the HPA-axis at all three levels; the hypothalamus, pituitary gland and the adrenal gland. This stimulates the release of cortisol from the adrenal gland, which results cortisol acting on immune cells and inhibiting the production pro-inflammatory cytokines. Blue lines represent stimulation and red dashed lines present inhibition. Taken from Silverman and Stemberg, 'Glucocorticoid regulation of inflammation and its functional correlates: from HPA axis to glucocorticoids receptor dysfunction.' Annals of the New York academy of sciences (2012)

Cortisol is able to inhibit the production of pro-inflammatory cytokines (figure 1.7), thus dampening the pro-inflammatory response (Bethin *et al.* 2000; Ray *et al.* 1990). The anti-inflammatory traits of glucocorticoids have been exploited for decades as treatment with synthetically produced glucocorticoids such as prednisolone as been used in treatment of many inflammatory conditions including autoimmune diseases. Disregulation of the HPA-axis has been reported in autoimmune diseases such as RA (Cutolo *et al.* 2003; Mastorakos and Ilias 2000), in which RA patients that are not undergoing treatment with glucocorticoid medication and patients with active disease have reduced ACTH synthesis and therefore produce very low levels of cortisol and adrenal androgen DHEA/DHEAS. This inefficient adrenal activity observed in RA results in reduced stress response signals to maintain

homeostasis, and therefore decreased suppression of elevated pro-inflammatory responses such as increased levels of IL-6 and TNF, which are in turn contributing to the chronic inflammation observed in RA. The reduced adrenal activity within the HPA-axis, which has shown to be also due to resistance to ACTH stimulation, has been shown to make individuals more susceptible to developing inflammatory and autoimmune conditions. Glucocorticoid resistance has also been demonstrated in autoimmunity; in which studies have shown that abnormal signalling by nuclear receptor glucocorticoid receptor (GR) expressed in various cells (including immune cells), thus preventing glucocorticoids mediated the effects (Silverman and Sternberg 2012).

Gonadotropin-releasing hormone (GnRH) has also been investigated in the development of inflammatory and autoimmune conditions, as it has been shown to be immunostimulatory. The importance of GnRH in thymic maturation has been demonstrated, as treatment of neonatal rats with GnRH antagonists resulted in significantly reduced thymic proliferative responses, and with a decrease in $CD4^+$ population observed and reduced antigen stimulated antibody responses in adult rats (Tanriverdi *et al.* 2003). In vitro studies, GnRH has shown to increase T cell responses through upregulation of IL-2R and increasing production of IFN-γ after treatment with GnRH. In models for autoimmunity, there was reduced disease severity and decreased anti-DNA antibodies after GnRH antagonist treatment in SLE-prone mice (Jacobson *et al.* 1994), which was observed in both castrated and intact mice suggesting the effects of GnRH are independent of gonadal sex hormones.

1.4.7 Sex hormones and autoimmunity

The predominance of autoimmune conditions in females has initiated a vast amount of research into the role sex hormones play in immune and inflammatory responses, especially on the effect of oestrogen on immune responses due to this increased susceptibility in females. However, male sex hormones known as androgens have also been shown to mediate immune responses. Sex hormone levels are shown to have a significant effect on the severity of autoimmune diseases. Any drastic changes in levels of sex hormones can have a profound effect on the immune system; pregnancy enhances disease activity in SLE individuals; furthermore, menopause, chronic stress, and steroid hormonal replacements may affect susceptibility to autoimmunity (Angstwurm *et al.* 1997; Hirano *et al.* 2007; Oliver and Silman 2009; Verthelyi and Klinman 2000). Decreasing levels of DHEA are characteristic of the ageing process and may contribute to an increased susceptibility to some autoimmune diseases (Hammer *et al.* 2005; Straub *et al.* 1998). Androgens and oestrogens are known to have conflicting effects on the immune system; androgens such as testosterone and the most potent androgen 5α-dihydrotestosterone (DHT) have an anti-inflammatory effect (Liva and Voskuhl 2001), whereas oestrogens such as 17β-estradiol and oestrone enhance immune responses (Maret *et al.* 2003).

1.4.8 Effect of sex hormones on the immune system: mice studies

Oestrogen has been identified as an immune enhancer especially in responses involving the humoral immune system. This revelation could potentially explain the increased susceptibility of autoimmunity in females compared to males, thus many studies have investigated the role of oestrogen in the development of autoimmune conditions in both animal models and in humans. In animal models, treatment of New Zealand brown/New Zealand white F_1 female mice (model used for SLE in mice), with 17β-estradiol enhanced

disease activity (Grimaldi *et al.* 2006). Physiological concentrations of 17β-estradiol increase production of pro-inflammatory cytokines such as IL-1, IL-6 and TNF (Garidou *et al.* 2004).

Various studies have investigated the effects of oestrogen on dendritic cells, particularly the pDC subset due to its important role in the pathogenesis in SLE, as it is the major producer of IFN-α, a proinflammatory cytokine found to be significantly elevated in SLE patient sera. A study carried out by Seillet and colleagues investigating the role of oestrogen in the previously reported higher production of TLR7 mediated IFN-α by pDCs in females compared to males (Seillet *et al.* 2012). They found that transplanting human female $CD34^+$ hematopoietic progenitor cells into sublethally irradiated NOD/SCID/$\beta2m^{-/-}$ female and male mice, followed by subsequent *in vitro* stimulating of harvested human pDCs (identified as $CD45^+$ $CD123^+$ BDCA-4^+) with TLR7/TLR8 ligands showed that a significantly higher frequency of not only IFN-α producing pDCs but also an increase in TNF-α producing pDCs was observed in female mice in comparison to the male mice. This suggests that these differences in innate immune responses are not just linked to the X-chromosome, but are also influenced by sex hormones. This hypothesis was supported with the subsequent finding by Seillet *el al*, that supplementing postmenopausal women with 17β-estradiol increased TLR7/TLR-dependent production of IFN-α (Seillet *et al.* 2012). Stimulation of pDCs with NA-IC from SLE sera resulted in an increased IFN-α and TNF-α production by pDCs after 17β-estradiol treatment. These enhancing effects of oestrogen on innate immune responses were all dependent on ERα signalling in pDCs as demonstrated in the ERα knockout mice.

Scotland and colleagues demonstrated the importance of oestrogen in the maintenance of resident tissue leukocyte population and the recruitment of more leukocytes during infection

in an animal model of sepsis (Scotland *et al.* 2011). Ovariectomised mice had significantly reduced total resident leukocytes, which included $F4/80^+$ macrophages and $CD19^+$ B cells but no reduction in the numbers of $CD3^+$ and $CD8^+$ T cells. There was also a reduction in tissue expression of chemokines such as CCL2, CXCL1, CXCL12 and chemokine receptors CCR1, CCR2 and CXCR4, thus explaining the reduction in the resident leukocyte population. Resident leukocyte expression of TLRs such as TLR1, TLR2 and TLR3 has also shown to be significantly reduced in the ovariectomised mice, alongside reduced phagocytosis by macrophages in vitro.

However, in some studies oestrogen has been shown to have a protective effect in autoimmunity, particularly in T cell mediated autoimmune conditions, through activation of ER-α signalling (Yang *et al.* 2010). In cell-mediated immune responses high concentrations of oestrogen have been shown to have a dampening effect on immune responses as demonstrated in T cell driven disease MS, as oestrogen is able to decrease Th1 mediated responses and increase Th2 immune responses by decreasing Th1 cytokines such as IFN-γ. A study found that ER-α knockout mice induced with EAE did not respond to oestrogen (Garidou *et al.* 2004), thus suggesting ER-α signalling is required to mediate the protective effects of oestrogen.

In contrast to oestrogens, the effects of androgens on immune responses have been less thoroughly researched, however the small number of studies have shown androgens to be anti-inflammatory as there is evidence to suggest androgens are able to dampen inflammatory responses. In terms of autoimmune diseases, androgens have shown to reduce disease activity in humans and in animal models (Harbour, Pervez-Gill, et al 1995). Previous studies have demonstrated the protective role testosterone and DHT in autoimmunity as exogenous administration suppressed Th1 mediated autoimmune diseases by initiating Th2 cytokine

production. Animal studies showed that castration of male mice but not ovariectomy in females increases Th1 mediated autoimmunity and decreases Th2 cytokine profile, thus exacerbating EAE, in contrast female mice implanted with testosterone pellets reduced the severity of EAE. Therefore, low levels of testosterone initiate production of pro-inflammatory mediators (Gilliver *et al.* 2006). In a rat model of cutaneous wound healing, inhibition of 5α-reductase, which blocks the conversion of testosterone to its more active metabolite 5α-DHT, resulted in the upregulation of proinflammatory cytokines by macrophages (Gilliver *et al.* 2006).

A study looking into an inflammatory condition Orchitis, associated with low testosterone levels, which can develop into chronic testicular inflammation within the testes and is associated with high levels of pro-inflammatory cytokines leading to tissue damage and subsequent infertility in severe cases, used an animal model called experimental autoimmune orchitis (EAO) and found that implanting testosterone pellets into mice resulted in reduced EAO disease activity, positively correlating with the decrease in macrophage and CD4 T cell number, and decrease in proinflammatory cytokines such as IL-6, TNF-α, IFN-γ and IL-2. In contrast, there was a significant increase in the number of immunosuppressive $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells (Fijak *et al.* 2011), thus highlighting the importance of testosterone in resolving inflammation.

1.4.9 Effect of sex hormones on the immune system in humans

In terms of humoral immune responses, oestrogen has been shown to increase B cell proliferation and differentiation thus elevating IgG and IgM antibody production (anti-

dsDNA in SLE patients) in both males and females at physiological concentrations (Kanda and Tamaki 1999; Kanda *et al.* 1999).

In RA, changes in oestrogen metabolism have been demonstrated as increased metabolism of oestrogens into more potent and inflammatory oestrogen metabolites such as 16αOH-oestrone has been demonstrated in RA synovial cells, but not in OA patients (Schmidt *et al.* 2009). These potent oestrogen metabolites have shown to unsuccessfully inhibit TNF-α induced inflammation, thus suggesting a pro-inflammatory role in RA.

Some of the effects of oestrogen seem to be dose dependent, as high concentrations of oestrogen have also been shown to decrease IL-2R expression and IL-2 production in activated T cells from healthy controls (McMurray *et al.* 2001), but at low concentrations of oestrogen an opposite outcome is obtained. In innate immune reactions oestrogen has also been shown to decrease inflammatory mediators; oestrogen inhibits TNF-α production through TLR4 stimulation by LPS in human PBMCs (Rogers and Eastell 2001).

This suggests that oestrogens have conflicting roles on immune responses. Oestrogens have shown to have conflicting effects on different autoimmune diseases; in RA and MS, high levels of oestrogens cause disease remission which has been well demonstrated during pregnancy and the menstrual cycle (Latman 1983). These autoimmune conditions are also more common in older women often developing at menopausal age or postmenopausal. However, in SLE pregnancy exacerbates disease activity in which it is common for flares to occur (Ruiz-Irastorza *et al.* 1996) and onset of the disease usually occurs at puberty and child bearing ages 20-35yrs in which oestrogen levels are very high. These contrasting effects of

oestrogen observed in different autoimmune diseases are thought to be due to the cell types involved; RA and MS are predominately T cell (Th1) driven diseases, whereas SLE is thought have a higher level of B cell involvement. Thus, oestrogens have been shown to increase humoral responses and reduce Th1 driven pathways (Feng *et al.* ; Polanczyk *et al.* 2004), at high concentrations at least, which could be the reason for the difference observed.

Testosterone is also able to decrease IL-1 synthesis in synovial macrophages in vitro and potent androgen receptor agonist DHT is able to repress activity and expression of the IL-6 gene promoter in human fibroblasts (Weidler *et al.* 2005). In terms of humoral responses, there is a complete contrast between oestrogens and androgens as testosterone has shown to inhibit IgM and IgG production (Kanda *et al.* 1996). Enhanced DC proliferation and antigen stimulation has been shown in males with hypogonadism, suggesting that testosterone is important in regulating immune responses in a suppressive manner.

It is well established that SLE patients have low levels of androgens in serum, which inversely correlates with disease activity (Jungers *et al.* 1982). Male SLE patients have a significantly lower androgen levels and increased oestrogen levels in serum (Mok and Lau 2000; Sequeira *et al.* 1993). It is not well understood if low androgen levels predispose individuals to SLE or if the development of autoimmune disease itself is responsible for these reduced levels of androgens. In SLE, glucocorticoid based treatment drastically reduces androgen levels in serum thus it is extremely difficult to quantitate androgen levels in SLE patients while on medication. However, low androgen levels in men have been associated with the development of RA (Cutolo *et al.* 1988). Also, in male MS patients disease onset is much later then their female counterparts, which could be due to the age associated decrease

in DHEA and testosterone concentrations in men, thus the protective effect of androgens against autoimmune development is absent (Hammer *et al.* 2005).

Recently, it has been shown that the anti-inflammatory effects of androgens can be mediated through androgen activation of peroxisome proliferator activated receptors (PPARs) (Dunn *et al.* 2007) and have shown to protect male mice from developing autoimmunity. Therefore, PPARs may play an important role in mediating the sex differences observed in autoimmunity.

1.5 PPAR

1.5.1 Introduction

Peroxisome proliferator activated receptors (PPARs) are a group of transcription factors that are part of the nuclear receptors superfamily. PPARs have primarily been associated with regulating expression of genes involved in lipid and glucose metabolism. Three different isoforms have been discovered originally in Xenopus: PPAR-alpha, PPAR-gamma and PPAR-delta (also known as PPAR-beta). PPARs were first discovered in rodents as being responsible for peroxisome proliferation in the liver upon treatment with industrial chemical such as solvents; peroxisomes are subcellular organelles that are important in lipid metabolism via β-oxidation of fatty acids or hydrogen peroxide mediated respiration. PPARs are composed of five regions: C and E, which are the most conserved regions and contain the DNA-binding domain (DBD) and the ligand-binding domain; A/B region consists of the AF1 ligand-independent activation domain; the F region contains the AF2 ligand-dependent activation domain and finally the variable hinge region D (Daynes and Jones 2002).

1.5.2 PPAR ligand mediated activation

PPARs are activated in a ligand dependent manner, which in turn results in altering the role of PPARs to transcription factors, thus targeting specific genes. Generic for all the members of the nuclear receptor superfamily is the presence of a central DNA-binding domain essential for recognising PPAR-response elements (PPREs) in promoter regions of target genes. These PPRE regions are made up of repeated sequences separated by one nucleotide known as Direct Repeat-1 (DR1). Upon ligand binding, PPAR forms a heterodimeric complex with another member of the nuclear receptor family, retinoid X receptor (RXR)

(Clark *et al.* 2000). After PPAR activation, PPARs are able to dissociate from co repressor molecules nuclear receptor co-repressor/silencing mediator for retinoid and thyroid hormone receptor, collectively known as NCoR/SMRT. Histone deacetylases (HDACs) are also needed alongside NCoR/SMRT to suppress PPAR mediated gene transcription, however, this only applies to PPAR Isoforms alpha and gamma as PPAR-delta can bind PPREs still associated to the co-repressor complex. The dissociation of co-repressor molecules allows co-activators such as steroid receptor co-activator 1 (SRC1) and members of the DRIP/TRAP (vitamin-D-receptor interacting protein/thyroid-hormone-receptor-associated protein) complex and CREB-binding protein (CBP)/p300 to bind to the LBD in the E region of PPAR, thus able to translocate from the cytoplasm to the nucleus (Clark 2002). This then allows changes to be made to chromatin structure via histoneacetyltransferase surrounding target genes of PPAR thus initiating transcription.

1.5.3 PPARs and the immune system

Many studies have suggested a role of PPARs in the immune system as PPARs have shown to be expressed in multiple immune cell subsets, and have acted predominately as anti-inflammatory mediators. PPAR-α and PPAR-γ have both been investigated to vast depth in comparison to PPAR-δ as its effects are still widely unknown. In particular, studies in mice have shown that PPAR-α is able to modulate the anti-inflammatory effects of androgens. It has been suggested that males are less likely to develop autoimmune diseases due to higher expression of PPAR-α then their female counterparts. Early *in vitro* studies investigating the effect of PPAR-α on immune responses using mice models showed that PPAR-α knockout mice had a prolonged immune responses (Gocke *et al.* 2009), when treated with PPAR-α activating ligands such as LTB_4 and arachidonic acid, compared to the wild type mice, thus

suggesting PPAR-α is able to inhibit immune responses . The literature also suggests that supplementing experimental mice with ω-3 fatty acids or steroids such as DHT which are all agonists for PPAR-α, reduced the production of pro-inflammatory cytokines. A study exploring the role of PPAR-α in modulating cellular redox status in aged mice found that PPAR-α was able to reduce tissue lipid peroxidation, inhibition of NF-κB activity and production of pro-inflammatory cytokines, which are all involved in promoting oxidative stress in the ageing process (Hasler and Zouali 2005). PPAR-α can antagonise NF-κB and AP-1 signalling thus prevent expression of genes involved in inflammatory responses (Poynter and Daynes 1998). PPAR-α ligands are also able to induce apoptosis in activated macrophages but not in inactivated macrophages or undifferentiated monocytes.

It was a study by Dunn *et al* (2007) which suggested that PPAR-α expression was sensitive to testosterone, thus mediated the effect of androgens on T helper cell activity (Dunn *et al.* 2007). Androgen activated PPAR-α reduced TCR-induced NF-κB and c-jun activity. There was also an increase in Th2 mediated cytokine production. They also demonstrated a gender difference in PPAR-α expression as it was more abundant in male mice in comparison to female mice. Furthermore upon EAE induction, male PPAR-α knockout mice displayed more severe disease activity than male wild-type animals, whereas disease severity in female mice was not significantly different in knock-out and wild-type animals. MOG p35-55-reactive splenocytes from male PPAR-α knock-out mice produced increased levels of IFN-γ and TNF compared to their wild-type counterparts (Dunn *et al.* 2007). This suggests that males are less likely to develop Th1-mediated autoimmune diseases due to higher T cell expression of PPAR-α.

PPAR-γ is also expressed in a wide range of inflammatory mediators such as monocytes, macrophages and Th17 cells. PPAR-γ ligands such as thiazolidinediones have been used for the treatment of type-2 diabetes as they were able to lower blood glucose levels and levels of circulating fatty acids in insulin resistant mice (Szanto and Nagy 2008). In mice, PPAR-γ has been shown to be expressed in macrophages in which it was able to inhibit expression of inflammatory mediators such as nitric oxide synthase (iNOS), scavenger receptor A and matrix metalloproteinase-9 (MMP-9) (Cunard *et al.* 2002). These studies also demonstrated that PPAR-γ agonists were able to inhibit pro-inflammatory mediators LPS, IFN-γ and phorbol ester by interfering transcription factor signalling such as NF-κB AP-1 and signal inducers and activators of transcription 1 (STAT-1). This mechanism of PPAR-γ inhibition of gene transcription is similar to the action of the PPAR-α isoform.

PPAR-γ ligands are also able to have a pro-inflammatory effect on B cells by stimulating plasma cell differentiation and antibody production. PPAR-γ is also expressed in T cells; studies have shown that the two ligands 15d-PGJ_2 and thiazolidinediones were able to inhibit IL-2 production in murine T cell clones but not IL-2 mediated cell proliferation (Clark 2002). Also other studies demonstrated the enhancing effect of PPAR-γ ciglitazone on murine natural T regulatory cells (nTregs) and inducible Tregs (iTregs) (Szanto and Nagy 2008; Wohlfert *et al.* 2007). This suggests PPAR-γ ligands can be used in the treatment of autoimmune diseases by enhancing Treg activity, which is particularly important in modulating T-helper cells that may become autoreactive. PPAR-γ ligands are also able to induce apoptosis in activated T cells, which has been demonstrated in both the animal and human model (Tautenhahn *et al.* 2003). Recently it has been reported that PPAR-γ is expressed in the Th17 cells subset, which is has been branded as an important instigator in

developing autoimmune diseases. Klotz et al showed that PPAR-γ activation inhibited Th17 differentiation by preventing TGF-β/IL-6-induced expression of RORγt in T cells (Klotz *et al.* 2009), which is an important transcription factor, needed for Th17 differentiation.

Therefore, PPARs especially PPAR-α could be responsible for mediating the effects of androgens on the immune system, and thus could not only explain the observed robust immune responses in females during infection in comparison to males, but also may explain the increased incidence in autoimmunity development in females.

1.6 Objectives

Androgens and oestrogens have been attributed with conflicting effects on the immune system; androgens such as testosterone and the most potent androgen; 5α-dihydrotestosterone (DHT) have an anti-inflammatory effect, whereas oestrogens such as 17β-oestradiol and generally enhance immune responses. Therefore, these conflicting affects of sex hormones could potentially explain the increased susceptibility of females developing autoimmune diseases. The overarching hypothesis tested in this project was that both long-term and acute effects of sex hormones will affect T cell responses.

Firstly, this study sought to compare the way T cells from healthy males and females regulate the activation of androgens, and how T cells are able to regulate their exposure to sex hormones. For this purpose the mRNA and protein expression of sex hormone metabolising enzymes and sex hormone receptors in resting and *in vitro* stimulated naïve $CD4^+$ T cells from healthy male and female donors were investigated.

Secondly, we sought to investigate regulation 5 α-Reductase 1 in T cells and other immune cells from patients with SLE in comparison to healthy controls.

Thirdly, this study aimed to investigate the influence of androgens on *in vitro* T cell responses using physiological concentrations of hormones and to compare this effect between male and female donors.

2 MATERIALS AND METHODS

2.1 Peripheral blood collection from healthy controls and SLE patients.

Ethical approval to collect blood from healthy volunteers was obtained by the University of Birmingham ethics committee under the 'Regulation of immune cell activation by steroid hormones' study. Healthy volunteers gave consent for blood donation by a signing a written consent form before blood was taken by trained phlebotomists. Healthy volunteers recruited for this study were aged between 20-35 years and were not undergoing treatment for inflammatory conditions, and female volunteers recruited were not taking oral contraceptives. For the steroid receptors and 5α-reductase qPCR experiments, blood was obtained from female donors, who were 7-10 days into the follicular phase of the menstrual cycle.

Under an ethically approved study, blood samples from SLE patients were taken during Consultant Rheumatologist Dr Caroline Gordon's SLE clinic at Birmingham City Hospital. Patients fulfilling the American College of Rheumatology 1987 revised criteria for SLE were recruited for this pilot study and gave informed consent. Anti-dsDNA titer and disease activity (British Isles Lupus Assessment Group, BILAG) was determined at the time of sampling. A BILAG system score of B or more (≥5 points) was considered as a marker of active disease. Sex and age-matched healthy blood donors were recruited as controls. Signed informed consent was obtained from healthy blood donors and SLE patients prior to participation in the study, which was approved by the local research ethics committee.

2.2 Cell isolation from peripheral blood

2.2.1 Isolation of peripheral blood mononuclear cells (PBMCs).

Peripheral blood was obtained from healthy donors in tubes containing heparin at a final concentration of 1000 U/l. Peripheral blood taken from SLE patients and matching healthy controls were collected using heparin-coated (green-topped) blood collection tubes. Peripheral blood was diluted with RPMI 1640 medium (supplemented with 2nM glutamine, 1% 100U/ml penicillin G, and 10µg/ml streptomycin sulphate (GPS) (Invitrogen, Life technologies)) at a 1:1 ratio and then gradually layered on Ficoll-Paque (Amersham Biosciences, Bucks UK) at a ratio of 3:1 whole blood to Ficoll-Paque. Tubes were then centrifuged at 300 g for 30 minutes at 22°C, with no brake. After centrifugation, the mononuclear cells were removed from above the Ficoll-Paque layer using a plastic Pasteur pipette and then washed three times in 20ml RPMI, in which cells were then centrifuged at 300 g at 22°C for 8mins; the supernatant was removed after each wash and cells were resuspended in fresh medium. PBMCs were then counted using a haemocytometer and used in subsequent experiments. Cell viability of cell cultures were determined using flow cytometry as live cells had high forward scatter and side scatter fluorescence, unlike dead cells and debris, which appeared in the bottom left area of the plot with low forward scatter and side scatter fluorescence. Therefore, only samples with 98% live cells were included in subsequent experiments.

2.2.2 Isolation of $CD4^+$ T cells from PBMCs.

After isolation of PBMCs as described in section 2.2.1; $CD4^+$ T cells were isolated from a PBMC population via negative isolation using a $CD4^+$ T cell isolation kit (Miltenyi Biotech). Firstly, PBMCs were counted using a haemocytometer and resuspended in 40µl MACS

buffer (containing PBS at pH 7.2, 0.5% BSA and 2mM EDTA) per 10^7 cells in a 1.5ml eppendorf tube. 10µl of Biotin Antibody Cocktail per 10^7 cells was added and cells were then incubated at 4°C for 10 minutes. After the incubation period, cells were then washed with 500µl MACS buffer by centrifuging for 5 minutes at 3000 g. Cells were then resuspended in 30µl of MACS buffer per 10^7 cells and 20µl of Anti-biotin MicroBeads per 10^7 cells and incubated at 4°C for 15 minutes. Cells were then washed with 500µl MACS buffer by centrifuging for 5 minutes at 3000 g and resuspended in 500µl MACS buffer ready for magnetic cell separation.

An LS column (Miltenyi Biotech) was placed in the magnetic MACS separator. Firstly, the column was washed with 3ml MACS buffer three times and then the 500µl cell suspension was added to the top of the column. The column was then washed with 3ml of MACS buffer three times to elute the unlabelled $CD4^+$ cell population. Purity of the $CD4^+$ T cell population was determined via flow cytometry.

2.2.3 Isolation of $CD4^+$ RA^+ T cells from PBMCs.

The same protocol as described in section 2.2.2 for $CD4^+$ T cells isolation was used for $CD4^+$ RA^+ T cells isolation using a Miltenyi magnetic cell separation kit for $CD4^+$ RA^+ T cells via negative selection. PBMCs were resuspended in 40µl MACS buffer per 10^7 cells in a 1.5ml eppendorf tube. 10µl per 10^7 cells of Biotin Antibody Cocktail was added and cells were then incubated at 4°C for 10 minutes. After washing the cells, the cells were resuspended in 80µl of MACS buffer per 10^7 cells and 20µl of Anti-biotin MicroBeads per 10^7 cells and incubated at 4°C for 15 minutes. Cells were then washed and then preceded to the magnetic cell

separation steps described in section 2.2.2. Purity of the $CD4^+$ RA^+ T cell population was determined via flow cytometry.

2.3 Quantitative PCR (qPCR)

2.3.1 $CD4^+$ RA^+ T cells stimulation for subsequent qPCR analysis

Freshly isolated naïve $CD4^+$ CD45 RA^+ T cells with a purity of ≥95% were resuspended in 500µl of serum-free media (RPMI supplemented with 1%GPS) in duplicate in a 24 well flat-bottomed plate. 25µl of anti-CD3 and anti-CD28 activation beads (Dynabeads) (Invitrogen, Life technologies) were added per 1 x 10^6 of cells. Control cells that were not stimulated with activation beads were also cultured alongside stimulated cells. Cells were incubated for 24 hours at 37°C with 5% CO_2.

2.3.2 RNA extraction

RNA was extracted from unstimulated or stimulated $CD4^+$ RA^+ T cells (assay described in section 2.3.1) using the Qiagen RNeasy Mini Kit. Freshly isolated cells or cultured cells were lysed with 350µl RLT (lysis buffer) and stored at -81°C if not required immediately. Frozen cell lysate (allowed to slowly thaw) or non-frozen lysate was then homogenised by adding 1 volume of 70% ethanol (350µl), which was mixed well without the requirement of centrifugation. 700µl of the sample (including any precipitate that may have formed) was transferred to an RNeasy spin column and placed in a 2ml collection tube ,and centrifuged for 15 seconds at 10000 g. Flow through was discarded and 700µl buffer RW1 was added to the column and centrifuged again for 15s at 10000 g. The flow through was discarded and 500µl RPE buffer was added to the column to be centrifuged for another 15s at 10000 g; this step

was repeated twice. A new 1.5ml collection was then used and 30µl of RNA-free water was added to the column and centrifuged for 1 minute to collect the eluted RNA.

2.3.3 DNase I treatment

Extracted RNA was treated with DNA-free DNase treatment and removal kit (Ambion, Life technologies) to remove genomic DNA. 3.5µl 10x DNase I buffer and 1µl DNase I was added to RNA samples in 1.5ml eppendorf tubes and then incubated at 37°C for 30 minutes. 5µl DNase inactivation reagent was then added to each RNA sample and kept at room temperature for 2 minutes. Samples were centrifuged for 1 minute at 13000 g. The supernatant containing the RNA was then carefully removed without extracting any DNase inactivation reagent white coloured precipitate.

2.3.4 Reverse transcription (RT-PCR)

A Vilo reverse transcription Kit (Invitrogen, Life technologies) was used to reverse transcribes RNA samples into cDNA. For a 20µl total reaction volume the following reaction mix was made; 4µl 5x Vilo reaction mix; 2µl 10x Superscript enzyme mix and 14µl RNA (up to 2.5µg) was used. The samples were then transferred to a thermocycler, which was set up for the following protocol; 25°C for 10 minutes, 42°C for 2 hours and finally 85°C for 5 minutes. After the protocol was completed, cDNA samples were stored at -21°C until they were required.

2.3.5 PCR (cDNA) clean up

Buffers and enzymes used in the RT reaction (section 2.3.4) were removed from the cDNA samples using a NucleoSpin Extract II kit (Macherey-Nagel). Firstly, one volume of sample was mixed with two volumes of NT buffer. The sample was then loaded into a NucleoSpin extract II Column placed in a 2ml collection tube and centrifuged for 1 minute at 11000 g. The flow-through was discarded and 600µl of NT3 buffer was then added to the column, which was then centrifuged for 1 minute at 11000 g. The column was centrifuged for another two minutes at 11000 g to remove NT3 buffer quantitatively. The column was placed in a new 1.5ml microcentrifuge tube and 50µl elution buffer (NE) was added to the column and then incubated for 1 minute at room temperature. Finally, the samples were centrifuged for 1 minute at 11000 g to elute the cDNA.

2.3.6 Quantitative PCR (qPCR)

qPCRs were carried out for gene expression for the following; oestrogen receptor-α (gene ESR1) and oestrogen receptor-β (ESR2); androgen receptor (gene AR); 5α-reductase isoforms 1, 2, 3 (genes SRD5A1, SRD5A2, and SRD5A3); PPAR-α, PPAR-γ and PPAR-δ (genes PPARA, PPARG and PPARD) and CYP19A1 (aromatase). Taqman single gene expression assays (Applied Biosystems, Life technologies); containing a ready made mix of reverse, forward primers and probes, were used for all the genes investigated. A duplicate of each reaction was set up using a 96 well plate. Each 20µl reaction contained 12.5µl of 2X Taqman PCR Master Mix (Applied Biosystems, Life technologies), 30-50ng of cDNA, 1µl of Taqman assay and RNase-free water was added to make up the total reaction volume of 20µl. Ribosomal RNA (18S) was used as a reference gene and was used in separate reactions. Human gene reference cDNA (Stratagene), which contains most of the human genome and

prostate RNA (courtesy of the Arlt lab), was used as a positive control for all the genes of interest. A MX3000P QPCR System (Stratagene) machine was used and the protocol set was as follows; one cycle at 50°C for 2 minutes and one cycle at 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 sec and 60°C for 1 minute. After manually setting the threshold in the exponential area of the curve, the gene expression of the genes investigated was quantified relative to 18S expression (CT value of 18s subtracted from CT value of gene of interest to calculate the delta CT). Relative quantification (RQ value) was then calculated using the following formula; power (2,-Δct), in which the efficiency of the reactions is assumed to be 100%.

2.4 Liquid Chromatography Mass Spectrometry (LCMS)

2.4.1 5α-reductase enzymatic activity

To measure 5α-reductase enzymatic activity, a testosterone to DHT conversion assay was carried out and the concentration of DHT was measured using LCMS (liquid chromatography-mass spectrometry). 3 x 10^6 freshly isolated $CD4^+$ T cells were resuspended in 400µl media (Cellgro) (supplemented with 1% 100U/ml Penicillin G and 10µg/ml Streptomycin sulphate) and 50µl of foetal calf serum (FCS) was added to glass TLC tubes. Assays were supplemented with FCS to improve the kinetics of the conversion assay, as FCS supplementation prevented high numbers of cell death. 25µl (per 10^6 cells) of anti-CD3 and anti-CD28 stimulation beads (Invitrogen, Life technologies) was added (not to unstimulated control samples). For a final concentration of 200nM testosterone; 4.75µl of 20uM testosterone (made up in 100% ethanol, Sigma) was added to unstimulated and stimulated cells, and 4.75µl of 100% ethanol were added to control cell samples. Tubes containing no cells and just medium (with and without FCS) were used as control samples to detect any

background steroid hormones present in the medium or FCS. Testosterone was not added to these control samples. The TLR glass tubes were then placed in a horizontally-rotating incubator 37°C and incubated for 24 hours.

2.4.2 Steroid hormone extraction from cell media.

20 µl internal standards; testosterone-d_3 and DHT-d_3 were added to all samples before extraction, so that any differences between samples that may arise during the extraction process can be standardised to the internal standard. To extract the steroid hormones from the media, cells were pelleted by centrifuging the tubes at 300 g for 5 minutes and then the media supernatant was transferred into a fresh TLC tubes. 3ml dichloromethane was then added to each tube so that steroid hormones would move from the media into the dichloromethane layer to extract the steroids. The tubes were then centrifuged at 300 g for 5 minutes and allowed to stand for 10 minutes. The top layer (media) was removed and the remaining dichloromethane was evaporated to dryness using nitrogen gas and heating the tubes to 40-50°C. Finally, the steroid hormones were reconstituted in 100µl 50/50 methanol (LCMS grade)/distilled water and transferred into a 500µl glass vial ready for LCMS analysis.

Concentration of T and DHT (ng/ml)	Volume of testosterone (ml)	Volume of DHT (ml)	Internal standard Testosterone-d_3 (10µl) and DHT-d_3 (10µl)	Volume of 50/50 methanol (LCMS grade) Distilled water (µl)
200	200	200	20	580
100	100	100	20	780
50	50	50	20	880
25	25	25	20	930
10	10	10	20	940
5	5	5	20	970
1	1	1	20	978

Table 2.1 Calibration samples used to produce a standard curve to quantitate the concentration of DHT produced. The calibration series was produced using the following dilutions shown.

2.4.3 Mass spectrometry parameters

All samples and calibrators were prepared in 50/50 methanol/distilled water (LC/MS grade); 20µl of each sample was analysed. A Waters Xero mass spectrometer with Acquity uPLC system was used, which was fitted with a HSS T3, 1.8µm, and 1.2x50mm column. The column temperature remained constant throughout the experiments. The following settings were used: an electrospray source in positive ionisation mode, capillary voltage 2.0kV, cone voltage 14-30V, collision energy 8-26eV (depending on the mass transition), a source temperature of 150°C, and a desolvation temperature of 600°C.

2.5 Western blotting using SDS-PAGE

2.5.1 Gel electrophoresis

For a 12% resolving gel; 5.25ml of distilled water, 3.25ml of 1.5M Tris buffer pH 8.8 and 300µl of sodium dodecyl sulphate (SDS) was added to 6ml of Protogel (30% acrylamide and bis-acrylamide). Once the gel was ready to be poured into the cassette, 75µl of ammonium

persulphate (APS) and 15µl of tetramethylethylenediamine (TEMED) was added to the mixture and left to set in a cassette for 10 minutes. Distilled water was added to the top of the cassette to ensure a straight gel line formed and to also prevent the formation of air bubbles in the resolving gel. 5% staking gel was made with 1.3ml of protogel, 6.1ml of distilled water, 2.5ml of 0.5M Tris Buffer pH 6.8, 100µl of 10% SDS, 50µl of APS and 10µl of TEMED. Once the resolving gel had set, the distilled water was removed from the top of the cassette and staking gel was then loaded on top of the resolving gel. A comb was then immediately inserted into the stacking gel form wells. Once the stacking gel had set the comb was removed and SDS running buffer was then added to remove any air bubbles present in the wells. The white strip on the cassette was removed and subsequently inserted into a running tank filled with SDS running buffer. Freshly isolated naïve $CD4^+$ CD45 RA^+ T cells were resuspended in loading buffer blue heated at 100^0C for 10 minutes and centrifuged to pellet any undissolved protein. 10µl of Protogel protein ladder was loaded into the first lane using a Hamilton glass syringe. 20µl of sample (protein from 2 x 10^5 cells per well) was loaded in subsequent lanes. The electrodes from the gel take were attached to a voltage supplier and the gel ran for 2 hours at 190 volts.

2.5.2 Transfer to PVDF membrane

Once the proteins had been separated using gel electrophoresis, they were then transferred onto a PVDF membrane via a wet blotting system. This system involves the formation of a membrane sandwich in a tray of blotting buffer. In the tray, a sponge and then filter paper soaked in blotting buffer were placed on the black side of a large cassette. A piece of PVDF membrane was cut 1cm larger in diameter then the gel. The membrane was activated in methanol for 30 seconds and then rinsed in distilled water and blotting buffer. The activated membrane was then placed on the filter paper soaked in blotting buffer in the cassette. The

gel was then placed on the membrane and covered with filter paper and a sponge both soaked in blotting buffer and the cassette was subsequently sealed. This ensures that the PVDF membrane and the gel are packaged in a tight sandwich to allow transfer of the protein from the gel onto the PVDF membrane. A roller was used to remove air bubbles and folds. The cassette was placed in a tank with the black side of the cassette facing the black electrode in the blotting tank filled with blotting buffer, as this ensures that the protein from the gel transfers onto the PVDF membrane and not onto the filter paper. The transfer was run at 450 milliamps for 90 minutes.

2.5.3 Antibody probing

After the transfer, the membrane was removed from the cassette and the gel was discarded. To prevent any unspecific binding of the antibody on the membrane, the membrane blot was soaked in 1% TBS-Tween (tween 0.5%) with 5% milk, which is used as a blocking reagent, for 1 hour before probing the blot with the primary antibody. The blot was then washed three times with 1x TBS-Tween for 15 minutes. The blot is probed with the primary antibody mouse anti-human androgen receptor (Southern Biotech) at a dilution of 1:5000 (made up in 0.5% milk TBS-tween) overnight with gentle agitation at 4°C.

The blot was washed three times with 1x TBS-Tween for 15 minutes. The blot was then probed with a HRP conjugated secondary sheep anti-mouse IgG antibody (GE healthcare) at dilution of 1:2000 for 1 hour at room temperature with gentle agitation. The blot was then washed three times with 1x TBS-Tween for 15 minutes.

2.5.4 Enhanced chemiluminescence (ECL plus)

The proteins were visualised using ECL plus (GE healthcare): the blot is incubated with ECL for 1min with gentle agitation. The blot was then wrapped in saran wrap and a roller was used to remove air bubbles or folds. The blot was placed in a cassette and taped down ready to expose the blot. The blot was exposed onto a film for 1 minute (autoradiography against X-ray film-Kodak) and developed using a developer in a dark room.

2.5.5 β-actin antibody probing

For a protein loading control, the membrane was subsequently probed for β-actin protein. Firstly, the membrane was washed in three times 1x TBS-Tween for 15 minutes. The blot was probed with a primary antibody mouse anti-human β-actin (Southern Biotech) at a 1:5000 dilution (made up in 0.5% milk TBS-tween) and left overnight with gentle agitation at 4°C. The membrane was then probed with a secondary HRP conjugated antibody using the protocol described previously for androgen receptor and also visualised using ECL (as described above).

2.6 Flow cytometry

2.6.1 Cell membrane surface staining

Firstly, the wells of a 96 well round-bottomed flexiplate were coated with FCS. Cells were then resuspended in FCS and 5 x 10^5 cells were added to each well, and then centrifuged at 300 g at 4°C for 4 minutes. The supernatant was then removed leaving the cells pelleted in the wells. Cells were resuspended in directly conjugated antibody (specific for the protein of

interest) Isotype and species-matched control antibody, which was made up in final volume of 50µl MACS buffer (as used in section 2.2.2). For staining panels containing more than one fluorochrome, compensation samples for single colour staining were required. Optimal dilutions for all antibodies were determined by titration experiments. One sample was stained with species, concentration and isotype-matched controls (irrelevant samples) for the specific antibodies to detect any background signals. Cells were then incubated for 15 minutes at 2-4°C in the dark. The cells were then washed twice in 50µl MACS buffer and centrifuged at 300 g at 4°C for 4 minutes, and then transferred into FACS tubes and resuspended in 500µl of MACS buffer. Flow cytometry was performed using a Dako Cyan flow cytometer (Beckman Coulter Ltd). Summit 4.4 software (Dako) was used to analysis the data.

2.6.2 Intracellular staining

Firstly, the cells underwent surface staining as described in section 2.6.1. Cells were then resuspended in 50µl of medium A (fixation) (Invitrogen, Life technologies) and incubated for 10 minutes at 2-4°C. The cells were then washed twice in 50µl MACS buffer and centrifuged at 300 g at 4°C for 4 minutes. Cells were resuspended in 50µl of medium B (for cell permabilisation) (Invitrogen, Life technologies) and incubated for 10 minutes at 2-4°C. The cells were then washed twice in 50µl MACS buffer and centrifuged at 300 g at 4°C for 4 minutes. Cells were then resuspended in 50µl antibody (made up in MACS buffer) that were specific for intracellular proteins (including control samples stained with concentration, Isotype-matched irrelevant antibodies) and were incubated for 15 minutes at 2-4°C. The cells were then washed twice in 50µl MACS buffer and centrifuged at 300 g at 4°C for 4 minutes. 50µls of secondary antibody (made up in MACS buffer) was added to wells and incubated for 15 minutes at 2-4°C. Secondary antibodies were only used for unconjugated primary

antibodies. Cells were then washed twice in 50µl MACS buffer and centrifuged at 300 g at 4°C for 4 minutes. Finally, cells were resuspended in 200µl MACS buffer and transferred into FACS tubes containing 300µl MACS buffer. Flow cytometry was performed using a Dako Cyan flow cytometer (Beckman Coulter Ltd). Summit 4.4 software (Dako) was used to analysis the data.

Table 2.2 shows the antibodies used in intracellular staining for PPARs and 5α-reductase 1. Dilutions for the PPARs antibodies were made from a stock concentration of 100µg/ml and for the 5α- reductase 1antibody, the dilutions were made from a stock concentration of 200µg/ml.

	Dilution	Antibody Clone	Supplier
Primary antibodies			
Mouse anti-human CD4 APC	1:100	EDU-2	Immunotools
Mouse anti-human CD45 RA PE	1:100	4KB5	Dako
Mouse anti-human PPAR-α	1:50	H0723	R&D systems
Mouse anti-human PPAR-γ	1:20	K8713	R&D systems
Mouse anti-human PPAR-δ	1:40	K9436	R&D systems
Mouse anti-human AR	1:40	523339	R&D systems
Rabbit anti-human 5α-reductase 1	1:100	H-105	Santa Cruz Biotech
Isotype-matched control antibodies			
Mouse IgG1 APC	*	M1-14D12	ebiosciences
Mouse IgG1 PE	*	PPV-04	Immunotools
Mouse IgG2a (for PPARs)	*	DAK-GO5	Dako
Mouse IgG2b (for AR)	*	DAK-GO9	Dako
Rabbit serum IgG	*	-	Dako
Secondary antibodies			
Goat anti-mouse IgG2a FITC	1:100	-	Southern biotech
Goat anti-mouse IgG2b FITC	1:100	-	Southern biotech
Goat anti-rabbit FITC	1:100	-	Southern biotech

Table 2.2 Antibodies used for intracellular staining of PPARs, AR and 5α-reductase 1. The same concentration used for the specific antibodies were also used for the Isotype and species-matched controls (represented by the asterisks)

Table 2.3 shows the antibodies used for 5α-reductase staining in SLE patients and healthy controls; using the protocol in section 2.6.1 and 2.6.2. Two separate staining panels were used; the first panel includes antibodies used for surface staining; mouse anti-human CD3 pacific blue, mouse anti-human CD4 APC, mouse anti-human CD45 RO PE, mouse anti-human CD8 PeCy7, and rabbit anti-human 5α- reductase 1 antibody or the isotype-matched

control antibody (rabbit IgG) for intracellular staining. The second panel includes the antibodies used for surface staining of; mouse anti-human CD14 PeCy7, mouse anti-human CD19 pacific blue, and intracellular staining with rabbit anti-human 5α-reductase 1 antibody or the isotype-matched control antibody (rabbit IgG).

	Dilution	Antibody Clone	Supplier
Primary antibodies			
Mouse anti-human CD3 pacific blue	1:200	UCHT1	Biolegend
Mouse anti-human CD4 APC	1:100	EDU-2	Immunotools
Mouse anti-human CD45 RO PE	1:100	UCHL1	Dako
Mouse anti-human CD8 PeCy7	1:100	RPA-T8	ebioscience
Mouse anti-human CD14 PeCy7	1:100	61D3	ebioscience
Mouse anti-human CD19 pacific blue	1:200	HIB19	Biolegend
Rabbit anti-human 5α-reductase 1	1:100	H-105	Santa Cruz Biotech
Isotype-matched control antibodies			
Mouse IgG1 pacific blue (for CD3 and CD19)	*	MOPC-21	Biolegend
Mouse IgG1 APC	*	M1-14D12	ebioscience
Mouse IgG1 PE	*	PPV-04	Immunotools
Mouse IgG2a PeCy7 (for CD8 and CD14)	*	eBM2a	ebioscience
Rabbit serum IgG	*	-	Dako
Secondary antibody			
Goat anti-rabbit FITC	1:100	-	Southern biotech

Table 2.3 Antibodies used for intracellular staining of 5α-reductase 1 in SLE patients and healthy controls.

Table 2.4 shows the antibodies used for 5α-reductase staining in B cell subsets from SLE patients and healthy controls using the protocol in sections 2.6.1 and 2.6.2.

	Dilution	Antibody Clone	Supplier
Primary antibodies			
Mouse anti-human CD19 pacific blue	1:200	HIB19	Biolegend
Mouse anti-human CD27 APC	1:50	O323	ebioscience
Mouse anti-human CD38 PE Texas-Red	1:100	HIT2	Invitrogen (Life tech)
Mouse anti-human IgD PeCy7	1:50	IA6-2	Biolegend
Rabbit anti-human 5α-reductase 1	1:100	H-105	Santa Cruz Biotech
Isotype-matched control antibodies			
Mouse IgG1 pacific blue	*	MOPC-21	Biolegend
Mouse IgG1 APC	*	M1-14D12	ebioscience
Mouse IgG1 PE Texas-Red	*	-	Invitrogen (Life tech)
Mouse IgG2a PeCy7	*	eBM2a	ebioscience
Rabbit serum IgG	*	-	Dako
Secondary antibody			
Goat anti-rabbit FITC	1:100	-	Southern biotech

Table 2.4 Antibodies used for intracellular staining of 5α-reductase 1 in B cell subsets from SLE patients and healthy controls.

2.6.3 Sex hormone treatment and cytokine production assays

5 x 10^5 freshly isolated PBMCs (section 2.2.2) were resuspended in 482µl of serum-free media (RPMI supplemented with 1% GPS) per well in a 24 well flat-bottomed plate Cells were then incubated for 20 hours at 37°C with 5% CO_2. For cytokines IL-10 and IL-17, which are produced much later than IL-2 and IFN-γ after stimulation, cells were stimulated with CD3 and CD28 activation beads for 20 hours before the addition of sex hormones and Brefeldin A (Sigma). After overnight incubation, 18µl of anti-CD3 and anti-CD28 activation beads were added to each well except in wells for control cells that were not stimulated with

activation beads. Brefeldin A was added to all wells (both cells with or without stimulation beads) at a final concentration of 10μg/ml. Both control and stimulated cells were then treated with different concentrations of either testosterone; 0.1nM, 1nM, 7nM and 15nM, or treated with different concentrations of DHT; 0.0nM, 0.1nM, 0.5nM and 2nM, or different concentrations of 17β-estradiol; 0.07nM, 0.13nM, 0.29nM and 0.95nM. In control wells, which were not treated with any sex hormones, 1μl 100% ethanol was added instead as a vehicle control. All the stock solutions of the sex hormones were made up in 100% ethanol; in all the treatments the volume of the sex hormones added to the cells was less than 1.5% of the total assay volume. Cells were incubated for 4 hours at 37°C with 5% CO_2. After the 4 hours incubation, the cells are ready to be stained for intracellular cytokines using the methods in sections 2.6.1 and 2.6.2 and analysed by flow cytometry.

Table 2.5 shows the antibodies used for intracellular staining of cytokines after treatment with sex hormones using the protocol in section 2.6.1 and 2.6.2. Four separate staining panels were used; the first panel included surface staining with antibodies; mouse anti-human CD3 pacific blue, mouse anti-human CD4 FITC, mouse anti-human CD45 RO PE and intracellular staining with antibodies anti-human IFN-γ APC and IL-2 PeCy7 or the Isotype-matched control antibody. The second panel included surface staining with antibodies; mouse anti-human CD3 pacific blue, mouse anti-human CD8 PE, mouse anti-human CD45 RO FITC, and intracellular staining with antibody anti-human IFN-γ APC and IL-2 PeCy7. The third panel (and fourth panel) were used for cells stimulated for 24 hours, which included surface staining antibodies; mouse anti-human CD3 pacific blue, mouse anti-human CD4 APC, mouse anti-human CD8 PeCy7, and intracellular staining with antibodies anti-human IL-10 FITC and anti-human IL-17 PE. The fourth panel included surface staining antibodies; mouse

anti-human CD3 pacific blue, mouse anti-human CD4 APC, mouse anti-human CD8 PeCy7, and intracellular staining with antibody anti-human TNFα PE.

	Dilution	Antibody Clone	Supplier
Surface antibodies			
Mouse anti-human CD3 pacific blue	1:200	UCHT1	Biolegend
Mouse anti-human CD3 PE	1:100	UCHT-1	Immunotools
Mouse anti-human CD4 FITC	1:100	MEM-115	Immunotools
Mouse anti-human CD45 RO PE	1:100	UCHL1	Dako
Mouse anti-human CD45 RO FITC	1:100	UCHL1	Dako
Mouse anti-human CD8 PeCy7	1:100	RPA-T8	ebioscience
Mouse anti-human CD8 PE	1:100	UCHT-4	Immunotools
Intracellular antibodies			
Mouse anti-human IL-2 PeCy7	1:100	M-A251	BD biosciences
Mouse anti-human IL-10 FITC	1:25	BT-10	ebioscience
Mouse anti-human IL-17A PE	1:25	SCPL-1362	BD biosciences
Mouse anti-human IFN-γ APC	1:500	B27	BD biosciences
Mouse anti-human TNF-α PE	1:50	6401.1111	BD biosciences
Isotype-matched control antibodies			
Mouse IgG1 pacific blue	*	MOPC-21	Biolegend
Mouse IgG1 PE	*	M1-14D12	ebioscience
Mouse IgG1 FITC	*	203	Immunotools
Mouse IgG2a PeCy7	*	eBM2a	ebioscience
Mouse IgG1 APC	*	M1-14D12	ebioscience

Table 2.5: Antibodies used for intracellular staining of cytokines using flow cytometry after treatment with sex hormones.

2.6.4 Testosterone and 4-hydroxytamoxifen treatment

5 x 10^5 freshly isolated PBMCs (section 2.2.2) were resuspended in 482µl of serum-free media (RPMI supplemented with 1% GPS) per well in a 24 well flat-bottomed plate. Cells were then incubated for 20 hours at 37°C with 5% CO_2. After overnight incubation, 18µl of anti-CD3 and anti-CD28 activation beads were added to each well except in wells for control cells that were not stimulated with activation beads. Brefeldin A was added to all wells (both cells treated with or without stimulation beads) at a final concentration of 10µg/ml. Both control cells and stimulated were then treated with a range of testosterone concentrations; 0.1nM, 1nM, 7nM and 15nM. Cells treated with different concentrations of testosterone were treated with 2nM 4-hydroxytamoxifen (control samples included cells treated with testosterone with no further 4-hydroxytamoxifen treatment). Cells were incubated for 4 hours at 37°C with 5% CO_2. After the 4 hours incubation, the cells are ready to be stained for intracellular cytokines using the methods in sections 2.6.1 and 2.6.2 and analysed by flow cytometry.

2.6.5 Sex hormone treatment and CFSE proliferation assay

To measure cell proliferation, cells were labelled with carboxyfluorescein diacetate, succinimidyl ester (CFSE); as the number of cell divisions increases, the CFSE signal decreases. Freshly isolated PBMCs were resuspended in 25µl per 1 x 10^6 cells in PBS 0.1% BSA. 5µM of CFSE (Invitrogen, Life technologies) was diluted in the same volume, in which the cells were suspended in, and then added to the cell suspension for a CFSE final concentration of 2.5µM. Cells were then incubated in the dark at 37°C for 10 minutes. After the incubation period, the cells were quenched with 5 volumes of ice-cold medium and incubated on ice for 5 minutes. The cells were then washed twice by adding 15ml medium

and centrifuging for 5 minutes at 20°C. 5 x 10^5 cells were resuspended in 482µl of medium (RPMI supplemented with 1% GPS and FCS) per well in a 24 well flat-bottomed plate. 18µl of anti-CD3 and anti-CD28 activation beads were added to each well except in wells for control cells that were not stimulated with activation beads. Both control cells and stimulated were then treated with a range of concentrations of testosterone; 0.1nM, 1nM, 7nM and 15nM. Cells were then cultured for 4 days at 37°C with 5% CO_2. After 4 days, cells were stained for flow cytometry analysis of the CFSE signal using the protocol outlined in section 2.6.1. Cells were stained with mouse anti-human CD4 APC and mouse anti-human CD8 pacific blue antibodies (both at 1:100 dilutions). Cells were then analysed for cell proliferation on the flow cytometer using the CFSE signal (FITC channel).

2.7 Statistical Analysis

Statistical analysis was carried out using non-parametric student T tests such as Mann-Whitney and paired Wilcoxon T tests. All statistical analysis was performed using Graph Pad Prism version 5 software. Data analysis producing a p value of ≤ 0.05 was deemed statistically significant.

3 GENDER DIFFERENCES IN HORMONE METABOLISM DURING T CELL ACTIVATION.

3.1 Introduction

Androgens and oestrogens have been attributed with conflicting effects on the immune system; androgens such as testosterone and DHT have an anti-inflammatory effect, whereas oestrogens such as 17β-oestradiol and oestrone generally enhance immune responses. Sex hormone levels have shown to have a profound effect on the severity of chronic inflammatory conditions and autoimmune diseases, therefore it is important to investigate the effects of sex hormones on the immune system. It is important to consider that the immune system in each individual has developed in the context of the hormone environment it is exposed to. So we need to distinguish between the effects of male or female hormones have throughout the development of the individuals T cell pool and thus investigate the long term and short-term effects of sex hormone exposure on T cells in the context of an in-vitro experiment over a short time duration. Research into the specific differences in the way T cells respond to hormones may help our understanding of autoimmune diseases and could potentially lead to new therapeutic developments.

Enzymes involved in sex hormone metabolism such as 5α-reductase have been proposed to play an important role in the regulation of immune responses; predominately in a suppressive manner. In mouse models for trauma-haemorrhage, 5α-reductase expression in T cells was found to be increased compared to controls, which resulted in an increase in DHT production and thus suppression of T cell responses (Samy *et al.* 2001). Androgens and oestrogens mediate their effects through binding to nuclear receptors; AR; ER-α and ER-β. These sex

hormone receptors have been shown to be expressed in various immune cell subsets (Brechenmacher *et al.* 2008), therefore this suggests that immune cells may be responsive to the actions of endogenous or exogenous androgens and oestrogens. AR expression has been shown to be higher in macrophages from males in comparison to female macrophages (McCrohon *et al.* 2000); this has been linked to androgen-mediated development of atherosclerosis, and higher susceptibility to this disease in males.

One of the aims of this project was to investigate differences in the way T cells from healthy males and females regulated the production of androgens and oestrogens, and how T cells were able regulate their exposure to sex hormones by investigating the expression of sex hormone metabolising enzymes and sex hormone receptors. To accomplish this, we explored the differences in mRNA expression of sex hormone receptors genes and sex hormone metabolising enzymes between male and female naïve $CD4^+$ T cells at baseline and after *in vitro.* We investigated expression of 5α-reductase isoforms 1, 2 and 3 (genes being SRD5A1, SRD5A2, SRD5A3 respectively); as the activity if this enzyme results in the production of the most androgen, DHT. We also investigated the mRNA expression of sex hormone receptors; AR, ER-α, and ER-β (gene names ESR1 and ESR2 respectively) at baseline and after stimulation.

We also wanted to explore the role of PPARs in mediating sex differences observed in immune responses as Dunn and colleagues found in mice that androgen activated PPAR-α suppressed Th1 cell mediated responses (Dunn *et al.* 2007). They also demonstrated a sex difference in PPAR-α expression, as it was more abundant in male mice in comparison to female mice. This was found to protect males in the development of autoimmunity.

Therefore, we wanted to investigate if this higher expression of PPARs in males (in particular PPAR-α) in comparison to females was present in human naïve $CD4^+$ T cells.

3.2 mRNA and protein expression of androgen receptor, and mRNA expression of oestrogen receptors; ER-α and ER-β in naïve $CD4^+$ T cells in males and females.

We found no significant differences the expression of ER-α or AR in naïve $CD4^+$ T cells between males and females (figure 3.1A, C). There was a trend towards a higher expression of ER-β in naïve $CD4^+$ T cells from males compared to female naïve $CD4^+$ T cells, however this was not statistically significant (p=0.09). Also, we observed no significant differences in ER-α, ER-β and AR expression after *in vitro* stimulation between males and females. However, there was a significant upregulation of ER-β expression in female stimulated naive $CD4^+$ T cells in comparison to unstimulated $CD4^+$ T cells (p=0.003) (figure 3.1B). Both Western blots (figure 3.2A) and flow cytometry (figure 3.2C, D) confirmed the protein expression of AR in naïve $CD4^+$ T cells. In contrast to the mRNA data, we did detect a significant upregulation of AR expression upon stimulation in naive $CD4^+$ T cell in comparison to the unstimulated cells on the protein level, which was observed in both males (p=0.04) and females (p=0.04) (figure 3.2D). Overall, there were few significant differences in sex hormone receptor expression between males and females on the baseline or upon *in vitro* stimulation.

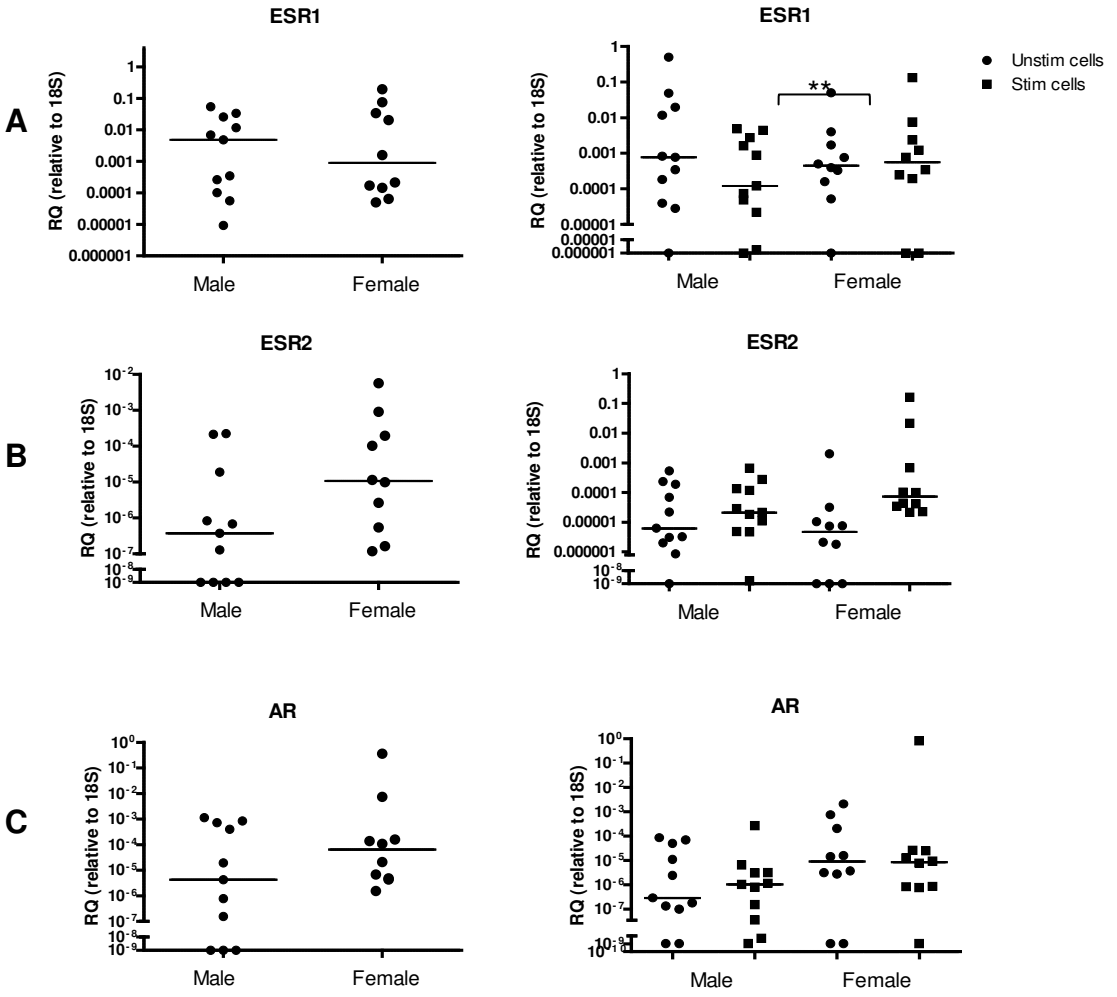

Figure 3.1 mRNA gene expression of sex hormone receptors in naïve CD4$^+$ T cells. Freshly isolated naive CD4$^+$ T cells were separated into three different conditions: freshly isolated cells that were immediately lysed, cultured unstimulated cells and cells stimulated with anti CD3 anti CD28 activation beads for 24 hours prior to cell lysis. Freshly isolated cells were used as control for the unstimulated-cultured cells as artefacts may arise from culturing the cells. Relative quantification (RQ) to ribosomal RNA 18s are shown for **A**) ER-α (ESR1), **B**) ER-β (ESR2) and **C**) AR. Horizontal lines present the median. Wilcoxon matched pairs t test; * $p<0.05$, ** $p<0.001$.

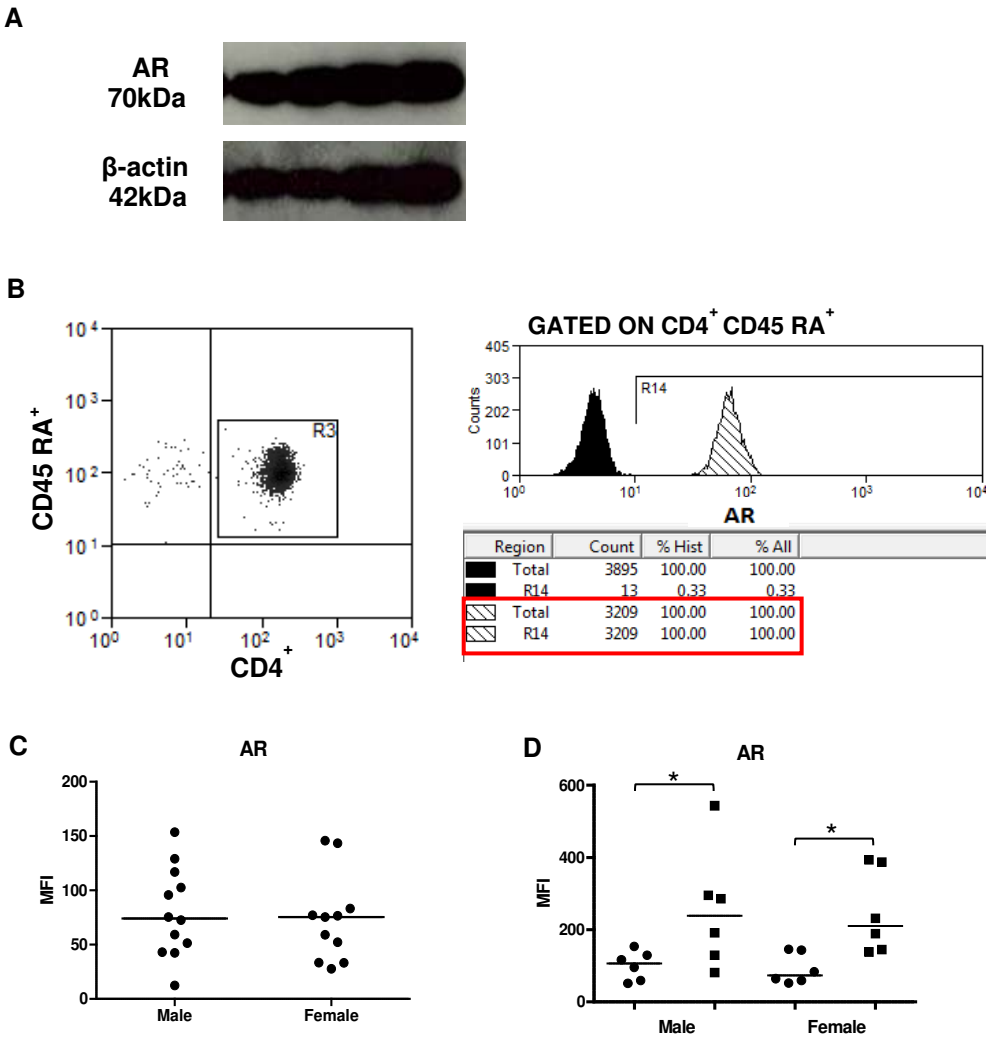

Figure 3.2 Protein expression of AR in naïve CD4$^+$ CD45 RA$^+$ T cells.
A) Protein expression of androgen receptor (AR) in isolated naïve CD4$^+$ CD45 RA$^+$ T cells from four donors was detected using Western blotting; β-actin was used as a loading control. Naive CD4$^+$ CD45 RA$^+$ T cells were stained for intracellular AR and analysed using flow cytometry. All the cells gated as CD4$^+$ CD45 RA$^+$ expressed AR as highlighted in the histogram for AR expression (**B**). **C**) Accumulative data for AR expression in naive CD4$^+$ CD45 RA$^+$ T cells from males (n=10) and females (n=10). **D**) Naïve CD4$^+$ CD45 RA$^+$ T cells from 6 males and 6 females were stained for intracellular AR expression after no stimulation (circles) or stimulation with anti CD3 and anti CD28 activation beads for 24 hours (squares) and analysed using flow cytometry. Horizontal lines present the median. MFI: median fluorescence intensity. Wilcoxon matched pairs t test; * $p<0.05$.

3.3 mRNA expression of 5α-reductase isoforms in naïve $CD4^+$ T cells from males and females.

We could not detect mRNA expression of 5α-reductase isoform 2 in naïve $CD4^+$ T cells or PBMCs, however in the prostate RNA positive control, did detect SRD5A2 expression (data not shown), and therefore the assays were working efficiently. However, we could detect mRNA expression for 5α-reductase isoforms 1 and 3 (figure 3.3A, B). On the baseline, there were no significant differences between males and females in 5α-reductase 1 or 3 expression in naïve $CD4^+$ T cells. Additionally, there were no significant differences in 5α-reductase 1 and 3 expression between males and females naïve $CD4^+$ T cells after *in vitro* stimulation. However, we did observe a significant upregulation in of both 5α-reductase 1 and 3 upon stimulation in female naive $CD4^+$ T cells in comparison to the unstimulated female naïve $CD4^+$ T cells. However, this was not observed in male naive $CD4^+$ T cells upon stimulation.

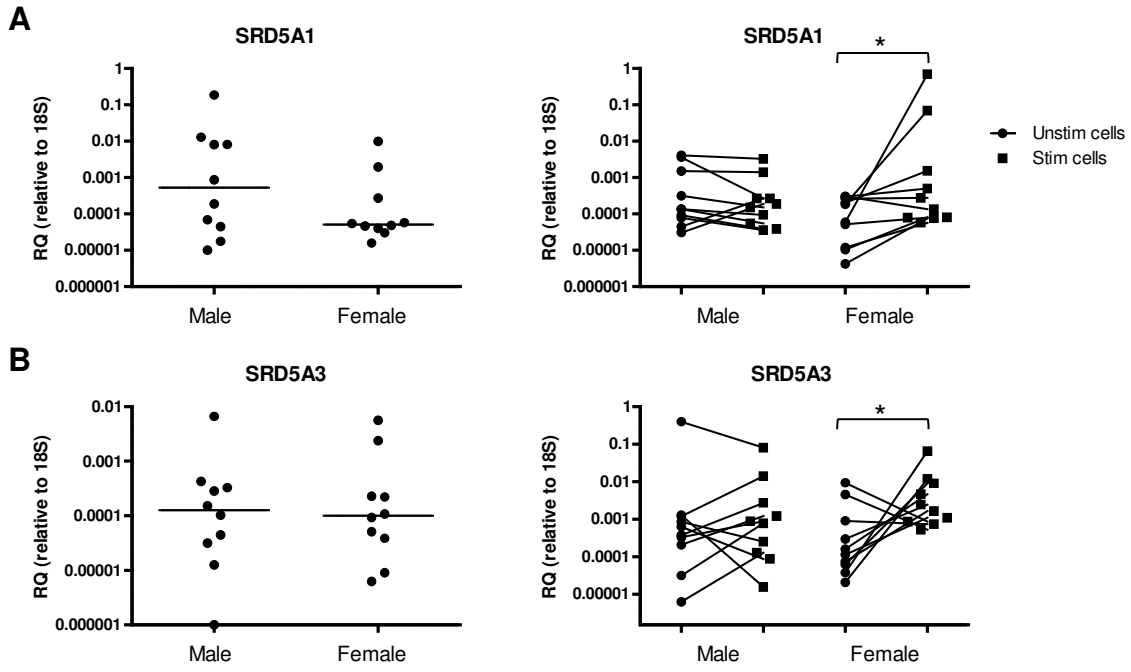

Figure 3.3 mRNA gene expression of 5α-reductase isoforms.
Freshly isolated naive $CD4^+$ T cells were separated into three different conditions: freshly isolated cells that were immediately lysed, cultured unstimulated cells and cells stimulated with anti CD3 and anti CD28 activation beads for 24 hours prior to cell lysis. Relative quantification (RQ) to ribosomal RNA 18s is shown for **A**) 5α-reductase 1 (SRD5A1) and **B**) 5α-reductase 3 (SRD5A3). Horizontal lines represent the median. Wilcoxon matched pairs t test; * $p<0.05$.

3.4 Protein expression and enzymatic activity of 5α-reductase in naïve $CD4^+$ T cells.

We wanted to confirm our mRNA findings by measuring 5α-reductase 1 on the protein level. We repeated the same experiments used for detecting mRNA expression and instead used flow cytometry as a readout for protein expression. $CD45RA^+$ $CD4^+$ T cells were stained for intracellular expression for 5α-reductase 1 (figure 3.4B). The protein data confirmed the mRNA data, as we observed an upregulation of 5α-reductase 1 expression in female stimulated $CD4^+$ T cells in comparison to the unstimulated $CD4^+$ T cells. This upregulation of 5α-reductase 1 expression was not observed in stimulated male $CD4^+$ T cells on the protein level, which was in line with the observations made on the mRNA level. A stimulation time

course for 5α-reductase 1 protein expression showed an upregulation of 5α-reductase 1 expression was observed at the 24 hours stimulation time point in both naïve (CD45 RO^-) and memory (CD45 RO^+) $CD4^+$ T cells (figure 3.4C).

We then wanted to investigate whether the increased gene and protein expression levels of 5α-reductase 1 in female $CD45RA^+$ $CD4^+$ T cells was reflected in 5α-reductase enzymatic activity. 5α-reductase conversion assays determined reduction of the substrate testosterone to DHT to as a readout for 5α-reductase activity and DHT concentrations were then measured using LCMS. These assays are particularly complicated and took several months to set up as extremely low levels of DHT are detected (figure 3.5B), and relatively insensitive assays are only available. The DHT levels detected were at the lower end of the detection range and therefore there were doubts regarding the validity of the analysis. We did detect a trend towards higher DHT production in the stimulated $CD4^+$ T cells compared to the unstimulated, but unfortunately we obtained extremely low concentrations of DHT detected by LCMS in these conversion assays. Therefore, while we could confirm a trend towards higher 5α-reductase activity in the stimulated female $CD4^+$ T cells compared to the unstimulated cells, because of the low DHT concentration levels a definite judgement cannot be made yet. We detected conversion of testosterone to androstenedione (figure 3.5C) but a standard curve for androstenedione concentration was not used in the LCMS experiments therefore exact concentrations of androstenedione were unknown.

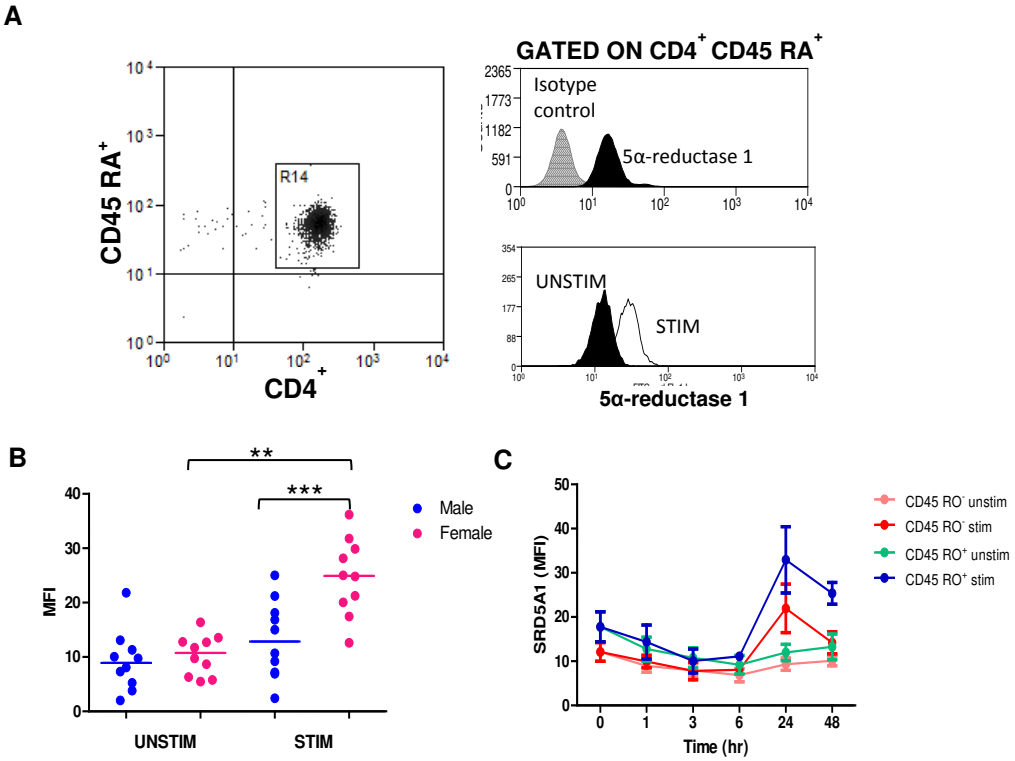

Figure 3.4 5α-reductase 1 protein expression in naïve CD4$^+$ T cells.
A) FACS plots for one donor showing naive CD4$^+$ CD45 RA$^+$ T cells that were stained for intracellular 5α-reductase 1 expression (shown in histogram) after no stimulation (unstimulated) or stimulation with anti CD3 and anti CD28 activation beads for 24 hours and analysed using flow cytometry. 5α-reductase 1 expression in CD4$^+$ CD45 RA$^+$ T cells was measured in 10 males and 10 females (**B**). Horizontal lines present the median. **C**) Female CD4$^+$ T cells (n=3) were stimulated for various time durations and then stained for intracellular 5α-reductase 1 expression in naïve CD4$^+$ CD45 RO$^-$ and memory CD4$^+$ CD45 RO$^+$ T cells. MFI: median fluorescence intensity. MFI of isotype-matched controls were subtracted from the 5α-reductase MFI. Error bars represent SEM. Wilcoxon matched pairs t test; ** $p<0.001$; *** $p<0.0001$.

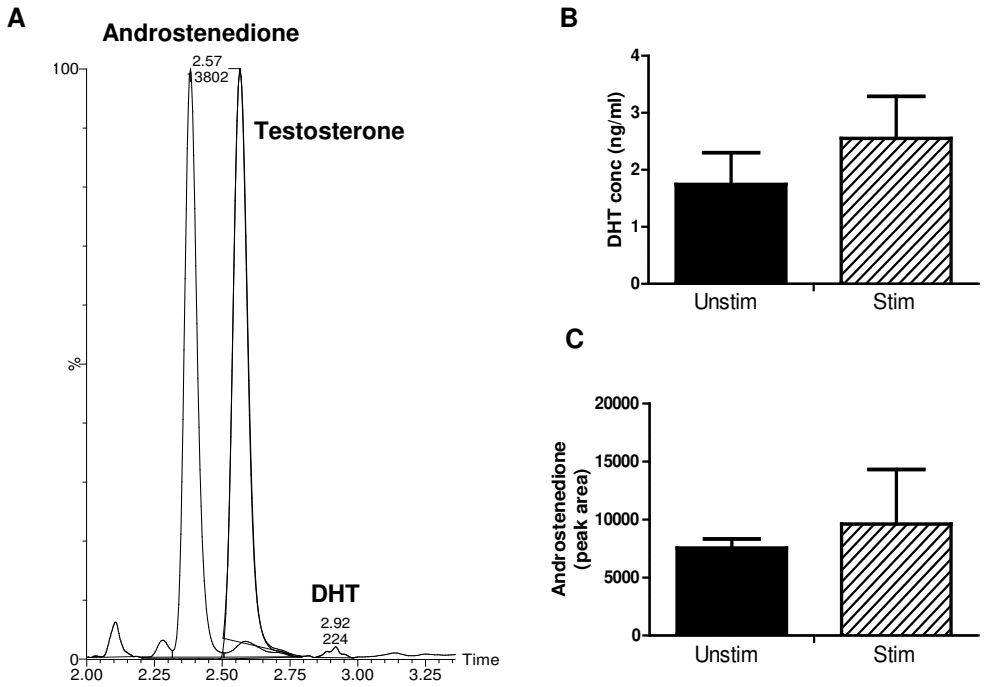

Figure 3.5 5α-reductase activity in female naïve CD4$^+$ T cells.
5α-reductase activity in female unstimulated or *in vitro* anti CD3/anti CD28-stimulated naïve CD4$^+$ T cells was measured using a conversion assay of substrate testosterone to DHT. DHT concentration was measured using LCMS after 24 hours conversion assays, **A**) shows detected LCMS peaks for testosterone, DHT and androstenedione. **B**) The concentration of DHT detected (n=3). **C**) Androstenedione was also detected in the assays and is represented as area under the curve (n=3). Error bars represent SEM.

3.5 mRNA and protein expression of PPARs in naïve CD4$^+$ T cells in males and females.

We observed no significant differences in baseline mRNA or protein expression of PPAR-γ and PPAR-δ in naïve CD4$^+$ T cells between males and females. There was a trend observed, in which baseline expression of PPAR-α was higher in male naïve CD4$^+$ T cells compared to female naïve CD4$^+$ T cells (figure 3.6A), however this finding was not statically significant on the mRNA (p=0.08) or on the protein level (figure 3.6E). There were no significant differences in all three PPAR isoforms expression after stimulation between males and females on both the mRNA (figure 3.6A, B, C) and protein level (see appendix). However, there was a significant upregulation of PPAR-γ in female stimulated CD4$^+$ T cells compared

to the unstimulated female $CD4^+$ T cells; however this was not observed on the protein level (see appendix). Overall, there were no significant differences in the expression of PPARs between males and females on the baseline and upon stimulation on both the mRNA and protein levels.

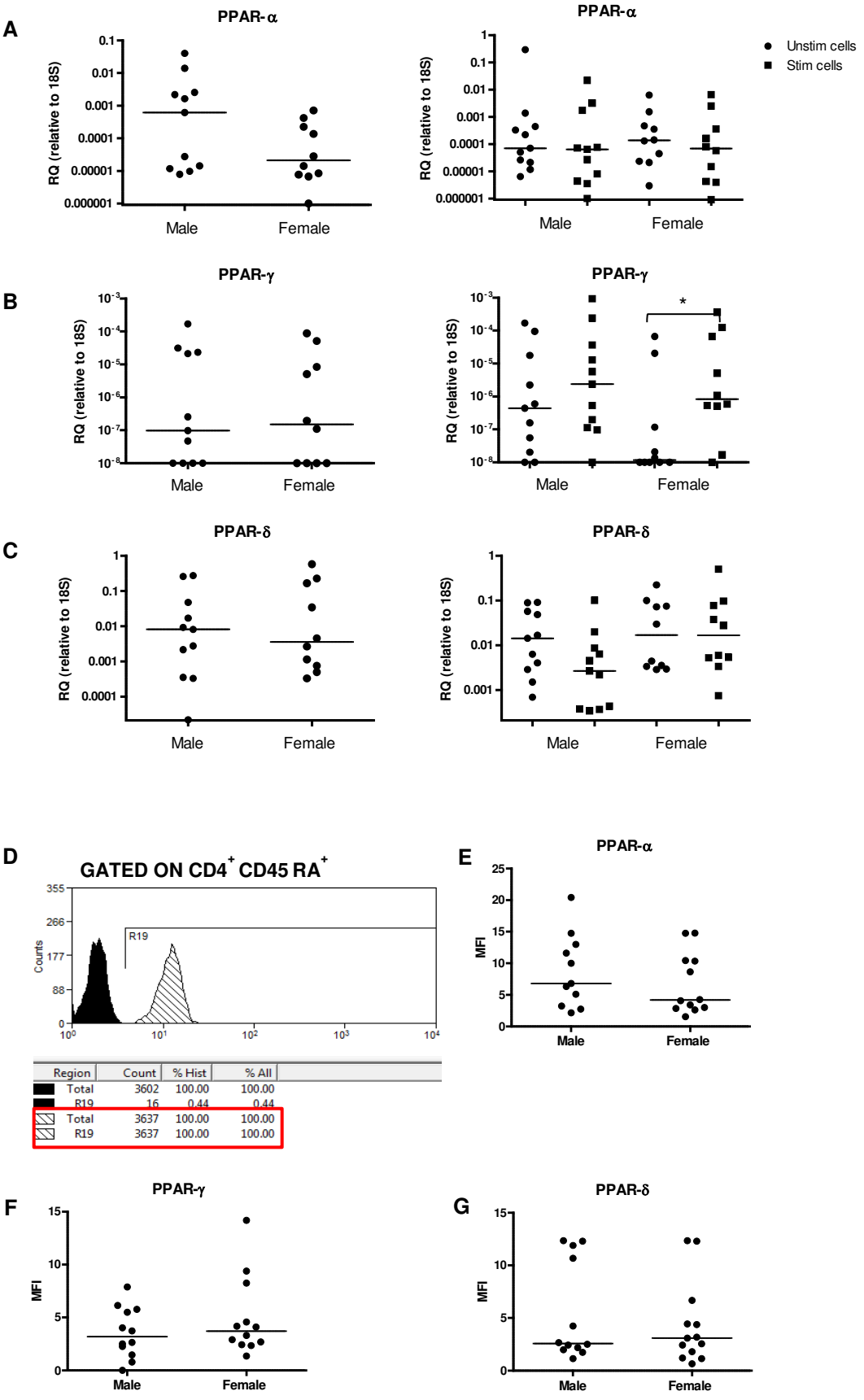
A
PPAR-α
RQ (relative to 18S)
Male
Female
Unstim cells
Stim cells
B
PPAR-γ
*
C
PPAR-δ
D
GATED ON CD4+ CD45 RA+
Counts
R19
Region Count % Hist % All
Total 3602 100.00 100.00
R19 16 0.44 0.44
Total 3637 100.00 100.00
R19 3637 100.00 100.00
E
MFI
F
G

Figure 3.6 mRNA and protein expression of PPARs in naive $CD4^+$ T cells.
Freshly isolated naive $CD4^+$ T cells from males (n=10) and females (n=10) were separated into three different conditions: freshly isolated cells (baseline) that were immediately lysed, cultured un-stimulated cells and cells stimulated with anti CD3 and anti CD28 activation for 24 hours prior to cell lysis. mRNA gene expression was measured as relative quantification (RQ) to housekeeping gene 18s for **A**) PPAR-α, **B**) PPAR-γ and **C**) PPAR-δ. Naive $CD4^+$ CD45 RA^+ T cells were stained for intracellular PPARs and analysed using flow cytometry. All the cells gated as $CD4^+$ CD45 RA^+ expressed all three PPAR isoforms including PPAR-α as highlighted in the histogram for PPAR-α expression (**D**). Accumulative data from males (n=10) and females (n=10) are represented as dot graphs for **E**) PPAR-α, **F**) PPAR-γ and **G**) PPAR-δ. Horizontal lines present the median. MFI: median fluorescence intensity. Wilcoxon matched pairs t test; * $p<0.05$.

3.6 Discussion

One of the aims of this project was to investigate differences in the way T cells from male and female healthy individuals regulate response to androgens and oestrogens, and how naïve $CD4^+$ T cells are able regulate their exposure to sex hormones. We did this by investigating the expression of sex hormone metabolising enzymes and sex hormone receptors.

The role of sex hormone receptors is important for our understanding of the effects of sex hormones on inflammatory responses, as sex hormones are able to exert their influence by interaction and activation of sex hormone receptors, which have been previously shown to be present in various immune cell subsets (Brechenmacher *et al.* 2008). Our mRNA gene expression data showed there were no significant differences in the baseline expression of ER-α, ER-β or AR in naïve $CD4^+$ T cells between males and females (figure 3.1A, B, C). This suggests that naïve $CD4^+$ T cells from both males and females are able regulate their exposure to sex hormones in a similar manner. Upon *in vitro* stimulation, the data showed a significant increase in ER-β expression in female naïve $CD4^+$ T cells in comparison to the unstimulated naïve $CD4^+$ T cells. ER-β can form heterodimers with ER-α, which has shown to result suppressed ER-α transcriptional activity (Lindberg *et al.* 2003). Evidence of this modulating effect of ER-β on ER-α activity has been demonstrated in murine models, in which ER-β knock-out mice showed an increased ER-α regulation of progesterone receptor expression, and subsequently resulted in increased epithelial cell proliferation in the uterus and an exaggerated response to 17β-oestradiol via progesterone receptor activation (Weihua *et al.* 2000). Also, the immune enhancing effects of oestrogens have been shown to be ER-α dependent (Maret *et al.* 2003; Yang *et al.* 2010). Therefore, a possible explanation for the upregulation of ER-β upon naïve $CD4^+$ T cells activation in females could be to prevent the induction of inflammatory responses that are dependent on ER-α signalling pathways, and

could then be mechanism for preventing the development of chronic inflammatory diseases including autoimmune diseases. Increased ER-α expression and reduced ER-β has been demonstrated in SLE PBMCs (Lin *et al.* 2011) and interestingly, the expression of ER-β had a significant inverse correlation with disease activity (Inui *et al.* 2007). We did not see this upregulation of ER-β in male stimulated $CD4^+$ T cells; the reason for this could be due to the lower circulating oestrogen levels in males compared to females, therefore male $CD4^+$ T cells may not need to use this mechanism to reduce the effects of oestrogens exerted on T cell responses, as the levels of oestrogen exposure to male $CD4^+$ T cells would be considerably lower compared to females. The down regulation of ER-α expression in male stimulated $CD4^+$ T cells was not a statistically significant finding; however it does suggest that this could be a mechanism to inhibit pro-inflammatory effects exerted by oestrogen, therefore it would be interesting to confirm if this finding is significant on the protein level. As interesting as these findings are, it would be important to look on the protein level before drawing any conclusions from this mRNA data as previous studies have shown that oestrogen receptors are susceptible to post-translational modifications (Cheng *et al.* 2000; Cheng and Hart 2000; Saceda *et al.* 1998).

Very low levels of AR mRNA gene expression were observed in the qPCR experiments (the signal became detectable at around cycle 35-40), however subsequent flow cytometry analysis found high expression of levels AR protein in naïve $CD4^+$ T cells (figure 3.2A, C, D). The qPCR data suggests there is a trend towards higher expression of AR mRNA in female $CD4^+$ T cells compared to male $CD4^+$ T cells, however this is not statistically significant. In contrast, macrophages from males have been shown to have higher AR expression compared to females (McCrohon *et al.* 2000). However, we did detect a

significant upregulation of AR expression upon stimulation in naive $CD4^+$ T cell in comparison to the unstimulated cells on the protein level, which was observed in both males and females (figure 3.2D). One explanation for this contrasting mRNA and protein data could be that the AR gene does not need to be continually expressed if the protein is relatively stable. Also, expression of androgen and oestrogen receptors has been shown to be regulated by epigenetic modifications (Imamura 2011), therefore these genes might be silenced by these modifications, and post-translational modifications could also be responsible of the differences in mRNA and protein expression data. The observation that naïve $CD4^+$ T cells are increasing their exposure to androgens after stimulation by upregulating AR expression particularly in female $CD4^+$ T cells, where there is lower exposure to circulating androgens in comparison to males, shows that female $CD4^+$ T cells could be increasing their exposure to the immunosuppressing effects of androgens, but also simultaneously reducing their exposure to the immune enhancing effects of circulating oestrogens by upregulating ER-β expression after stimulation. Subsequent functional experiments on these sex hormone receptors would have to be carried out to investigate the outcome of these differences in expression on T cell responses.

5α-reductase is an important enzyme in androgen metabolism as it is responsible for the conversion of testosterone into DHT, which is the most potent androgen in the body; as it has a 10 times higher affinity for the AR in comparison to testosterone. The importance of DHT is demonstrated in 5α-reductase deficiency disorder, in which normal development of male external sex organs is obstructed (Andersson *et al.* 1991; Forti *et al.* 1996). Therefore, we wanted to investigate the expression of 5α-reductase in naïve $CD4^+$ T cells to detect any differences androgen metabolism between males and females. 5α-reductase 1 (SRD5A1) and 5α-reductase 2 (SRD5A2) have both been shown to be involved in androgen metabolism and

have distinctly different expression patterns in specific tissues and organs (Berman and Russell 1993; Silver *et al.* 1994). However, unlike SRD5A1 and SRD5A3 we could not detect SRD5A2 in naïve $CD4^+$ T cells or PBMCs, but it was detected in the positive control, therefore we can conclude that 5α-reductase 2 is not expressed in naïve $CD4^+$ T cells, which supports a previous finding in mice (Zhou *et al.* 1998). In the qPCR experiments, we found no significant differences in the baseline mRNA expression of the both isoforms between the sexes. There was no correlation between 5α-reductase expression and the different stages of the menstrual cycle (data not shown). However, it did show a significant upregulation of SRD5A1 and SRD5A3 mRNA expression in female stimulated naïve $CD4^+$ T cells in comparison to the female unstimulated $CD4^+$ T cells. Interestingly, this significant upregulation of SRD5A1 and SRD5A3 expression was only observed in female stimulated $CD4^+$ T cells and not in male stimulated $CD4^+$ T cells. This finding was also confirmed on the protein level, as we found 5α-reductase 1 was upregulated in only female stimulated $CD4^+$ T cells and a stimulation time course confirmed that 5α-reductase 1 expression peaked at 24 hours. Also, on the protein level we found the expression of 5α-reductase 1 in female stimulated T cells was significantly higher compared to 5α-reductase 1 in male stimulated T cells. There is evidence to suggest that 5α-reductase expression can be epigenetically regulated, as it has been shown that 5α-reductase is susceptible to DNA methylation, in particular cytosine methylation, in human lymphocytes (Rodriguez-Dorantes *et al.* 2002). Therefore, female $CD4^+$ T cells could be undergoing reduced cytosine methylation of 5α-reductase upon stimulation, which increases the gene expression, as this type of methylation results in the decrease in gene expression.

We did not confirm the 5α-reductase 3 findings on the protein level as it is not clear from the literature if this isoform is involved in androgen metabolism. We also found an upregulation

of SRD5A3 mRNA expression in female stimulated T cells only. During the initial discovery of the 5α-reductase 3, on the basis of sequence similarity to 5α-reductases 1 and 2 it was initially thought that 5α-reductase 3 was also able convert testosterone to DHT (Uemura *et al.* 2008). However, in the meantime the role of 5α-reductase 3 in steroid metabolism has been questioned (Stiles and Russell 2010). A more recent role has been identified for 5α-reductase 3, as it has been shown to have a vital role in the initial steps in N-glycosylation (Cantagrel *et al.* 2010). Post-translational modifications to antigens (i.e. glycosylation) have been linked to T-cell mediated autoimmunity (Purcell *et al.* 2008). Therefore, this possible increase in 5α-reductase 3 activity in female stimulated $CD4^+$ T cells could be defected in self reactive T cells, and therefore abnormalities in glycosylation that may arise from insufficient 5α-reductase 3 activity could lead to the development of autoimmune diseases.

Unfortunately, we were unable to have a definite answer to the question whether the increased expression leads to higher 5α-reductase enzymatic activity, as our testosterone conversion assays detected very low concentrations of DHT produced by female stimulated $CD4^+$ T cells. However, we did see a trend towards an increase in DHT production in the stimulated $CD4^+$ T cells compared to the unstimulated cells. We did detect conversion of testosterone to androstenedione by $CD4^+$ T cells, therefore suggesting that 17β-HSD 2 activity is active in $CD4^+$ T cells, which has been previously reported (Milewich *et al.* 1982; Zhou and Speiser 1999). A possible explanation for this increase in 5α-reductase 1 expression in stimulated female $CD4^+$ T cells and not in male stimulated $CD4^+$ T cells could be that male $CD4^+$ T cells are being exposed to higher levels of testosterone in the long term. Therefore, the absence of 5α-reductase 1 upregulation in male $CD4^+$ T cells suggests that DHT production may be controlled by a negative feedback mechanism to prevent the excess production of more active androgens. Interestingly, 5α-reductase deficiency in pregnant

female mice resulted in higher foetal deaths, which was associated with chronically elevated levels of oestrogen (Mahendroo *et al.* 1997). The castration of male mice has been shown to increase the mRNA levels and activity of 5α-reductase, and restoration of testosterone decreased mRNA levels and activity of 5α-reductase (Pratis *et al.* 2003). The levels of testosterone are lower in females, therefore the upregulation of 5α-reductase 1 in female $CD4^+$ T cells allows the potential increase production of DHT the more potent androgen. As previously stated, androgens are mainly immunosuppressive and have a protective role against the development of autoimmune diseases as observed in many studies (Bebo *et al.* 1999; Booji *et al.*1996; Kanda *et al.* 1997). Therefore, the upregulation 5α-reductase 1 expression in female $CD4^+$ T cells could increase the production of androgens, therefore enhancing the anti-inflammatory effects of androgens, thus protecting against development of autoimmunity. This observation leads to the question whether this mechanism may be defected in patients with autoimmune diseases, in particular those with a strong gender bias. In the following chapter, we therefore investigated 5α-reductase 1 regulation in patients with SLE.

It was a study by Dunn et al that suggested that PPAR-α expression was sensitive to testosterone and thus mediated the effect of androgens on Th1 mediated responses. They also demonstrated a sex difference in PPAR-α expression as it was more abundant in male mice in comparison to the females, which in turn protected males from developing EAE (Dunn *et al.* 2007). One of the aims of this project is to investigate if a sex difference in PPAR-α expression was present in human $CD4^+$ T cells. The qPCR data showed there were no statistically significant differences in any of the PPARs expression at baseline between the sexes. This finding suggests that the observations made by Dunn *et al* in mice are not extendable to the human system. However, recently Dunn and colleagues extended their work

on PPAR-α into humans and found that PPAR-α mRNA in male $CD4^+$ T cells was significantly upregulated (Zhang *et al.* 2012), this was in contrast to our findings as we found there were no statistically significant differences between the unstimulated and stimulated $CD4^+$ T cells for PPAR-α and PPAR-δ or in both males and females on the mRNA level. The expression of PPAR-γ female stimulated $CD4^+$ T cells was significantly higher in comparison to female $CD4^+$ T cells unstimulated cells, however this was not reflected on the protein level, even though there was a trend towards an increase in all three PPAR isoforms upon stimulation, in both males and females, however these findings were not statistically significant (see appendix). Dunn *et al* also found that female $CD4^+$ PPAR-α expression was upregulated upon treatment with DHT (Zhang *et al.* 2012), however the concentration of DHT used was 100 times more than the physiological levels of DHT found in both males and females.

Therefore, according to our findings, the gender differences in susceptibility to autoimmunity are not likely to be due to the expression PPAR-α or other PPAR isoforms in human $CD4^+$ T cells. It would be interesting to compare the expression of PPARs in autoimmune diseases compared to the healthy controls to see if there is a decrease in expression of PPARs in $CD4^+$ T cells in patients with autoimmune diseases and therefore resulting in reduction of the anti-inflammatory properties of PPARs.

4 5α-REDUCTASE EXPRESSION IN SLE PATIENTS

4.1 Introduction

There is a significant gender bias in SLE with females being more susceptible to developing the disease with a female to male ratio of 9:1. The predominance of autoimmune conditions, particularly SLE in females has initiated a vast amount of research into the role of sex hormones in immune and inflammatory responses, especially the effect of oestrogen on immune responses. Drastic changes in sex hormone levels are shown to have a significant effect on the severity on disease activity in SLE patients. During pregnancy, when oestrogen levels are high, disease activity is enhanced in SLE patients (Ruiz-Irastorza *et al.* 1996). Furthermore, the onset of the disease is predominately observed during the child-bearing ages. Oestrogen has been shown to increase B cell proliferation and differentiation, and is able to elevate IgG and IgM antibody production (anti-dsDNA in SLE patients) in both males and females at both high and physiological concentrations (Kanda *et al.* 1999).

In contrast to oestrogens, androgens have shown to affect immune responses in a suppressive manner, as it has been demonstrated that testosterone can inhibit IgM and IgG production by decreasing the production of IL-6 by monocytes (Kanda *et al.* 1996). It is well established that SLE patients have low levels of androgens in serum, which inversely correlates with disease activity (Jungers *et al.* 1982; Lahita *et al.* 1987; Overman *et al.* 2012). Male SLE patients have significantly lower androgen levels and increased oestrogen levels in serum compared to healthy males; this is also observed in RA (Cutolo *et al.* 1991) . However, it is not clear if low androgen levels are able to predispose individuals to developing

autoimmunity or if the progression of the autoimmune disease itself is responsible for these reduced levels of androgens. Furthermore, glucocorticoid treatment given to patients with severely active disease has shown to reduce androgen levels (MacAdams *et al.* 1986). In the experimental work described in the previous chapter, we found that 5α-reductase 1 expression was significantly upregulated in stimulated $CD4^+$ CD45 RA^+ T cells from healthy females in comparison to the unstimulated cells, which was detected both on the mRNA and protein level. However, this upregulation of 5α-reductase 1 expression after stimulation was not observed in male $CD4^+$ CD45 RA^+ T cells. Therefore, this possible increase in 5α-reductase 1 activity in female $CD4^+$ T cells upon stimulation could be a mechanism to increase the production of androgens, which have shown to have anti-inflammatory effects and suppress T cell responses after activation, thus potentially protect against development of autoimmunity. We wanted to investigate if this upregulation of 5α-reductase 1 expression after stimulation was defected in SLE patients, in which the majority are female, and therefore partially explain the autoreactive responses demonstrated in SLE T cells. If $CD4^+$ T cells from SLE patients are incapable of upregulating 5α-reductase 1 expression after stimulation, this could potentially prevent the anti-inflammatory effect of androgens on T cell stimulation, and in turn limit the suppression of pro-inflammatory responses in autoreactive T cells. We also wanted to investigate the expression of 5α-reductase 1 in $CD8^*$ T cells, $CD19^+$ B cells and $CD14^*$ monocytes from SLE patients and compare expression levels to healthy controls.

4.2 5α-reductase 1 expression in immune cells from SLE patients and age and gender matched controls.

Using flow cytometry, we measured the intracellular expression of 5α-reductase expression in T cells gated on $CD3^+$, $CD4^+$ $CD45\ RO^+$ or $CD45\ RO^-$, $CD8^+$ $CD45\ RO^+$ or $CD45\ RO^-$ (figure 4.1B), $CD14^+$monocytes (figure 4.1C), and finally $CD19^+$ B cells (figure 4.1D) from SLE patients (n=26) and age and gender-matched healthy controls (n=14). We found no significant differences in 5α-reductase expression in both naïve $CD4^+$ $CD45\ RO^-$ and memory $CD4^+$ $CD45\ RO^+$, and in naïve $CD8^+$ $CD45\ RO^-$ and memory $CD8^+$ $CD45\ RO^+$ between healthy controls and SLE patients. Also, no significant difference in 5α-reductase expression in $CD14^+$ monocytes between healthy controls and SLE patients was observed. Also, we did not observe any significant differences in 5α-reductase expression T cell subsets (figure 4.2A-D) and $CD14^+$ monocytes (figure 4.2E) between SLE patients with inactive and active disease. However, the results did show that 5α-reductase expression was significantly higher in the memory $CD4^+$ $CD45\ RO^+$ T cell subset population in comparison to the naïve $CD4^+$ $CD45\ RO^-$ T cell subset (figure 4.1B). This observation was made in both healthy controls and SLE patients.

Interestingly, we also found a significant increase in 5α-reductase expression in SLE $CD19^+$ B cells compared to the healthy controls and this positively correlated with disease activity (figure 4.1D), as SLE $CD19^+$ B cells from patients with active disease had significantly higher expression of 5α-reductase 1 in $CD19^+$ B cells compared to healthy controls.

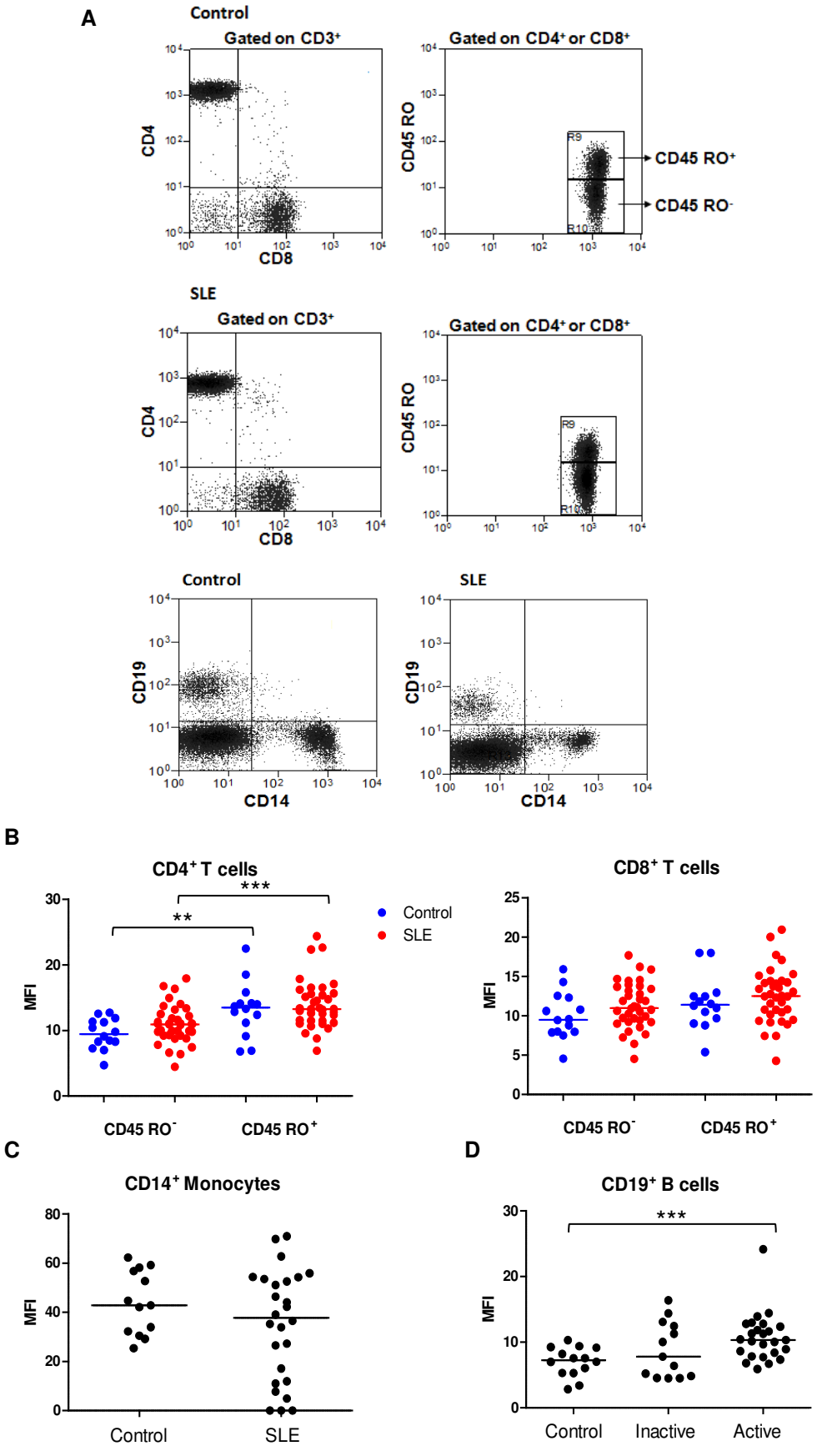
A
Control
Gated on CD3+
CD4
CD8
Gated on CD4+ or CD8+
CD45 RO
CD45 RO+
CD45 RO-
SLE
Gated on CD3+
CD4
CD8
Gated on CD4+ or CD8+
CD45 RO
Control
CD19
CD14
SLE
CD19
CD14
B
CD4+ T cells
MFI
Control
SLE
CD45 RO-
CD45 RO+
CD8+ T cells
MFI
CD45 RO-
CD45 RO+
C
CD14+ Monocytes
MFI
Control
SLE
D
CD19+ B cells
MFI
Control
Inactive
Active

Figure 4.1 5α-reductase expression in immune cells from SLE patients and age matched controls.

PBMCs from SLE patients (n=26) and age and gender-matched healthy controls (n=14) were stained for intracellular 5α-reductase 1 expression in different immune cell subsets using flow cytometry. **A**) FACS plots show T cells were gated on $CD3^+$, $CD4^+$or $CD8^+$ cells, and CD45 RO high or low cells, and B cells as $CD19^+$ and monocytes as $CD14^+$. **B**) 5α-reductase 1 expression in $CD4^+$ RO^- and $CD4^+$ RO^+ T cells, $CD8^+$ RO^- and $CD8^+$ RO^+ T cells (blue dots for healthy controls and red dots for SLE patients), **C**) $CD14^+$ monocytes, **D**) $CD19^+$ B cells divided into patients with inactive (n=13) or active (n=25) disease. Mann-Whitney T test; ** $p<0.001$; *** $p<0.0001$

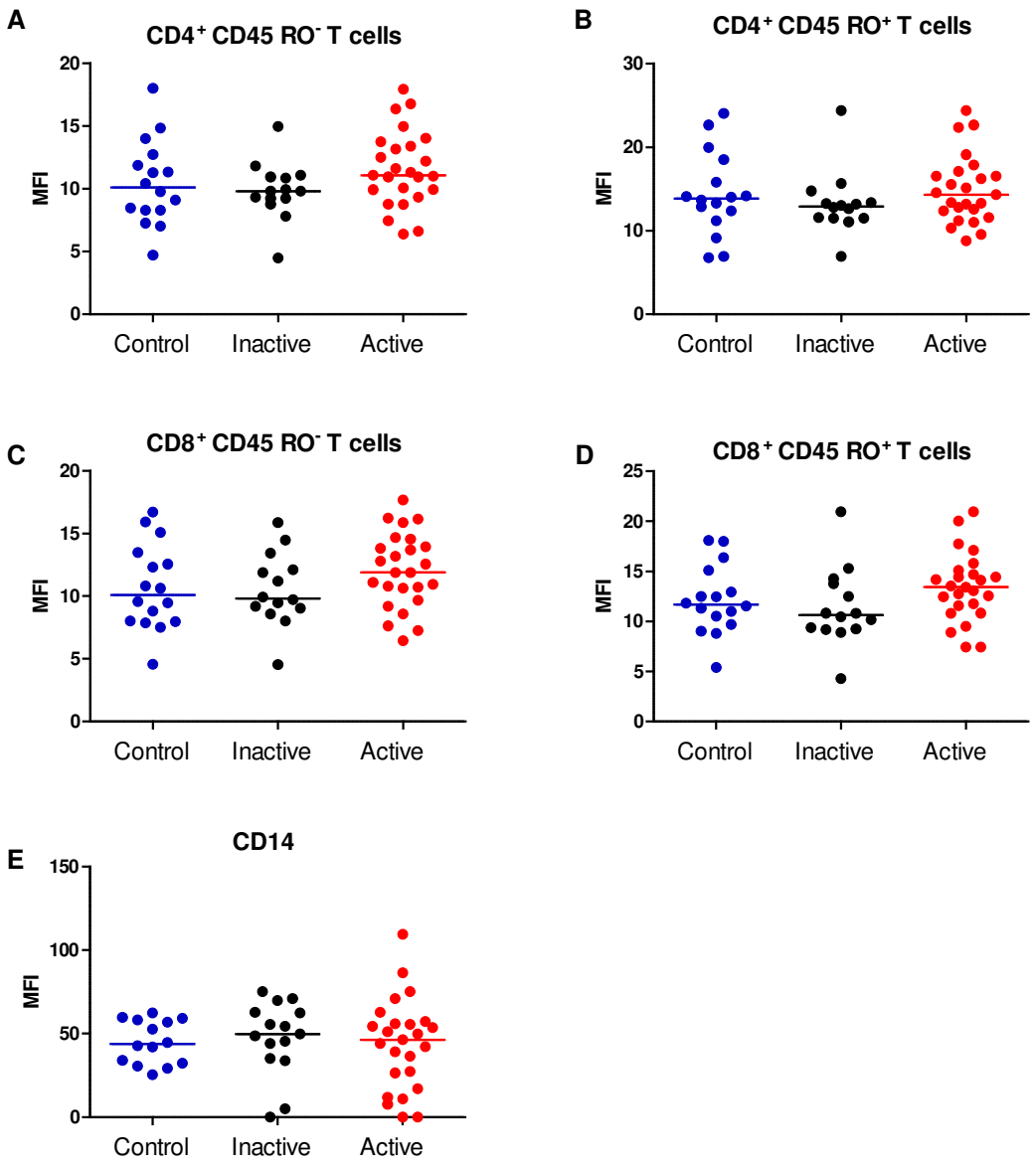

Figure 4.2 5α-reductase expression in T cells and monocytes from SLE patients with inactive and active disease, and age-gender matched controls.
PBMCs from SLE patients and age and gender-matched healthy controls (n=16) were measured for intracellular 5α-reductase 1 expression using flow cytometry. T cells were gated on $CD3^+$, $CD4^+$or $CD8^+$, and CD45 RO high or low cells (as illustrated in figure 4.1), **A**) $CD4^+$ $CD45\ RO^-$ **B**) $CD4^+$ $CD45\ RO^+$ T cells, **C**) $CD8^+$ $CD45\ RO^-$, **D**) $CD8^+$ $CD45\ RO^+$ T cells, **E**) $CD14^+$ monocytes. Blue dots represent the healthy controls; black dots represent SLE patients with inactive disease (n=14) and red dots for SLE patients with active disease (n=26). Horizontal lines represent the median. MFI: median fluorescence intensity.

4.3 5α-reductase 1 expression in $CD19^+$ B cell subsets from SLE patients and healthy controls.

We then wanted to investigate which B cell subset population was responsible for this significant difference in 5α-reductase expression in SLE patients (figure 4.3B). We used two B cell subset classification panels; CD27/IgD and CD38/IgD (figure 4.3A). In the CD27/IgD classification naïve B cells were classed as $CD27^-IgD^+$, unswitched memory being $CD27^+IgD^+$, switched memory were represented as $CD27^+IgD^-$ (intermediate) (have undergone somatic hypermutation), plasmablasts classed as $CD27^{++}IgD^-$ (high), and finally double-negative memory B cells were described as $CD27^-IgD^-$.The CD38/IgD panel uses the Bm1-Bm5 classification system (Sanz *et al.* 2008), in which naïve cells are classed as $CD38^-IgD^+$ (Bm1), pre-germinal cells (GC) and GC presented as $CD38^+IgD^+$ (Bm2), memory cells as $CD38^{++}IgD^-$ (high) (Bm3 and Bm4), $CD38^+IgD^-$ (early Bm5) and $CD38^-IgD^-$ (late Bm5). We did not detect any significant differences in 5α-reductase expression between SLE patients and healthy controls any of the B cell subsets. However, we did find significant differences between the B cell subsets in both SLE patients and healthy controls (figure 4.3B). However, after receiving the disease activity data for the patients we found that only two SLE patients out of 13 sampled had active disease. After reanalysing the data we did not detect any trends in the active and inactive patients and did not observe any differences between the healthy controls, inactive and active SLE patients (graphs not shown). There was a significantly higher expression of 5α-reductase in $CD27^{++}IgD^-$ B cells from SLE patients compared to; $CD27^+IgD^-$ (intermediate) ($p=0.03$); $CD27^-IgD^+$ ($p=0.01$) and $CD27^+IgD^+$ ($p=0.03$) (figure 4.3D). There was also a significantly higher expression of 5α-reductase in $CD27^{++}IgD^-$ (high) B cells from healthy controls compared to $CD27^+IgD^-$ ($p=0.04$); $CD27^-IgD^+$ ($p=0.007$) and $CD27^+IgD^+$ ($p=0.007$) (figure 4.3C). In SLE patients, there was a significantly higher expression of 5α-reductase in $CD38^{++}IgD^-$ (high) B cells from

SLE patients compared to; $CD38^{-}IgD^{-}$ (p=0.0003); $CD38^{+}IgD^{-}$ (intermediate) (p=0.01); $CD38^{-}IgD^{+}$ (p=0.001) and $CD27^{+}IgD^{+}$ (p=0.003). In healthy controls, there was also a significantly higher expression of 5α-reductase in $CD38^{++}IgD^{-}$ B cells from SLE patients compared to; $CD38^{-}IgD^{-}$ (p=0.03); $CD38^{+}IgD^{-}$ (p=0.03); $CD27^{-}IgD^{+}$ (p=0.007) and $CD27^{+}IgD^{+}$ (p=0.01).

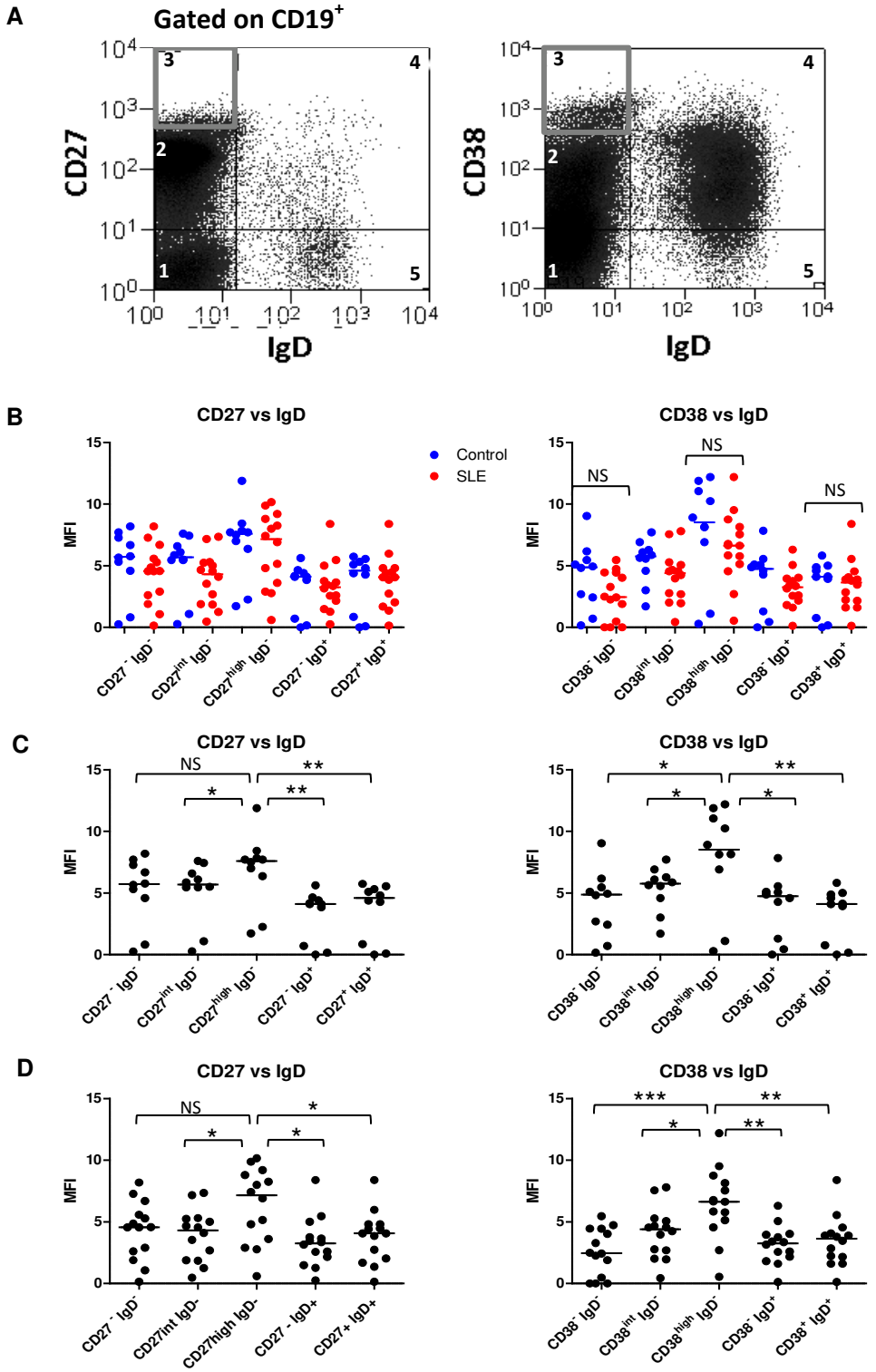

Figure 4.3 5α-reductase expression in B cell subsets from SLE patients and age matched controls.

PBMCs from SLE patients (n=13) and age and gender-matched healthy controls (n=10) were stained for intracellular 5α-reductase 1 expression using flow cytometry**. A**) FAC plots showing the gating strategy for B cell subsets using the markers CD27 vs. IgD and CD38 vs.

IgD. Using CD27 vs. IgD, cells were split into the following subsets; double-negative memory B cells (1); switched memory (2); plasmablasts (3); unswitched memory (4) and naive B cells (5). Using CD38 vs. IgD, cells were split into the following subsets; late memory B cells (1); early memory (2); memory B cells (3); pre-germinal and germinal cells (4), and naïve and memory B cells (5). **B**) Intracellular 5α-reductase 1 expression was measured in $CD19^+$ B cell subsets using classified markers; CD27 vs. IgD and CD38 vs. IgD from SLE patients (blue dots) and healthy controls (red dots). **C**) 5α-reductase 1 expression in healthy controls B cell subsets; CD27 vs. IgD and CD38 vs. IgD and **D**) 5α-reductase 1 expression in SLE patients B cell subsets; CD27 vs. IgD and CD38 vs. IgD. Horizontal lines represent the median. MFI: median fluorescence intensity. Mann-Whitney T test; * $p<0.05$; ** $p<0.001$; *** $p<0.0001$.

4.4 5α-reductase expression in T cells upon stimulation from SLE patients and age matched controls.

We wanted investigate if female T cells from SLE patients could also upregulate 5α-reductase 1 expression after stimulation as demonstrated in healthy females in the previous chapter. Freshly isolated PBMCs from both SLE patients and healthy controls were stimulated with anti-CD3 and anti-CD28 activation beads for 24 hours (alongside unstimulated controls). After the 24 hour incubation period, cells were then measured for 5α-reductase expression using flow cytometry. The results showed that both $CD4^+$ (figure 4.4B,C) and $CD8^+$ T cells (figure 4.4D,E) (naïve CD45 RO^- and memory CD45 RO^+) cells from SLE patients were able to upregulate 5α-reductase 1 expression after stimulation as seen in the healthy controls, and the majority of the SLE patients and healthy controls were female. Also, we did not observe any significant differences in 5α-reductase expression in unstimulated and stimulated T cell subsets between SLE patients with inactive and active disease for both $CD4^+$ (figure 4.5A,B) and $CD8^+$ T cells (figure 4.5C,D)

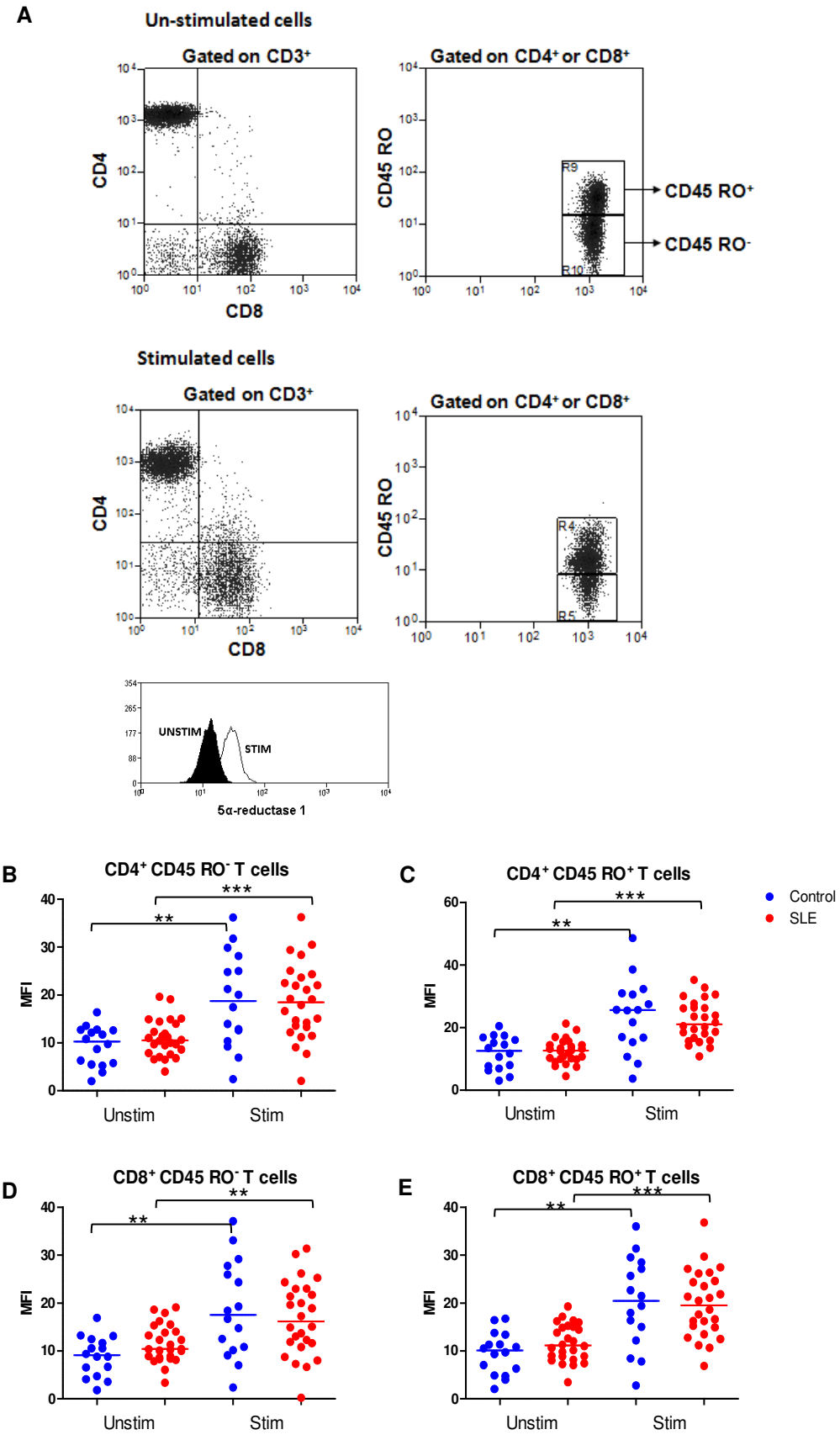

Figure 4.4 5α-reductase expression 1 in unstimulated and stimulated T cells from SLE patients and age matched controls.

PBMCs from SLE patients (n=26) and age and gender-matched healthy controls (n=16) were stimulated with anti-CD3 and anti-CD28 activation beads for 24 hours (alongside unstimulated controls). Cells were then measured for intracellular 5α-reductase 1 expression using flow cytometry. **A**) FACs plots showing unstimulated and stimulated T cells were gated on $CD3^+$, $CD4^+$or $CD8^+$ cells, and CD45 RO high or low. Accumulative data for intracellular 5α-reductase 1 expression in **B**) $CD4^+$ CD45 RO^- **C**) $CD4^+$ CD45 RO^+ T cells, **D**) $CD8^+$ CD45 RO^-, and **E**) $CD8^+$ CD45 RO^+ T cells. Blue dots represent the healthy controls and red dots for SLE patients. Horizontal lines represent the median. MFI: median fluorescence intensity. Mann-Whitney T test; ** p<0.001; *** p<0.0001

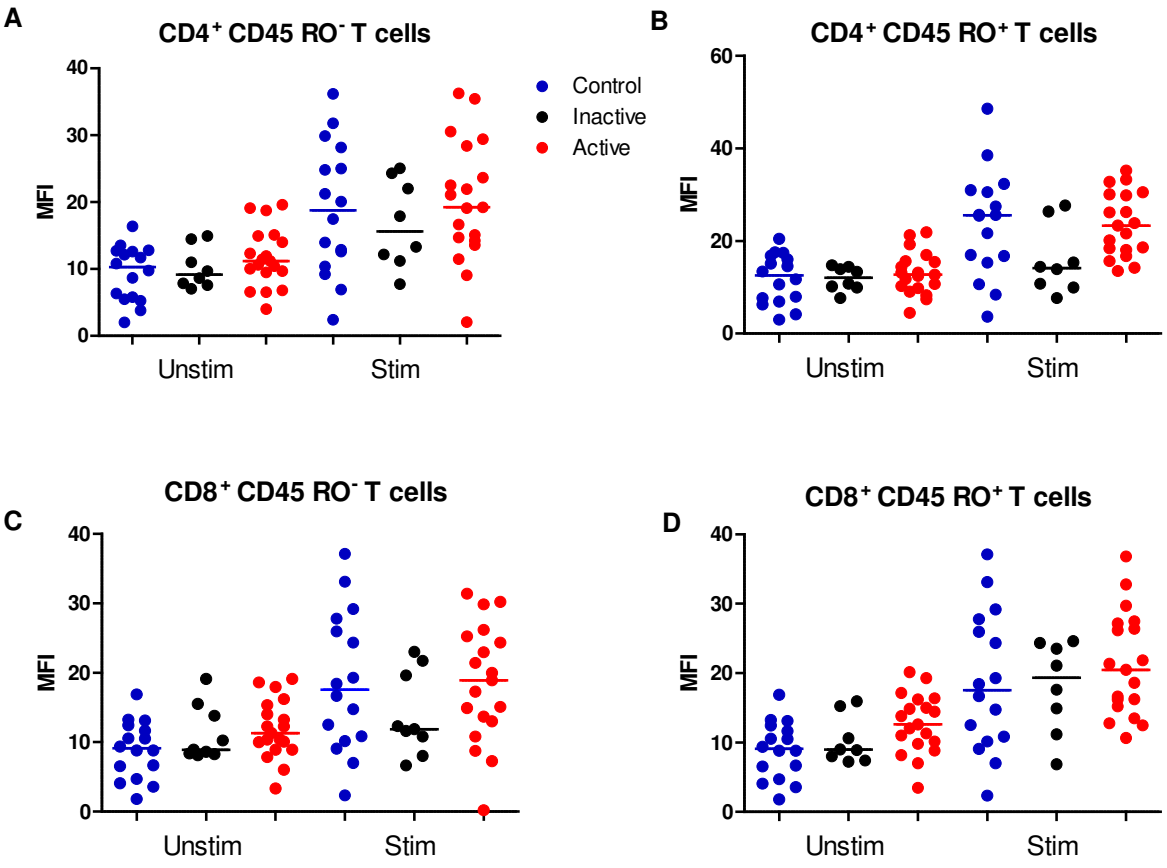

Figure 4.5 5α-reductase expression in unstimulated and stimulated T cells from SLE patients with inactive and active disease, and age-gender matched controls.
PBMCs from SLE patients and age and gender-matched healthy controls (n=16) were stimulated with anti-CD3 and anti-CD28 activation beads for 24 hours (alongside unstimulated controls). Cells were then measured for intracellular 5α-reductase 1 expression using flow cytometry. T cells were gated on $CD3^+$, $CD4^+$or $CD8^+$ cells, and CD45 RO high or low cells (as illustrated in figure 4.4), **A**) $CD4^+$ CD45 RO^- **B**) $CD4^+$ CD45 RO^+ T cells, **C**)

$CD8^+$ CD45 RO^-, **D**) $CD8^+$ CD45 RO^+ T cells. Blue dots represents healthy controls; black dots represent SLE patients with inactive disease (n=8) and red dots for SLE patients with active disease (n=19). Horizontal lines represent the median. MFI: median fluorescence intensity.

4.5 Discussion

We wanted to investigate if there is a dysfunction in the upregulation of 5α-reductase 1 expression after stimulation in SLE patients. If $CD4^+$ T cells from SLE patients are not able to upregulate 5α-reductase 1 expression after stimulation, this could prevent the anti-inflammatory effect of androgens on T cell stimulation and in turn limit the suppression of pro-inflammatory responses in autoreactive T cells. We also wanted to investigate the expression of 5α-reductase in $CD8^*$ T cells, $CD19^+$ B cells and $CD14^*$ monocytes from SLE patients and compare expression levels to healthy controls. There were no significant differences in 5α-reductase expression the T cell subsets (figure 4.1B) or in $CD14^+$ monocytes between healthy controls and SLE patients (figure 4.1C). Also, we did not see any significant differences between healthy controls and SLE patients with inactive or active disease for both T cells (figure 4.2A, B, C, D) and $CD14^*$ cells (figure 4.2E) However, the results did show that 5α-reductase expression was significantly higher in the memory $CD4^+$ CD45 RO^+ T subset in comparison to the naïve $CD4^+$ CD45 RO^- T cell subset. This observation was made in both healthy controls and SLE patients. This is interesting as it suggests that $CD4^+$ T cells that have encountered antigen, and therefore respond more rapidly to antigens, are potentially upregulating their production of DHT. This could a mechanism used by memory T cells to suppress pro-inflammatory immune responses, in particular the production Th2 cytokines such as IL-4 and IL-5; as DHT has shown to inhibit the production of these cytokines, which are important in mediating subsequent humoral responses. We then

investigated the effect of stimulation on 5α-reductase expression in T cells, and found that both $CD4^+$ and $CD8^+$ (naïve CD45 RO^- and memory CD45 RO^+) cells from SLE patients were able to upregulate 5α- reductase expression after stimulation as seen in healthy controls. This shows that T cells from SLE patients are not defected in increasing their exposure to immunosuppressive androgens.

However, in $CD19^+$ B cells we found a significant increase in 5α-expression in SLE $CD19^+$ B cells compared to the healthy controls and this positively correlated with disease activity (figure 4.1D), as SLE $CD19^+$ B cells from patients with active disease had significantly higher expression of 5α-reductase in $CD19^+$ B cells compared to healthy controls. SLE is considered to be a B cell driven disease as the production of autoantibodies to components of the nuclei including dsDNA are key to the loss of tolerance and pathogenesis of the disease (Kanda *et al.* 1997). The upregulation of 5α-reductase and the subsequent increase in intracellular androgen production could be an intracellular mechanism used by B cells in SLE to suppress their immune responses, as androgens have been shown to suppress antibody production by B cells. SLE patients have significantly lower levels of androgens in serum compared to healthy females, in which the levels of circulating androgen are already low due to being of the female sex. It is however not clear if females with low levels of androgens are predisposed to developing SLE as early diagnosed SLE patients are rapidly treated with corticosteroids, which reduces the levels of serum androgens. Therefore, B cells in SLE patients could be compensating for the reduced exposure to androgens in the serum and synthesising endogenous androgens, which can then directly act on androgen receptors within the cell cytosol causing translocation of the androgen receptor into the nuclei, where it can target genes involved in mediating inflammatory responses. This upregulation of 5α-reductase expression in B cells could be a mechanism to counteract the pro-inflammatory effects of oestrogens, which have shown to promote humoral responses in both B cells

healthy females and in models of autoimmune diseases (Erlandsson *et al.* 2003; Feng *et al.* ; Grimaldi *et al.* 2001). The production of DHT, which is more potent then testosterone would result in direct interaction with the androgen receptor and promote anti-inflammatory responses, whereas testosterone can be converted into oestrogen via the enzyme aromatase and therefore promote an enhanced inflammatory response. This observation could also give us some insight into the regulation of 5α-reductase, as this suggests that low levels of androgens present in SLE patients results in a higher 5α-reductase expression, whereas in PCOS patients (who have abnormally high levels of androgens) previous data from our group found that PBMC expression of 5α-reductase was reduced (unpublished). A previous study has also shown that DHT negatively regulates 5α-reductase activity, thus supporting this hypothesis (Samy *et al.* 2001). We wanted to then investigate the B cell subset that was responsible for the upregulation of 5α-reductase expression in SLE B cells from patients with active disease, but unfortunately we did not detect any significant differences in 5α-reductase expression between SLE patients and healthy controls in any of the B cell subsets (figure 4.3B). However, after receiving the disease activity data we found that only two SLE patients out of 13 sampled had active disease. We did not detect any trends in the active and inactive patients and did not find any differences between the healthy controls, inactive and active SLE patients. It was the SLE patients with active disease that upregulated 5α-reductase expression, therefore it would be essential to repeat these experiments for the B cell subsets with more SLE patients with active disease, and we then might see a difference in 5α-reductase expression in B cell subsets between SLE patients with active and control.

However, we did find significant differences between the B cell subsets in both SLE patients and healthy controls (figure 4.3C, D). There was a significantly higher expression of 5α-

reductase in $CD27^{++}IgD^{-}$ and $CD38^{++}IgD^{-}$ B cells in comparison to the other B cell subsets investigated from both SLE patients and healthy controls. This is particularly interesting as has been previously mentioned that androgens are able to decrease the production of antibodies and also prevent the generation of autoantibodies against dsDNA. This population of $CD27^{++}IgD^{-}$ B cells known as plasmablast cells, which are a subset of short lived IgM secreting plasma cells, have shown to be present in increased numbers in SLE patients (Dorner *et al.* 2011). This high expression of 5α-reductase in this B cell subset could be mechanism to prevent excessive antibody production and suppress potential production of autoantibodies. Androgens have been shown to modulate early B cell development in a suppressive manner (Altuwaijri 2009; Viselli *et al.* 1997); in the bone marrow, androgens are able to suppress B lymphopoiesis through AR signalling in bone marrow stromal cells, thus resulting in TGF-β production (Olsen *et al.* 2001). Androgens have also shown to have a key role in maintaining B cell tolerance, as mice with global AR deficiency and specifically absent AR signalling in B cells have resulted in the presences of anti dsDNA autoantibodies (Altuwaijri 2009). Therefore, it would be interesting to investigate the role of testosterone and more importantly endogenous production of DHT on antibody production by $CD27^{++}IgD^{-}$ and $CD38^{++}IgD^{-}$ B cells by using 5α-reductase inhibitors such as dutasteride and finasteride.

It is extremely difficult to investigate the direct effects of sex hormones on SLE disease pathogenesis as predominately all the patients with active disease are on glucocorticoid treatment, which influences the levels of sex hormones in the treatment. Therefore, this observation of 5α-reductase expression in B cells could be influenced by the glucocorticoid treatment the SLE patients were undergoing. We wanted to measure the levels of serum

androgens in these patients, however glucocorticoid treatment has a drastic effect on serum androgen levels. It would also be important to take into account the availability of sex hormones in the serum by investigating levels of SHPB, which are responsible for transporting sex hormones in the periphery, as high levels of SHPB in SLE patients would decrease to bioavailability of androgens to immune cells. A previous study showed that a particular genetic polymorphism of SHPB is associated with SLE and that it has a stronger affinity for androgens then oestrogens, therefore reducing the bioavailability to peripheral cells in an already low androgen reserve environment (Piotrowski *et al.* 2010).

Overall, we found a significant increase in 5α-reductase expression in SLE $CD19^+$ B cells compared to the healthy controls and this positively correlated with disease activity, as SLE $CD19^+$ B cells from patients with active disease had significantly higher expression of 5α-reductase in $CD19^+$ B cells compared to healthy controls. We also showed there was a significantly higher expression of 5α-reductase in $CD27^{++}IgD^-$ and $CD38^{++}IgD^-$ B cells in comparison to the other B cell subsets from both SLE patients and healthy controls. Therefore, it would be important to follow up these experiments with functional assays investigating the effect of inhibiting 5α-reductase activity, thus preventing the production of endogenous DHT within B cells, on B cell responses.

5 THE EFFECTS OF ANDROGENS ON T CELL RESPONSES

5.1 Introduction

In the human system, the influence of androgens on inflammatory responses both in homeostatic and disease states is not very well understood. The higher incidence of autoimmune conditions in females may not just be due to the immune enhancing properties of oestrogens, but may also be attributable to the low physiological androgen levels in females. In addition, the even lower androgens levels found in individuals suffering from autoimmune diseases may contribute to immune imbalance. Treatment with glucocorticoids is an additional confounding factor as these reduce systemic androgen levels (MacAdams *et al.* 1986).

In the context of the humoral response, androgens such as testosterone have been shown to inhibit IgM and IgG production. On the other hand, enhanced DC proliferation and antigen stimulation has been shown in males with hypogonadism, suggesting that testosterone is important in regulating immune responses in a suppressive manner (Corrales *et al.* 2012). In mice, testosterone has been shown to directly act on $CD4^+$ T cell to increase the production of IL-10 (Liva and Voskuhl 2001). Also, DHT has been previously shown to suppress Th2 derived cytokines IL-4 and IL-5 but had no effect on IL-2 (Araneo *et al.* 1991). Gender dimorphism is not only evident in autoimmunity but also in trauma cases, as injured men experience much more severe immunosuppression compared to females, leading to a slower recovery rate and higher trauma related death rates. Murine models investigating the role of sex hormones in trauma-haemorrhage found that oestrogens helped maintain immune

responses; in contrast androgens were shown to suppress Th1 cytokine production while castration or supplementation with 17β-estradiol in male mice elevated immune responses (Angele *et al.* 2001; Samy *et al.* 2001). It was also shown that 5α-reductase activity was increased post-trauma resulting in an increased production of DHT, thus further suppressing immune responses.

However, most of the studies investigating the effects of androgens were carried out in animal models and not in humans, and usually involve in vivo treatment of androgens in animal models or castration, therefore not fully representing physiological concentrations of androgens circulating normally present in humans. As we had previously demonstrated that 5α-reductase expression is upregulated in T cell subsets upon stimulation in both healthy individuals and SLE patients, we wanted to then investigate the effects of androgens on human T cell cytokine production and proliferation to understand what effects increased androgen levels would have on T cell responses. We wanted use physiological concentrations of androgens found in circulation to get a better understanding on the influence of androgens on T cell responses. In circulation, sex hormones (particularly the most potent sex hormones such as 17β-oestradiol and DHT) are bound to SHPB with a strong affinity or bound to albumin to a lesser extent, thus very low levels of active free (unbound) sex hormones are detected in the circulation. Therefore, we took this into account when deciding on androgen concentrations to use in our in vitro assays.

A range of concentrations were chosen that reflected both the average concentrations of free and total (bound and unbound) androgens present in circulation; for testosterone the highest concentration we used was 15nM and for DHT the highest concentration used was 2nM, as

this reflects the average total concentration of testosterone and DHT found in circulation in healthy males. 7nM of testosterone and 0.5nM of DHT was used to reflect the levels found in serum from polycystic ovary syndrome (PCOS) patients, in which females have significantly higher androgen levels compared to healthy females. 1nM testosterone and 0.1nM of DHT were also used to replicate the average levels of these androgens found in healthy females. Finally, 0.1nM of testosterone and 0.01nM of DHT was used to reflect the low concentrations of androgens found in the serum of SLE patients.

5.2 Cytokine production by T cells in males and females.

We found that the percentage of $CD4^+$ T cells producing IL-2 and IFN-γ were significantly higher in females compared to males (n=6) (figure 5.1B,C), however this was not reflected in the $CD8^+$ T cells (figure 5.2A,B). There were no significant differences in the amount of IL-10, IL-17 and TNF-α produced by either $CD4^+$ or $CD8^+$ T cells between the sexes, however this could be due to the low number of replicates for these cytokines (n=3).

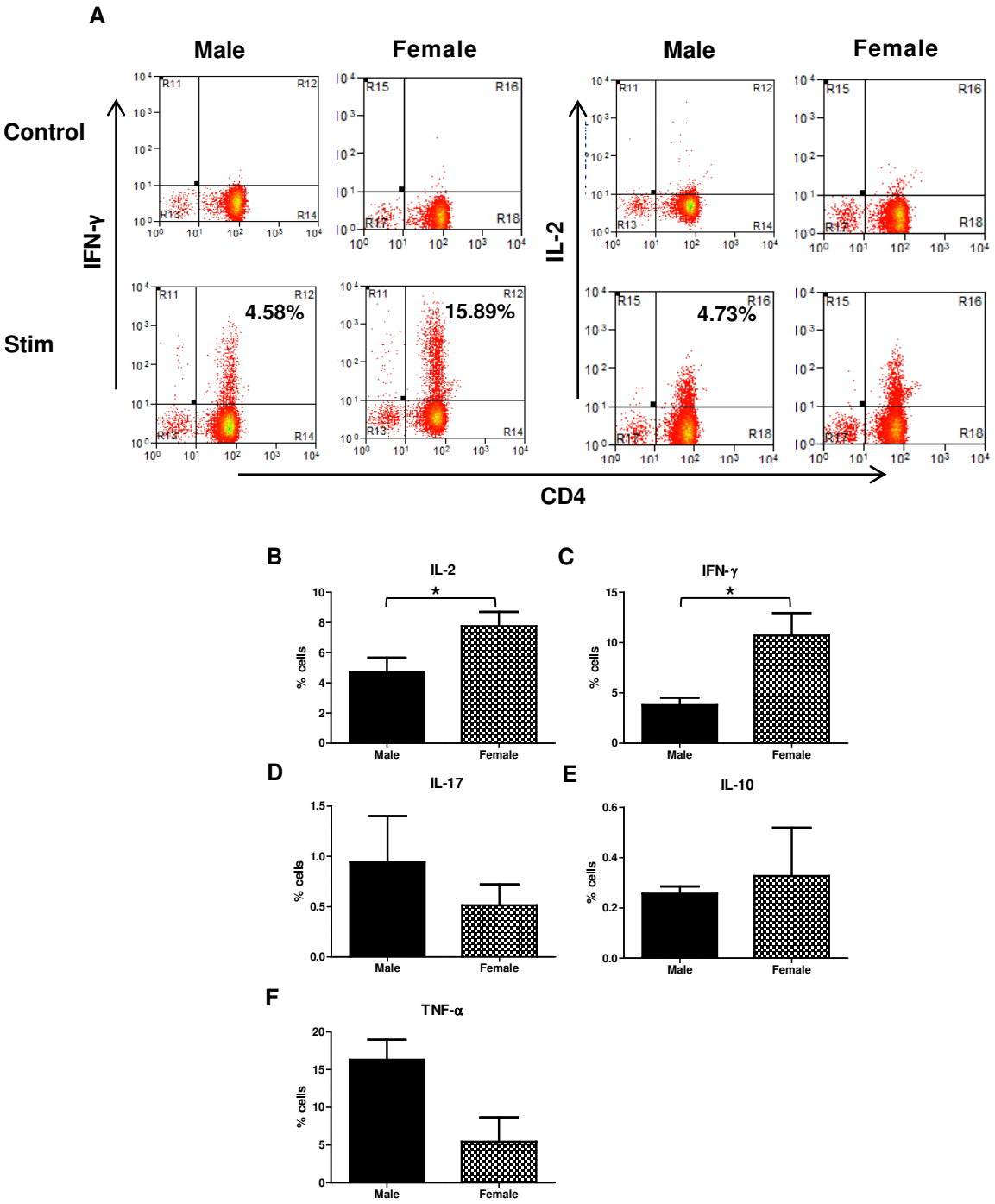

Figure 5.1 Cytokine production by CD4$^+$ T cells from males and females.
PBMCs from females and males (n=6) were stimulated with anti CD3 and anti CD28 activation beads and cultured for 4 hours in the presence of Brefeldin A to measure intracellular production of cytokines. **A**) The FACs plots for % CD4$^+$ T cells producing IL-2 and IFN-γ for one male and female donor. The graphs summarise data from all six experiments **B**) IL-2, **C**) IFN-γ, **D**) IL-17, **E**) IL-10, and **F**) TNF-α from CD4$^+$ T cells (n=3) using flow cytometry. Error bars represent SEM. Mann-Whitney T test; * $p<0.05$.

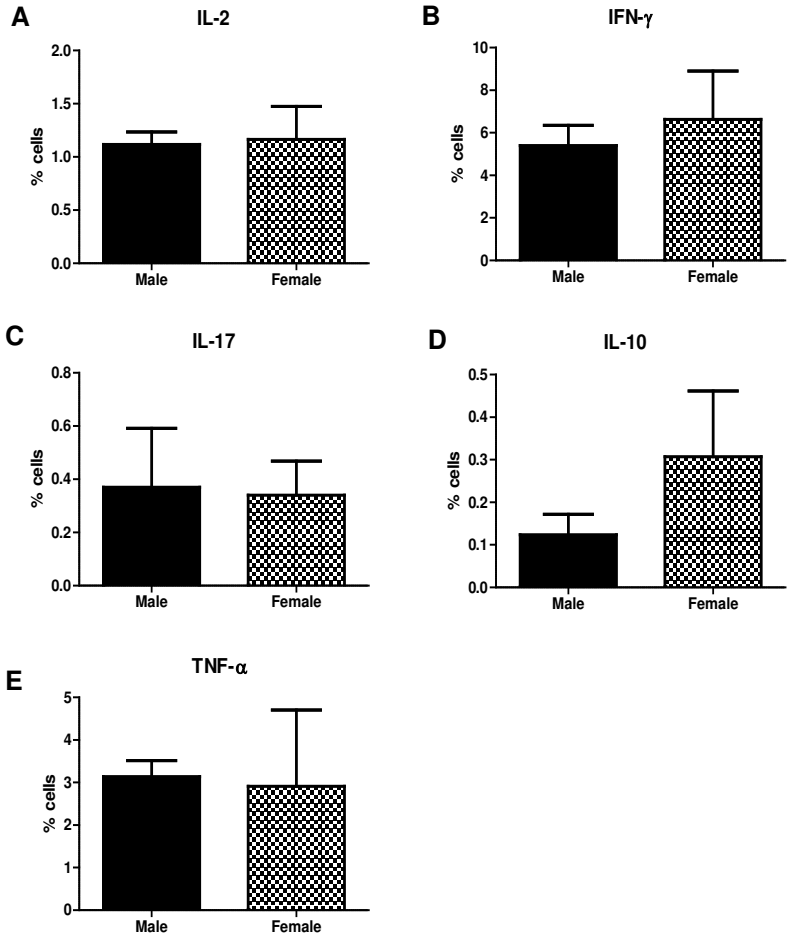

Figure 5.2 Cytokine production by CD8$^+$ T cells from males and females.
PBMCs from females (n=6) and males (n=6) were stimulated with anti CD3 and anti CD28 activation beads and cultured for 4 hours in the presence of Brefeldin A to assess the proportion of CD8$^+$ T cells producing cytokines **A**) IL-2, **B**) IFN-γ. (n=3): **C**) IL-17, **D**) IL-10, and **E**) TNF-α using flow cytometry. Error bars represent SEM.

5.3 T cell cytokine production after testosterone and DHT treatment.

We observed a significant increase in the proportion of IL-2 producing cells in $CD4^+$ T cells from females treated with 1nM testosterone (levels found in healthy women)compared to the untreated control (figure 5.3A). There was also a significant decrease in IL-2 production when $CD4^+$ T cells from females were treated with 15nM of testosterone (levels found in healthy men) compared to the 1nM testosterone treatment. In male $CD4^+$ T cells (figure 5.3B), there was no significant increase in IL-2 levels between the untreated control and 1nM testosterone, however an significant decrease in IL-2 levels was observed between cells treated with 1nM testosterone and 15nM testosterone. There was a similar trend in $CD4^+$ T cells producing IFN-γ (figure 5.4A) and $CD8^+$ T cells producing IL-2 after testosterone treatment, however the differences observed were not significant. We found no significant differences on IL-2 production in both males and females when cells were treated with a range of physiological concentrations of DHT. There were no significant differences in the production of IL-10, IL-17 or TNF-α by $CD4^+$ and $CD8^+$ T cells from both males and females upon treatment with testosterone or DHT (see appendix).

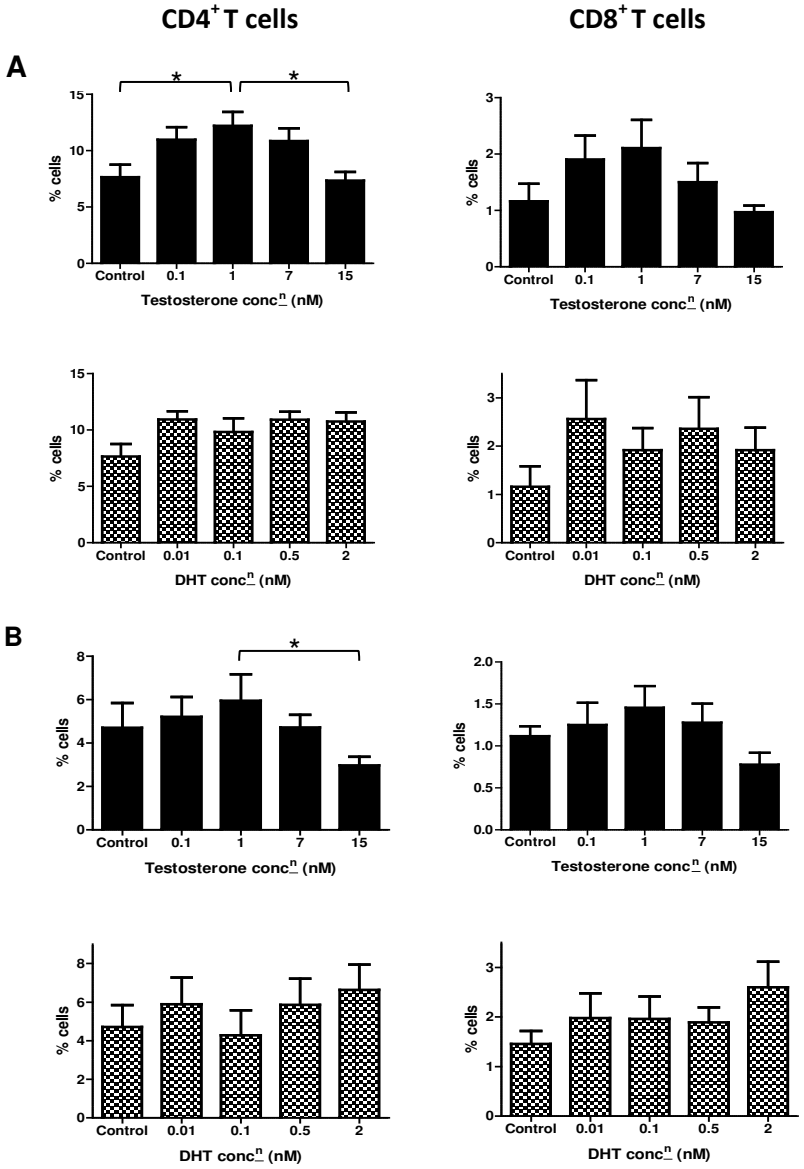

Figure 5.3 IL-2 production by T cells after testosterone and DHT treatment.
PBMCs were stimulated with anti CD3 and anti CD28 activation beads and cultured for 4 hours with different concentrations of testosterone (black bars) and DHT (checkered bars) in the presence of Brefeldin A to measure the proportion of IL-2 producing T cells from **A**) females (n=6) and **B**) males (n=6) using flow cytometry. Mann-Whitney T test; * $p<0.05$.

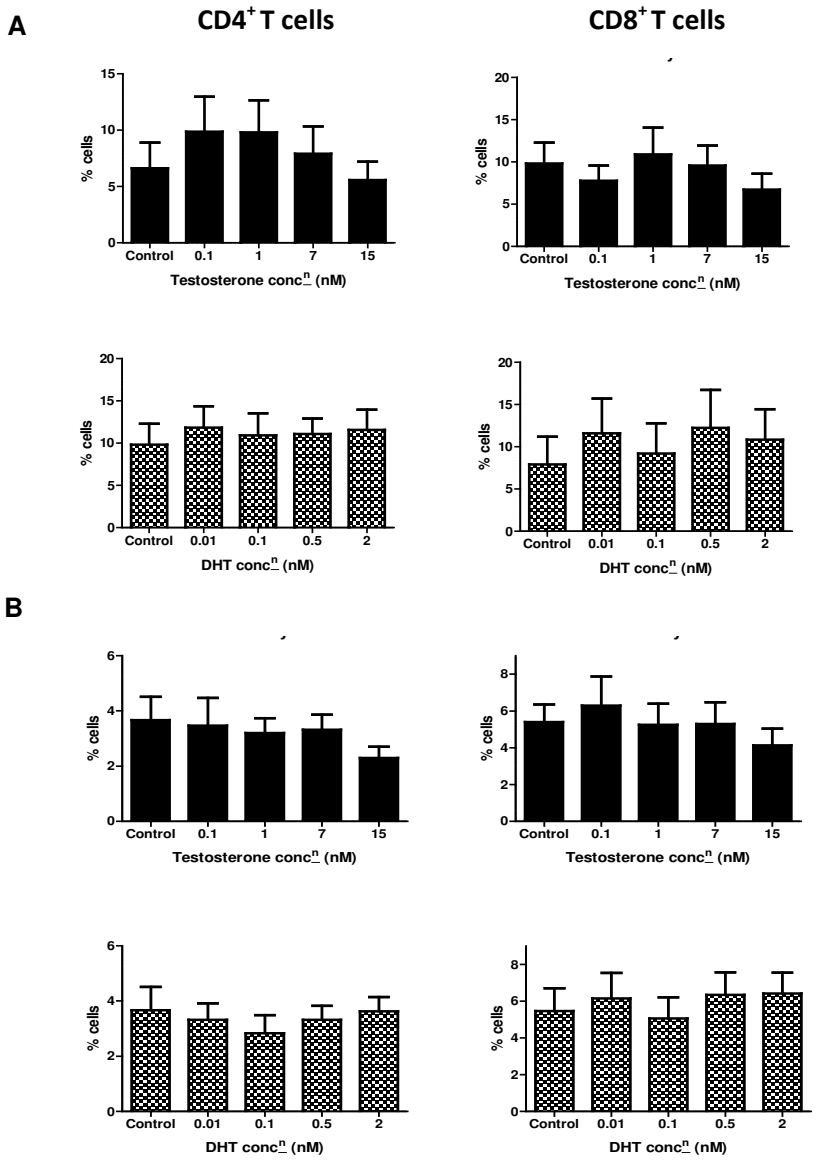

Figure 5.4 IFN-γ production by T cells after testosterone and DHT treatment.
PBMCs were stimulated with anti CD3 and anti CD28 activation beads and cultured for 4 hours with different concentrations of testosterone (black bars) and DHT (checkered bars) in the presence of Brefeldin A to measure the proportion of IFN-γ producing T cells from **A**) females (n=6) and **B**) males (n=6) using flow cytometry. Error bars represent SEM.

5.4 IL-2 production after treatment with 17β-oestradiol and 4-hydroxytamoxifen

We wanted to investigate if the increase in the proportion of IL-2-producing female $CD4^+$ T cells at the low concentrations of testosterone (0.1nM and 1nM) was actually due to 17β-oestradiol activating oestrogen receptors, as testosterone can be converted into 17β-oestradiol

by aromatase. Firstly, we investigated the effect of 17β-oestradiol treatment on the proportion of IL-2-producing $CD4^+$ T cells from females. A range of concentrations were chosen that reflected average concentrations of 17β-oestradiol present in circulation; the highest concentration we used was 0.95nM as this reflects the average total concentration of 17β-oestradiol found in healthy females during the preovulatory phase of the menstrual cycle. 0.29nM of 17β-oestradiol was used to reflect the levels found during the luteal phase of the menstrual and 0.13nM of 17β-oestradiol was used to reflect the concentration found during the follicular phase of the menstrual cycle. Finally, 0.07nM of 17β-oestradiol was used to reflect the serum concentrations found in men and post-menopausal women. We found a dose dependent increase in the percentage of $CD4^+$ T cells producing IL-2 (figure 5.5A), which was close to reaching statistical significance ($p=0.055$). We also observed this trend in IFN-γ; however this observation was not significant.

We used an oestrogen receptor inhibitor called 4-hydroxytamoxifen, which is the active metabolite of tamoxifen to investigate whether 17β-oestradiol was affecting the proportion of IL-2-producing $CD4^+$ T cells (figure 5.5C). Treatment of female $CD4^+$ T cells with 4-hydroxytamoxifen with different concentrations of testosterone abolished the increase in the proportion of IL-2 producing cells previously observed when treated with the lower concentrations of testosterone. We also wanted to investigate what immune cell type in the PBMC population expressed aromatase, which converts testosterone into 17β-estradiol, as the literature suggests that T cells express very low levels of aromatase (Zhou and Speiser 1999). qPCR experiments were carried out for aromatase expression on the mRNA level (gene name CYP19A1) and showed that macrophages had the highest expression of aromatase and this observation was statistically significant in comparison to $CD4^+$ T cells ($p=0.02$), but not

compared monocytes (figure 5.5D). Both $CD4^+$ T cells and monocytes had very low levels of expression with a signal detectable after cycle 40.

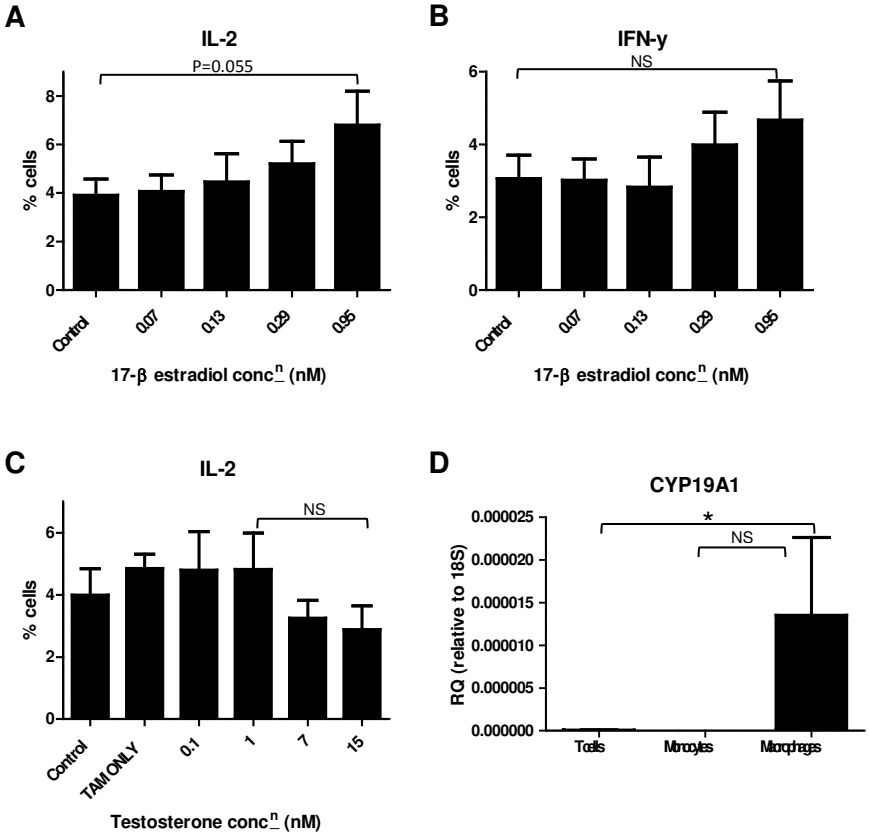

Figure 5.5 IL-2 production by female $CD4^+$ T cells after 17β-estradiol treatment and 4-hydroxytamoxifen.

PBMCs from female donors (n=4) were stimulated with anti CD3 and anti CD28 activation beads and cultured for 4 hours with different concentrations of **A**) 17β-estradiol to assess subsequent percentage cells producing IL-2 and IFN-γ using flow cytometry **B**) different concentrations of testosterone with additional treatments with **C**) 2nM 4-hydroxytamoxifen to assess the percentage cells producing of IL-2 by $CD4^+$ T cells using flow cytometry. **D**) Gene expression for aromatase (CYP19A1) was measured as relative quantification to 18s in $CD4^+$ T cells, monocytes and in vitro differentiated macrophages cultured for 6 days in the presence of GM-CSF (courtesy of Nichola Adlard) (n=3) by qPCR. Error bars represent SEM. NS (not significant). Mann-Whitney T test; * $p<0.05$.

5.5 $CD4^+$ T cell proliferation after testosterone treatment.

After observing the significant effects of testosterone on IL-2 production by female $CD4^+$ T cells we then investigated the effect of testosterone treatment on female $CD4^+$ T cells proliferation (figure 5.6), as IL-2 is an important growth factor for T cell proliferation. However, we did not detect any significant differences in the number of cell divisions after treatment with different concentrations of testosterone.

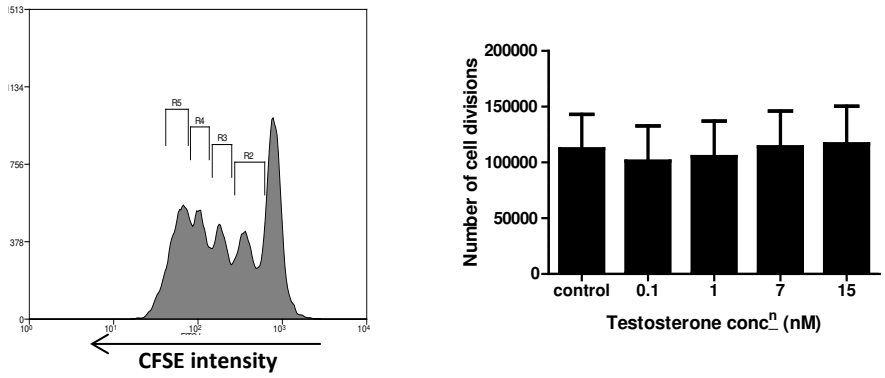

Figure 5.6 Female $CD4^+$ T cell proliferation after testosterone treatment.
PBMCs from female donors (n=4) were labelled with CFSE and stimulated with anti CD3 and anti CD28 activation beads and cultured for 4 days with different concentrations of testosterone. At day 4 PBMCs were stained for $CD4^+$ cells and CFSE fluorescence intensity was measured using flow cytometry. **A**) FACs plots for CFSE profiles, in which each bar presents a round of division. **B**) Number of divisions was calculated by multiplying the number of cells in each peak by the number of rounds of divisions. Error bars represents SEM.

5.6 Discussion

We demonstrated in the previous chapters that 5α-reductase expression is upregulated in female T cell subsets upon stimulation in both healthy individuals and SLE patients, thus potentially increasing their production of DHT. We therefore wanted to investigate what the effects of increased androgen production would have on human T cell cytokine production and proliferation. We wanted use physiological concentrations of androgens found in circulation to get a better understanding on the influence of androgens on T cell responses. A series of concentrations were chosen that reflected physiological concentrations of androgens present in circulation in healthy males and females, but also lower and higher concentrations found in disease. Many of the previous studies investigating the effects of androgens on immune response used animal models, which predominately involved the castration and then supplementation of androgen or the treatment with 5α-reductase inhibitors such as finasteride (an 5α-reductase 2 inhibitor) (Fijak *et al.* 2011; Gilliver *et al.* 2006). We wanted to investigate the potential consequence of upregulating 5α-reductase 1 in female T cells after stimulation on T cell responses. Therefore, we initially investigated the effect of dutasteride (a 5α-reductase 1 inhibitor), which is commonly used for the treatment of benign prostatic hyperplasia and prostate cancer, on the proportion of IL-2 and IFN-γ-producing $CD4^+$ T cells (see appendix). However, we only observed a decrease in the proportion of $CD4^+$ T cells producing these cytokines at a concentration of 10μM dutasteride, which is much higher than concentrations reached therapeutically (Schröder *et al.* 2013). To my knowledge, there has not been any studies investigating differences in immune function in patients being treated with 5α-reductase inhibitors, however finasteride treatment before induction of trauma-haemorrhage and sepsis in mice has been shown to inhibit the immunosuppressive effect of DHT (Zeckey *et al.* 2011). Therefore, we then investigated the effect of testosterone and

DHT on cytokine production by T cells, as previous *in vitro* studies have often non-physiological concentrations (often considerably higher concentrations) of androgens (Zhang *et al.* 2012). We found significant differences between males and females in the percentage of cells producing certain cytokines; there were significantly higher percentage of $CD4^+$ T cells producing IL-2 and IFN-y from females in comparison to the male $CD4^+$ T cells. This observation is in agreement with the published literature (Araneo *et al.* 1991; Zhang *et al.* 2012) and confirms that female $CD4^+$ T cells produce higher levels of these Th1 derived cytokines in comparison to males in response to stimulation. We did not investigate the differences in Th2 derived cytokines such as IL-4 and IL-5 between the sexes, but previous studies in humans have found that women produce higher levels Th2 cytokines compared to men, which is consistent with the findings that women have higher antibody titers (Giron-Gonzalez *et al.* 2000). However, there is some conflicting data generated from mice as a study found that male $CD4^+$ T cells produced higher levels of IL-10 compared to females (Liva and Voskuhl 2001). We did carry out some preliminary experiments on the production of IL-10 by T cells and found a slightly higher level of IL-10 produced by female $CD4^+$ T cells and $CD8^+$ T cells compared to the males, which would support the higher Th2 responses in females as IL-10 is a potent stimulator of B cell proliferation and differentiation (Cai *et al.* 2012). Therefore, higher IL-10 production in females could make them more susceptible to increased autoreactive B cells responses, as elevated IL-10 levels detected in SLE patient serum positively correlates with serological disease activity and anti-DNA antibodies (Csiszar *et al.* 2000). However, we would need to carry out more IL-10 experiments to investigate if this observation holds up. Also, we obtained very low proportions of $CD4^+$ T cell producing IL-10, which is most likely due to the short 24 hours stimulation assays, as 72 hour assays are usually required to detect higher levels of IL-10. Also, it may be important to investigate the Treg lacking FoxP3 population as they are the main producers of IL-10 within

the $CD4^+$ T cell population (Feuerer *et al.* 2009). We also investigated the levels of pro-inflammatory cytokines IL-17 and TNF-α and found a trend towards a higher production of these cytokines by male $CD4^+$ T cells, however this was not statistically significant, possibly due to the low number of repeats as previously mentioned. However, if this trend is confirmed then this would be in line with the literature as human whole blood stimulated with LPS results in higher TNF-α in males compared to females (Aulock *et al.* 2006). The role of IL-17 has been clearly demonstrated in the pathogenesis of T cell driven autoimmune diseases (Nakae *et al.* 2003; van den Berg and Miossec 2009), therefore it would be expected that IL-17 production is higher in females then in males, as females are more susceptible to developing these autoimmune conditions.

We then wanted investigate the effects of testosterone and DHT on production of these cytokines, particularly DHT as we had previously found that female T cells were upregulating 5α-reductase 1 expression after stimulation, therefore we wanted to investigate what effects this increase in DHT levels would have on T cell cytokine responses. For IL-2, we observed an interesting pattern creating a bell-shaped curve, in which low concentrations of testosterone (0.1nM to 1nM) significantly increased the percentage of IL-2 producing female $CD4^+$ T cells compared to the untreated control and in contrast the higher testosterone concentrations (7nM and 15nM) in particular 15nM significantly decreased the IL-2 levels in comparison to the $CD4^+$ T cells treated with 1nM testosterone. This pattern was also observed in female $CD8^+$ T cells; however the differences observed were not statistically significant. Also, a similar trend was seen in female $CD4^+$ T cells producing IFN-γ; however none of the differences were statistically significant. In male $CD4^+$ T cells, an increase in IL-2 levels between the control and 1nM testosterone was not observed, however a significant

decrease in IL-2 levels in cells treated with 7nM and particularly 15nM testosterone as seen in female $CD4^+$ T cells was also observed in male $CD4^+$ T cells. Therefore, we observed what appears to be a dual effect of hormones on IL-2-producing $CD4^+$ T cells; firstly a long term effect on the T cells that leads to a different behaviour of T cells depending on whether they have been generated in a "female" or "male" hormone environment and secondly a short term effect of hormones present at the time of stimulation. The long term effects are most likely to be involving hormone-dependent epigenetic modifications as hormones have been shown to influence sex hormone receptor expression through epigenetic modifications (Imamura 2011), while the short term effects are more likely to be mediated through the oestrogen or androgen receptors respectively. This effect of testosterone on proportion of IL-2-producing $CD4^+$ T cells suggests that low testosterone levels found in healthy females and SLE patients could be responsible for more robust immune responses seen in females in both health and disease. In contrast, high testosterone concentrations seen in healthy males and PCOS patients seem to be able to suppress the proportion of IL-2-producing $CD4^+$ T cells, thus potentially reducing subsequent $CD4^+$ T cell proliferation and differentiation. High levels of testosterone could also be protecting males from generating proinflammatory responses, and possibly prevent the development of chronic inflammation and autoimmunity. There is evidence to suggest testosterone is able to dampen immune responses in models of autoimmunity and trauma-haemorrhage, the latter resulting in a severe decrease in the rate of wound healing and increase mortality in males (Gilliver *et al.* 2006). The actions of androgens are mediated through the classical androgen receptor signalling pathway, in which androgen receptor translocation after subsequent activation leads to the transcription of target genes with androgen response elements in their promoter regions. DHT is the most potent androgen in the body as it has an affinity for the androgen receptor in comparison to testosterone. Therefore, we also investigated the effect of DHT on cytokine production,

however there were no significant differences in the treatment of DHT on $CD4^+$ and $CD8^+$ T cell cytokine production from both males and females, in contrast to the findings in the testosterone treatment on IL-2 production. As these differential effects of low and high testosterone concentrations on IL-2 production were not observed upon DHT treatment, which directly activates the androgen receptor, it questioned if a direct effect of testosterone on the androgen receptor could be responsible for the increase in IL-2 production at these lower concentrations. However, testosterone can be converted into 17β-oestradiol by aromatase, therefore is conceivable that the increased proportion of IL-2-producing $CD4^+$ T cells treated with the low but physiological concentrations of testosterone may be explained by conversion of testosterone to 17β-oestradiol. This would also be in agreement with the lack of effect of DHT, which cannot be converted to 17β-oestradiol. Therefore to investigate this further so we used an oestrogen receptor inhibitor, 4-hydroxytamoxifen (the active metabolite of tamoxifen) to investigate if 17β-oestradiol was responsible for the increase in IL-2 production in female $CD4^+$ T cells. Treatment of female $CD4^+$ T cells with 4-hydroxytamoxifen in conjunction with different concentrations of testosterone abolished the increase in proportion of IL-2-producing $CD4^+$ T cells previously observed with the lower concentrations of testosterone. This suggests that it was 17β-oestradiol and its activation of oestrogen receptors, which was responsible for this increase in proportion of IL-2-producing $CD4^+$ T cells at the lower testosterone concentrations. However, T cells have been shown to have very low expression of aromatase suggesting that T cells are not able to produce endogenous oestrogens, but can respond to oestrogens as they express both isoforms of oestrogen receptor. However, 4-hydroxytamoxifen treatment had no effect on the decrease of IL-2 production observed when $CD4^+$ T cells were treated with high concentrations of testosterone (7nM and 15nM), thus suggesting this observation was not mediated by oestrogens and could be testosterone activating the androgen receptor.

In experiments testing whether oestrogens have an effect in our cell culture system, we found that the percentage of female $CD4^+$ T cell producing IL-2 and IFN-y showed a concentration dependent increase under influence of 17β-estradiol. If the low testosterone effect was mediated by 17β-oestradiol, it remained to be tested whether the testosterone was converted in the T cells or in other cells in the PBMC cultures, as T cells have been reported to have very low aromatase activity. Previous publications by Straub and colleagues have suggested that monocytes and macrophages are able to convert testosterone to 17β-oestradiol (Schmidt *et al.* 2000). To answer this question we used qPCR to investigate aromatase gene expression (CYP19A1) in $CD4^+$ T cells, monocytes and in vitro differentiated macrophages. The results showed that both $CD4^+$ T cells and monocytes had very low levels of mRNA expression for aromatase; in contrast macrophages had significantly higher gene expression for aromatase compared to $CD4^+$ T cells. This is an interesting observation, but it does not explain why at higher levels of testosterone the effect is reversed. It has been previously shown that at high concentrations testosterone is able to activate androgen receptor with high affinity in a similar manner to DHT (Grino *et al.* 1990). Also, there is evidence to suggest that high concentrations of testosterone can inhibit aromatase activity; human synoviocytes isolated from RA patients that were treated with 250nM testosterone showed a significant decrease in testosterone conversion to oestrone and 17β-oestradiol due to decreased aromatase activity (Schmidt *et al.* 2005). Also, the treatment of healthy men with exogenous testosterone, which results in an increase in serum testosterone levels from 22nM to 35nM, showed a significant reduction in aromatase activity after testosterone treatment (Vottero *et al.* 2006). As our findings were only observed in female $CD4^+$ T cells, possible explanation may be differences in the aromatase activity in male and female individuals, however this is not reported in the literature. However, we did detect a higher aromatase expression in *in vitro* differentiated monocytes to macrophages compared to undifferentiated monocytes, which suggests that

differentiation of monocytes into macrophages results in an increased exposure of endogenous oestrogens. Oestrogen has shown to increase MHCII expression through the upregulation of IL-1 production in mice (Ruh *et al.* 1998). Therefore, it would be interesting to investigate when this upregulation of aromatase occurs in macrophage differentiation through time course analysis, and effect of aromatase inhibitors on macrophage responses.

IL-2 is an important cytokine and growth factor needed for T cell survival, proliferation and differentiation. The initial encounter of antigen by naïve T cells induces activation through TCR and co-stimulation signalling pathways, resulting in the rapid production of IL-2. IL-2 produced by the T cells in turn acts on the T cell itself in an intracine manner by interacting with the IL-2 receptor (IL-2R) on the surface of the T cells, thus driving subsequent proliferation and differentiation. To further investigate if the increase in IL-2 production was down to testosterone conversion into 17β-oestradiol, we treated female $CD4^+$ T cells with physiological concentrations of 17β-oestradiol detected at various stages of the menstrual cycle in order to get both low and high physiological levels present. We found that there was a dose dependent in increase in IL-2 and IFN-γ production, which positively correlated with increasing concentrations of 17β-oestradiol. In contrast to our initial findings, previous studies have shown oestrogen is able to decrease IL-2 production and IL-2 mRNA expression in a dose dependent manner (McMurray *et al.* 2001). There are several mediators involved in the transcription and therefore the synthesis of IL-2, the most important being the transcription factors; AP-1, NFAT and NF-κB, which bind to specific regions within the IL-2 gene promoter. Oestrogen receptors upon ligand activation have shown to not only target oestrogen response elements in promoter regions of specific genes but also target non-ERE regions such as AP1 enhancer elements specific for AP1 transcription factors thus suggesting

the influence of oestrogens on IL-2 transcription (Paech *et al.* 1997). The exact mechanisms of which oestrogen is able to mediate these effects is not very well understood, however McMurray and colleagues also showed that oestrogen activated oestrogen receptors are able to prevent the binding of transcription factors AP1 and NFκB to the promoter regions of the IL-2 gene by in part increasing the levels of IκBα, an cytoplasmic inhibitor of NFκB which prevents its translocation into the nucleus (McMurray *et al.* 2001). IL-2 transcription is also modulated by two transcription factors with contrasting roles that bind the same region of the IL-2R promoter (cyclic AMP response element (CRE); cyclic cAMP response element binding (CREB) in its phosphorylated form activates IL-2 transcription and in contrast cyclic AMP response element modulator (CREMα) once phosphorylated suppresses IL-2 transcription. An imbalance of these two conflicting transcription factors have been demonstrated in SLE T cells, in which CREMα transcriptional activity is elevated and levels of phosphorylated CREB are reduced, thus an increased suppression of IL-2 production is observed (Juang 2005; Solomou *et al.* 2001). Moulton and colleagues found that oestrogen was able to increase CREMα mRNA expression and activity in T cells (Moulton *et al.* 2012). However, in both of these studies extremely high and non-physiological concentrations of 17-β-oestradiol were used, ranging from 100nM to 10μM and both studies found a significant effect of 17-β-oestradiol at 10μM, which is significantly higher than the maximum physiological concentration range of 1nM-2nM found in women. Therefore, these inhibitory effects of oestrogen in these studies will need to be verified using physiological concentrations. It could be that at lower concentrations of oestrogens that the reverse is observed and that decreases IκBα levels and therefore elevating IL-2 transcription, which would support our observations of 17β-oestradiol treatment on proportion of IL-2-producing $CD4^+$ T cells. Also, there is evidence to suggest that CREMα is able to mediate silencing of the IL-2 gene through epigenetic modifications; histone deacetylation and cytosine phosphate

guanosine (CpP)-DNA hypermethylation (Hedrich *et al*, 2011). Therefore, if oestrogen is are able to decrease CREMα activity at physiological concentrations then it would be interesting to see if oestrogen could indirectly abrogate IL-2 gene silencing through epigenetic modifications.

To confirm our findings, an important experiment that needs to be done in future is to establish the levels of 17β-oestradiol in our culture system. Prof Arlt's laboratory is currently establishing the necessary techniques. It would also be necessary to confirm these findings by measuring the concentration of IL-2 produced by $CD4^+$ T cells after testosterone treatment. Tamoxifen, through its metabolite 4-hydroxytamoxifen, is a well established as a competitive antagonist binding to the oestrogen receptor. It is frequently prescribed to women with oestrogen sensitive breast cancer. The action of tamoxifen on oestrogen receptors has been previously questioned, therefore, it will be important to repeat the experiments with novel inhibitors which target aromatase directly such as anastrozole or exemestane.

After observing the significant effects of testosterone on the proportion of female $CD4^+$ T cells we then investigated the effect of testosterone treatment on female $CD4^+$ T cells proliferation (figure 5.5), as IL-2 is an important growth factor for T cell proliferation and the effect of androgens in humans is unclear. However, we did not detect any significant differences in the number of cell divisions after treatment with different concentrations of testosterone. In mice, castration has shown to increase the number of T cells in peripheral lymphoid tissues and in the thymus, which resulted in a significant increase in thymus mass as a result of castration, thus suggesting that androgens exert a suppressive affect on T cell proliferation. A possible explanation for our data is that there were vast amount of

heterogeneity between the proliferation assays as T cells from some donors had much higher number of cell divisions compared to others. Therefore it would be important to repeat these proliferation experiments with a higher number of donors. Also, it would be interesting to investigate T cell differentiation as IL-2 has a vital role in Th1 differentiation.

In summary, we found that the percentages of IL-2 and IFN-γ producing $CD4^+$ T cells from females were significantly higher compared to their male counterparts, which confirms previous findings in the current literature. The most interesting finding was the dual effect of testosterone treatment on proportion of IL-2 $CD4^+$ T cells from females only, in which at low concentrations of testosterone the proportion of IL-2 producing $CD4^+$T cells, which was not observed after DHT treatment. This was later confirmed to be oestrogen mediated (through potential conversion of testosterone into 17β-oestradiol by aromatase present in macrophages), as treatment with tamoxifen abrogated this increase in proportion of IL-2 $CD4^+$ T cells after treatment with low concentrations of testosterone suggesting that testosterone is being converted into but did not prevent the decrease the proportion of IL-2 producing $CD4^+$ T cells when cells were treated with high concentrations of testosterone, which suggests these are oestrogen independent affects. It would be important to confirm these findings using specific aromatase inhibitors and measuring 17β-oestradiol levels in these assays after testosterone treatment.

6 GENERAL DISCUSSION

6.1 Sex hormone metabolism and activation in T cells

The increased risk of autoimmune conditions such as SLE and RA in females has initiated a considerable amount of research into the role sex hormones play in immune and inflammatory responses. A large proportion of this research has focussed on the effect of oestrogen on immune responses. Sex hormones have substantial effects on the pathogenesis of chronic inflammatory disorders including autoimmunity, and this is clearly demonstrated during events of drastic changes in sex hormone levels (particularly oestrogens) including pregnancy, menstrual cycle and menopause. Endometriosis is a chronic inflammatory condition, in which oestrogen production in local tissues positively correlates with disease severity, and the onset of the disease occurs in predominately in woman of a reproductive age (Jones *et al.* 1995). Pregnancy has been associated with increased incidence of disease flares in SLE (Ruiz-Irastorza *et al.* 1996; Tengstrand *et al.* 2002). Androgen levels have also been associated with changes in immune responses and androgen deficiency has been associated with enhanced immunostimulatory responses and increased susceptibility to autoimmune diseases (Cutolo *et al.* 1991; Forsblad-d'Elia *et al.* 2009). Low androgen levels have been detected in both female and male SLE (and RA) patients (Lahita *et al.* 1987), and have been shown to be associated with disease activity . In contrast to oestrogens, the effects of androgens on immune responses have been less thoroughly researched, however the general consensus seems to be that androgens have predominately anti-inflammatory effects and therefore able to dampen inflammatory responses .

The initial aim of this project was to investigate differences in the way T cells from male and female healthy controls respond to androgens and oestrogens, and how they regulate their exposure to these hormones, as this could explain the enhanced T cell responses in females. To accomplish this, we explored the differences in the expression of a range of proteins including hormone receptors, downstream signalling molecules such as PPARs and enzymes involved in sex hormone metabolism between naïve CD4 T cells from healthy male and female donors. Our results showed that there were no significant differences the expression of sex hormone receptors in resting naïve $CD4^+$ T cells between males and females. We would have suspected that the different levels of sex hormones seen in males and female (higher androgen levels verses oestrogen in males and vice versa in females) might be able to influence to expression of sex hormone receptors within $CD4^+$ T cells, however this was not the case, thus suggesting that circulating sex hormone levels do not influence the expression of sex hormone receptors in T cells. Therefore, any differences in T cell responses between sexes are unlikely to be due to differences in sex hormone receptor availability; nevertheless, exposure of T cells to higher levels of androgens and oestrogens will increase overall sex hormone receptor mediated signals and therefore may influence T cell responses. It is well established that females and males have significantly different levels of androgens and oestrogens. These differences could be responsible for a large proportion of the differences in immune responses demonstrated between the sexes both in health and disease.

While we did not observe any significant differences in baseline expression of sex hormone receptors, we did observe an upregulation of ER-β in female $CD4^+$ T cells upon stimulation, which is interesting as others have shown that ER-β is able to mediate anti-inflammatory responses, in part by suppressing ER-α activation, which is the predominate isoform in

mediating the pro-inflammatory effects of oestrogens. Also, it has been shown that SLE $CD4^+$ T cells have significantly higher ER-β mRNA levels compared to healthy controls (Inui *et al.* 2007), suggesting that SLE $CD4^+$ T cells are suppressing their exposure to the pro-inflammatory affects of oestrogens.

In this study, we have largely focussed on the adaptive immune response. Sex hormones do not only exert their influences on adaptive immune responses, but have also been shown to influence innate immune responses (Seillet *et al.* 2012). Therefore it would be interesting to investigate any differences in sex hormone receptor expression and responses between the sexes in the cells of the innate immune system such as neutrophils, macrophages, NK cells and dendritic cells. As we did detect a significant upregulation in ER-β expression in female naïve $CD4^+$ T cells after stimulation it would be interesting to follow this up by investigating the effects of specific agonists and antagonists for ER-α and ER-β to investigate their individual roles in T cell responses.

One of the most important findings in this study was the upregulation of 5α-reductase in female T cells after stimulation, which was not observed in the male T cells. This is particularly interesting as 5α-reductase is responsible for the synthesis of the most potent androgen in the body, DHT. Circulatory levels of DHT are significantly lower in females compared to males, therefore this observation suggested it may be advantageous for the female organism if T cells can increase their exposure to androgens by producing their own DHT in an intracine manner. DHT is the most potent androgen receptor agonist, therefore any DHT produced within the cell can directly act on the androgen receptor within the cell, thus initiating transcription of target genes. DHT among other androgens such as testosterone have shown to exert mainly anti-inflammatory effects, therefore suggesting that the increase in 5α-

reductase 1 expression in female T cells may be a mechanism to prevent excessive stimulation. Clinical trials have been carried out for the treatment prostate cancer with 5α-reductase inhibitors (Schröder *et al.* 2013). No publications on the effect of this drug on the immune system have emerged from these trials. It would be interesting to investigate the effect of dutasteride in healthy males in order to get a *in vivo* insight of the influences of DHT on the immune system in a human model on immune responses such as T cell cytokine production and proliferation; innate responses such as phagocytosis and respiratory burst by phagocytes. 5α-reductase knockout mice have also been used in studies to investigate the effect of DHT (Osborne and Frye 2009), therefore 5α-reductase knockout mice could be used to investigate changes in immune responses.

An interesting future series of experiments could investigate changes in intracellular signalling after T cell stimulation that are able to regulate 5α-reductase 1 gene expression, and also if these mechanisms differ between males and females, as this might be responsible for this upregulation of 5α-reductase 1 in stimulated female T cells only. The regulation of 5α-reductase transcription has been shown to be dependent on DHT-activated androgen receptor in a cell-type specific manner (Berman and Russell 1993; Hellwinkel *et al.* 2000; Kuhnle *et al.* 1994), therefore differences in this mechanism could explain our observations. Although, we were able to confirm this mRNA finding on the protein level, unfortunately even after months of carrying out optimisation experiments, it was extremely difficult to detect high concentrations of DHT in 5α-reductase activity assays, as most of the testosterone substrate was converted into androstenedione via 17β–HSD and the total detectable levels of DHT were at the lower limit of the sensitivity of the assay. Furthermore these assays require high concentrations of substrate to detect any subsequent metabolites, these extremely high

concentrations of testosterone (200nM) would not be found in circulation and the assay is therefore not reflecting the physiological situation. Also, extremely high concentrations of testosterone have shown to inhibit 5α-reductase activity (Pratis *et al.* 2003). Therefore, an alternative method to detect DHT would have to be developed to further confirm the increased enzymatic activity of 5α-reductase in activated T cells from female donors.

This initial observation led us to hypothesise that this mechanism of upregulating androgen exposure by upregulation of 5α-reductase expression in immune cells could be deficient in SLE patients and might explain the deregulated immune responses in these patients.

6.2 5α-reductase expression and regulation in peripheral blood mononuclear cells from SLE patients.

We did not observe any significant differences in 5α-reductase expression in T cells on the baseline or after stimulation between SLE patients and healthy controls. However we did find a significantly higher expression of 5α-reductase in B cells from SLE patients compared to healthy controls. This is interesting for two reasons; firstly, B cell activation plays an important role in SLE, as our current understanding of this disease suggests that autoantibodies greatly contribute to the immune complex-driven complement activation seen in these patients (Stohl *et al.* 2012). Secondly, SLE patients have been shown to have very low androgen levels (positively correlating to disease severity) and in contrast, an increase in oestrogen metabolites has been observed in these patients. Most studies looking at the effects of sex hormones on B cells responses have mainly focused on oestrogen as it has shown to enhance humoral responses, however androgens have also shown to be able to exert immunosuppressant effects on B cells (Corrales *et al.* 2012).

In clinical trials for the potential use of sex hormones for the treatment of various autoimmune diseases, has been investigated. Most commonly DHEA has been used as many autoimmune disorders have shown an inverted correlation of DHEA/DHEA with disease severity (Straub *et al.* 2003). Low DHEA levels have been linked to high IL-6 levels in older people, and this has been suggested to play an important part in the increased incidence of autoimmune conditions such as RA as individuals over the ages of 60 (Hammer *et al.* 2005). However, the effects of DHEA, which have shown to be anti-inflammatory (Straub *et al.* 1998), is more likely due to the downstream metabolites of DHEA, as DHEA itself is an inactive precursor for active androgens and oestrogens. For this reason, testosterone has been investigated as a potential treatment for SLE. However, a trial treating SLE patients with testosterone patches did not see any significant differences in disease activity or patient symptoms (Gordon *et al.* 2008). Our observation of conversion of testosterone into oestrogens, which have shown to exacerbate disease severity in SLE patients, could be an explanation for the lack efficacy of testosterone therapy in SLE. However, the use of DHT as treatment for SLE may be beneficial as DHT is more potent than testosterone and therefore binds to the androgen receptor with greater affinity, and also cannot be converted in downstream to oestrogens.

6.3 Regulation of IL-2 production by sex hormones

In $CD4^+$ T cells from women, we observed an interesting pattern of regulation of IL-2 production at low concentrations of testosterone, which are physiologically found in women, the percentage of IL-2 producing female $CD4^+$ T cells significantly increased compared to the untreated control and in contrast the higher testosterone concentrations significantly decreased the IL-2 levels in comparison to the $CD4^+$ T cells treated with low concentrations

of testosterone. This decrease in IL-2 production at high testosterone concentrations was also observed in male $CD4^+$ T cells. We did not find this pattern with DHT. This observation of increased proportion of IL-2-producing $CD4^+$ T cells from females was completely unexpected as androgens have been shown to suppress pro-inflammatory responses. However, as testosterone can be converted into 17β-oestradiol, it suggested that testosterone was being converted into oestrogen at the lower concentrations. This was later confirmed as tamoxifen treatment abolished the increase of IL-2 at low testosterone concentrations. We also found that IL-2 and IFN-y showed a 17β-oestradiol dose dependent increase, however this just short of being statistically significant for IL-2, therefore it would be important to increase the number of repeats. However, the question still remains to why do only low, but not higher levels of testosterone increase production of IL-2. A possible explanation may be that the conversion of testosterone to estradiol by aromatase is saturated at these concentrations and the androgenic effect of testosterone tilts the balance towards an inhibitory signal, as this inhibition of aromatase activity by high concentrations of testosterone has been previously demonstrated (Schmidt *et al.* 2005). Other explanation could be due to higher aromatase activity in female macrophages compared to males, this as of yet has not been previously reported.

One of the key defects of SLE T cells is that they produce significantly lower levels of IL-2 in response to TCR stimulation and even after *in vitro* treatment with exogenous IL-2, SLE T cells remain unresponsive. IL-2 knockout mice have been shown to develop lethal autoimmune diseases, which has been partly explained by the reduction of regulatory T cells in an environment lacking IL-2, which has been shown to be vital for the development and survival of regulatory T cells (Malek and Bayer 2004). The interesting finding that Tregs

cannot produce IL-2 upon TCR stimulation and rely on IL-2 produced from other cells further supports this argument. Therefore, if the main producers of IL-2 (T cells) are defected and produce very low levels of IL-2 as seen in SLE, then this would result in reduced Treg responses, thus peripheral tolerance may be lost resulting in the development of autoimmunity. A possible explanation for this reduced IL-2 production by SLE T cells is the observation that CREMα transcriptional activity is elevated and levels of phosphorylated CREB are reduced in SLE T cells, thus an increased suppression of IL-2 production is observed (Juang 2005). It still remains unclear if oestrogen is able mediate this process of IL-2 transcription by modulating CREMα activity at physiological concentrations, but additionally the effect of androgens on CREMα and CREB activity should also be investigated, which to my knowledge has not been previously done and could explain the mechanism behind the contrasting affects of androgens and oestrogens on IL-2 transcription. Androgens are able to negatively regulate target gene expression in an androgen receptor-dependent manner, in which upon ligand activation of AR, AR is able to bind to negative-androgen responses elements (nARE) in the promoter regions of the target genes (Li *et al.* 2011; Qi *et al.* 2012). Interestingly, androgen receptors have shown to negatively regulate TGF-β transcription in prostate cells, as nARE regions are present in the TGF-β promoter region (Qi *et al.* 2012). Therefore, another interesting possibility could that nARE regions could be present in the IL-2 promoter and can mediate androgen receptor-dependent suppression of IL-2 transcription.

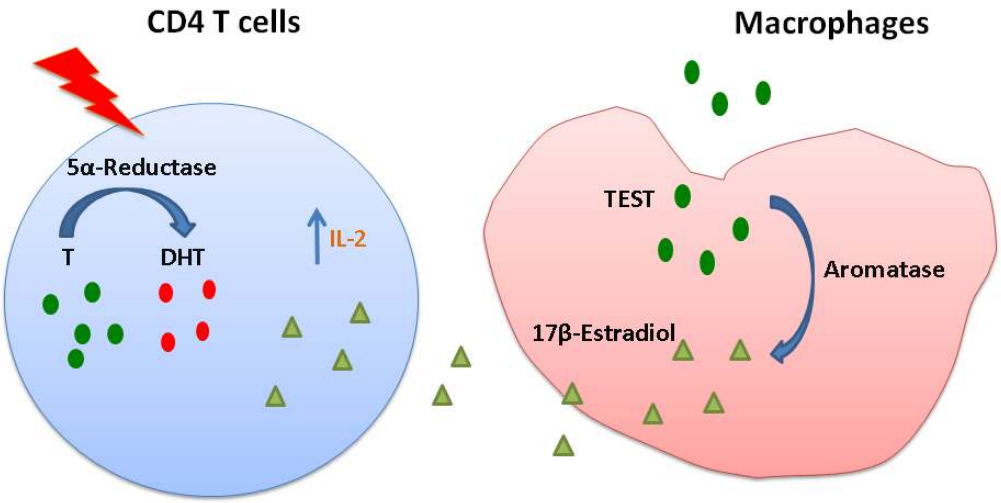

Figure 6.1 illustrates the main findings of the project.
Firstly, it was demonstrated that female not male T cells were able to upregulate 5α-reductase 1 expression upon stimulation, which could potentially lead to an increase in conversion of testosterone to the more potent androgen 5α-dihydrotestosterone (DHT). Secondly, this 5α-reductase 1 expression regulation upon stimulation was also observed in Systemic Lupus Erythematosus (SLE) T cells and interestingly SLE B cells expressed higher 5α-reductase 1 expression compared to healthy controls. Thirdly, *in vitro* treatment of female $CD4^+$ T cells with low concentrations of testosterone, which was being converted into 17β-estradiol by macrophages, resulted in an increase in IL-2 upon oestrogen receptor activation in female $CD4^+$ T cells by 17β-estradiol.

6.4 Future work

In this study, we made a novel discovery that female T cells were upregulating their expression of 5α-reductase 1 upon stimulation, and therefore may be increasing their exposure to the anti-inflammatory effects of androgens. It would be interesting to see if the inhibition of 5α-reductase 1 activity by transfection with dominant negative enzyme or knockdown by SiRNA has any effect on T cell responses. The differences we have seen in the gene regulation of 5α-reductase expression between T cells from male and female donors is most likely caused by gender specific epigenetic modifications. This could be further investigated in a follow-on project. 5α-reductase protein expression showed that expression

peaked at 24 hours; therefore it would be interesting to measure IL-2 production at this time point, which coincides with potentially increased DHT levels. However, our method limited our incubation duration to 4 hours, as our the time course for time period as brefeldin A is toxic and incubation longer than 6 hours is not recommended and in a cytokine time course (data not shown) we did see a decrease in both IL-2 and IFN-y producing cells after 24-48 hours treatment with brefeldin A, which was predominately due to the increase in dead cells. Another method such as detection by ELISA must be adopted in order to investigate effects of androgens on cytokine production for longer time durations.

To further characterise the outcomes of 5α-reductase upregulation in SLE B cells, it would be important to investigate the effects of DHT on B cell responses such as antibody production and antigen presentation to T cells, as previous studies have studied the effects of testosterone on B cell responses but not DHT (refs), which directly activates the androgen receptor; whereas testosterone can be converted to oestrogens, thus it would be important to establish if androgens are mediating these affects observed on B cell antibody production or oestrogens.

We did not see a dramatic effect of dutasteride treatment on IL-2 and IFN-γ production, very high concentrations were necessary to observe any changes. It is however difficult to establish how much dutasteride actually enters the cells. The concentration needed to reduce IL2 production are unlikely to be reached in vivo in patients treated for hormone dependent prostate cancer. It would also be interesting to repeat more IL-10, IL-17 and TNF-α assays to see if any of the preliminary differences observed after androgen or oestrogen treatment are statistically significant. Also, it would be interesting to see if dutasteride treatment has any

affect on these cytokines, as they might be more sensitive to this dutasteride treatment compared to IL-2 and IFN-γ.

To confirm the differences in IL-2 production observed after testosterone treatment, it would be important to use an aromatase inhibitor to confirm that the low concentrations testosterone is being converted into 17β-estradiol. It would also be important to use an androgen receptor inhibitor to completely dismiss the effects of androgens on IL-2 production at the lower concentrations, and confirm the role of androgen on IL-2 production at the higher concentrations of testosterone. Also, it would be important measure oestrogen levels in samples treated with androgens, to confirm the conversion into oestrogens. We currently have no technique to do this at the necessary level of sensitivity available to us, but the Arlt laboratory is currently working on establishing an assay. It would also be necessary to repeat the IL-2 and testosterone assays with purified T cells in the presence or absence of macrophages to confirm that these cells, through their aromatase activity, mediate the effects of low testosterone concentrations on IL-2 production. It would also be important to investigate the changes in signalling and transcription pathways that results in this increase and decrease IL-2 production in response to androgens/oestrogens treatment.

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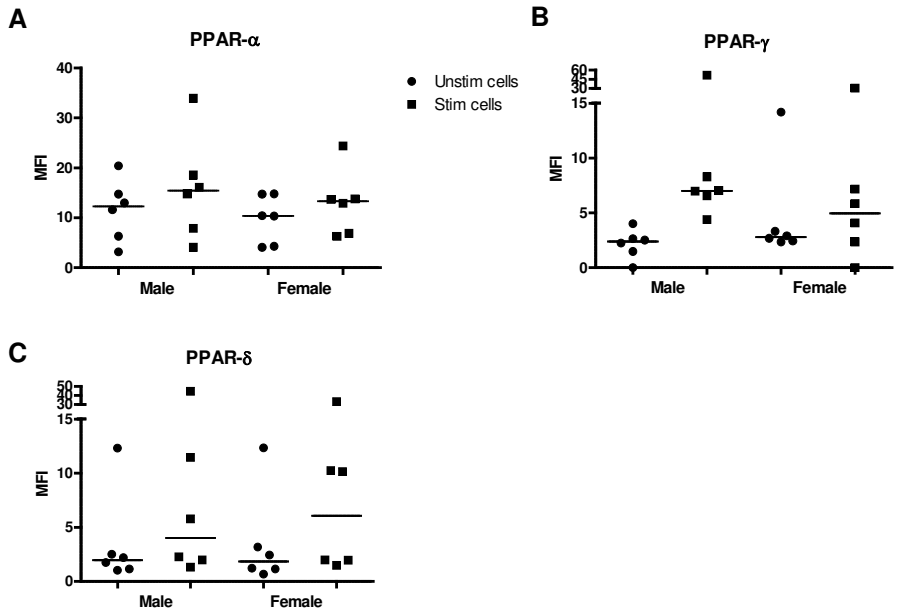
8 APPENDIX

8.1 Protein expression of PPARs naiveCD4+ T cells upon stimulation.



Naive $CD4^+$ CD45 RA^+ T cells from males (n=6) and females (n=6) were stimulated with anti CD3 and anti CD28 stimulation beads (alongside unstimulated cells) for 24 hours and then stained for intracellular expression of **A**) PPAR-α, **B**) PPAR-γ and **C**) PPAR-δ. Samples were analysed using flow cytometry. Horizontal lines represent the median. MFI: median fluorescence intensity.

Table 8.1 Clinical data for SLE patients. PBMCs taken from these patients were measured for 5α-reductase expression using flow cytometry (Chapter 3).

Age	Gender	GEN L	GEN N	MUC L	MUS N	NEU L	NEU N	MSK L	MSK N	CAR L	CAR N	VAS L	VAS N	REN L	REN N	HAE L	HAE N	COM P 3	COM P 4	DNA Quan	BILAG S
35	Female	D	0	C	1	D	0	B	5	D	0	D	0	D	0	C	1	1.12	0.26	0	7
59	Female	D	0	C	1	D	0	D	0	D	0	D	0	C	1	D	0	1.47	0.51	0	2
52	Female	D	0	C	1	D	0	B	5	D	0	D	0	D	0	D	0	1.19	0.36	0	6
64	Female	D	0	D	0	D	0	C	1	D	0	C	1	D	0	D	0	1.02	0.10	0	2
43	Female	C	1	C	1	C	1	C	1	D	0	D	0	D	0	C	1	1.19	0.20	30	4
53	Female	D	0	C	1	D	0	B	5	D	0	D	0	D	0	D	0	1.51	0.35	0	6
52	Female	D	0	C	1	D	0	D	0	D	0	D	0	D	0	C	1	0.39	0.05	383	2
37	Female	D	0	D	0	D	0	D	0	D	0	D	0	D	0	D	0	1.04	0.25	0	0
36	Female	D	0	B	5	D	0	D	0	D	0	D	0	D	0	D	0	1.16	0.42	0	5
67	Female	D	0	C	1	D	0	D	0	D	0	D	0	D	0	D	0	1.16	0.23	0	1
66	Female	D	0	C	1	D	0	D	0	B	5	D	0	D	0	C	1	1.17	0.33	42.9	7
32	Female	D	0	C	1	D	0	A	12	D	0	D	0	D	0	D	0	1.06	0.20	0	13
27	Female	D	0	D	0	D	0	D	0	D	0	D	0	D	0	D	0	0.99	0.13	0	0
41	Female	D	0	C	1	D	0	C	1	D	0	D	0	C	1	D	0	1.08	0.28	103.1	3
38	Female	D	0	C	1	D	0	C	1	D	0	D	0	C	1	D	0	1.12	0.34	0	3
42	Female	D	0	D	0	D	0	D	0	D	0	D	0	D	0	D	0	1.03	0.30	0	0
51	Female	D	0	C	1	D	0	C	1	D	0	D	0	C	1	B	5	ND	ND	ND	8
66	Female	D	0	D	0	D	0	D	0	D	0	D	0	D	0	B	5	0.91	0.16	0	5
47	Female	D	0	C	1	D	0	D	0	D	0	D	0	D	0	D	0	1.25	0.21	0	1
66	Female	D	0	C	1	D	0	C	1	B	5	D	0	C	1	C	1	0.92	0.24	41.5	9

35	Female	D	0	C	1	D	0	D	0	D	0	D	0	D	0	D	0	1.24	0.25	45.0	1
43	Female	D	0	B	5	D	0	B	5	D	0	B	5	D	0	C	1	1.32	0.20	22.1	16
32	Female	C	1	C	1	D	0	B	5	D	0	D	0	D	0	D	0	1.15	0.20	0	7
21	Female	D	0	D	0	D	0	C	1	B	5	D	0	D	0	B	5	0.94	0.15	91.9	11
38	Female	D	0	D	0	D	0	D	0	D	0	D	0	D	0	D	0	1.22	0.35	0	0
25	Female	B	5	B	5	D	0	B	5	D	0	D	0	D	0	D	0	1.50	0.29	0	15
49	Female	D	0	D	0	D	0	D	0	D	0	D	0	D	0	D	0	0.85	0.10	88	0
66	Female	D	0	C	1	D	0	C	1	C	1	D	0	D	0	C	1	0.98	0.27	33.8	4
41	Female	D	0	D	0	D	0	B	5	D	0	C	1	D	0	D	0	1.04	0.13	250.6	6
41	Female	D	0	D	0	D	0	C	1	D	0	D	0	D	0	C	1	0.86	0.19	0	2
38	Female	D	0	D	0	D	0	D	0	D	0	D	0	C	1	C	1	1.44	0.43	0	2
44	Female	D	0	D	0	D	0	D	0	D	0	D	0	D	0	D	0	0.95	0.17	0	0
60	Female	D	0	D	0	D	0	B	5	D	0	D	0	D	0	D	0	1.39	0.32	0	5
46	Female	D	0	D	0	D	0	D	0	D	0	D	0	D	0	D	0	1.22	0.33	0	0
47	Female	D	0	C	1	D	0	C	1	D	0	D	0	D	0	D	0	1.55	0.31	0	2
59	Female	D	0	D	0	D	0	D	0	D	0	D	0	D	0	D	0	1.21	0.18	0	0
46	Female	D	0	D	0	D	0	D	0	D	0	D	0	D	0	D	0	1.51	0.2	0	0
59	Female	D	0	C	1	D	0	C	1	D	0	D	0	D	0	D	0	1.59	0.55	0	2
26	Female	D	0	C	1	D	0	C	1	D	0	D	0	C	1	C	1	0.6	0.04	206.6	4
45	Female	D	0	C	1	D	0	C	1	D	0	D	0	D	0	D	0	1.41	0.42	0	2
67	Female	D	0	D	0	D	0	D	0	D	0	D	0	C	1	D	0	1.03	0.24	232.5	1
58	Female	D	0	C	1	E	0	D	0	D	0	D	0	D	0	C	1	1.13	0.19	257.6	2
52	Female	D	0	D	0	D	0	D	0	D	0	D	0	D	0	D	0	1.26	0.14	324.9	0
43	Female	E	0	D	0	E	0	D	0	E	0	E	0	E	0	D	0	1.07	0.22	0	0
33	Female	D	0	D	0	E	0	D	0	D	0	D	0	D	0	D	0	1.11	0.22	51.1	0
35	Female	D	0	D	0	D	0	D	0	E	0	D	0	D	0	D	0	1.11	0.22	20.8	0

47	Female	D	0	B	5	E	0	D	0	D	0	D	0	D	0	D	0	0.84	0.16	34.4	5
66	Male	E	0	C	1	E	0	D	0	E	0	E	0	E	0	C	1	1.28	0.20	0	2
45	Female	D	0	C	1	E	0	C	1	E	0	E	0	E	0	D	0	1.37	0.33	0	2
48	Female	D	0	D	0	E	0	C	1	E	0	E	0	E	0	C	1	1.24	0.18	0	2
43	Female	D	0	D	0	E	0	D	0	E	0	D	0	E	0	B	5	0.91	0.10	0	5
31	Male	D	0	D	0	E	0	D	0	D	0	E	0	D	0	C	1	1.13	0.17	24.9	1
36	Female	D	0	D	0	E	0	D	0	D	0	E	0	D	0	C	0	0.75	0.08	163	0
28	Female	D	0	D	0	E	0	D	0	E	0	D	0	E	0	D	0	0.93	0.11	0	0

BILAG scoring system, which examines multiple clinical features, was used to determine disease severity in patients. Abbreviations: General (GEN); Mucocutaneous (MUC); Musculoskeletal (MUS); Neurological (NEU); Cardiovascular (CAR); Vasculitis (VAS); Renal (REN); Haematology (HAE); Non-detectable (ND).

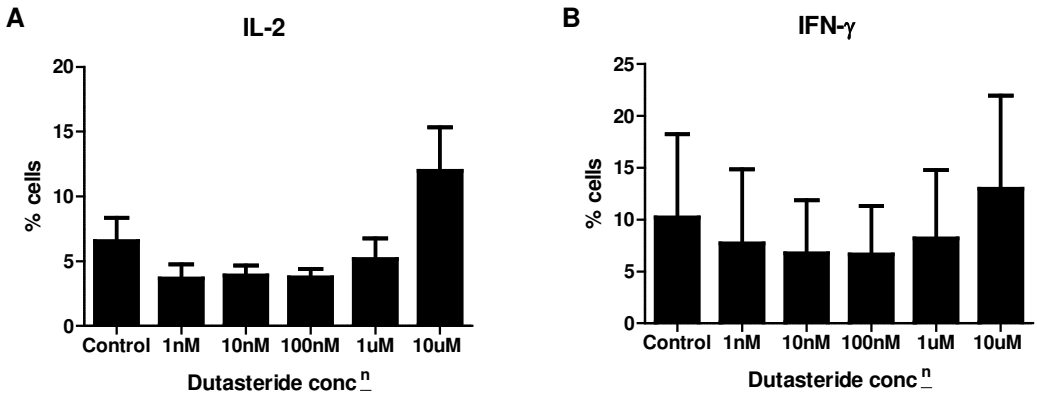
Table 8.2 Clinical data for SLE patients. Treatment information for SLE patients used in the study for 5α-reductase expression in immune cells (Chapter 3).

Age	Gender	Treatment
35	Female	Prednisolone
59	Female	Hydroxychloroquine, methotrexate
52	Female	Hydroxychloroquine, prednisolone, zathioprine
64	Female	Hydroxychloroquine, prednisolone, methotrexate
43	Female	Hydroxychloroquine, prednisolone, cyclophosphamide (3 weeks)
53	Female	Hydroxychloroquine, prednisolone, zathioprine
52	Female	Hydroxychloroquine
37	Female	Hydroxychloroquine, prednisolone, zathioprine
36	Female	Hydroxychloroquine, prednisolone, mycophenolate mofetil
67	Female	Hydroxychloroquine, prednisolone
66	Female	Hydroxychloroquine, prednisolone, zathioprine
32	Female	Prednisolone, mycophenolate mofetil
27	Female	Hydroxychloroquine, prednisolone
41	Female	Prednisolone, ciclosporin
38	Female	Hydroxychloroquine, prednisolone, zathioprine
42	Female	Hydroxychloroquine, prednisolone, mycophenolate mofetil
51	Female	Hydroxychloroquine, prednisolone, zathioprine
66	Female	Hydroxychloroquine
47	Female	Hydroxychloroquine, prednisolone

66	Female	Hydroxychloroquine, prednisolone, zathioprine
35	Female	Hydroxychloroquine, prednisolone, methotrexate
43	Female	Hydroxychloroquine, prednisolone, zathioprine
32	Female	Hydroxychloroquine, prednisolone, zathioprine
21	Female	Hydroxychloroquine, prednisolone
38	Female	Hydroxychloroquine
25	Female	Hydroxychloroquine, prednisolone
49	Female	Hydroxychloroquine, prednisolone
66	Female	Hydroxychloroquine, prednisolone
41	Female	Hydroxychloroquine, prednisolone
41	Female	Hydroxychloroquine, ciclosporin
38	Female	Hydroxychloroquine
44	Female	Hydroxychloroquine, prednisolone
60	Female	Hydroxychloroquine, prednisolone, azathioprine
46	Female	Hydroxychloroquine, prednisolone, methotrexate
58	Female	Hydroxychloroquine, prednisolone, azathioprine
52	Female	Hydroxychloroquine, prednisolone, methotrexate
43	Female	Hydroxychloroquine
33	Female	Hydroxychloroquine
35	Female	Hydroxychloroquine, prednisolone, azathioprine
47	Female	Hydroxychloroquine
66	Male	Hydroxychloroquine, prednisolone, mycophenolate mofetil
45	Female	Hydroxychloroquine

48	Female	Hydroxychloroquine, prednisolone, methotrexate
43	Female	Hydroxychloroquine, ciclosporin
31	Male	Hydroxychloroquine
36	Female	Hydroxychloroquine, prednisolone, azathioprine
28	Female	Hydroxychloroquine, mycophenolate mofetil

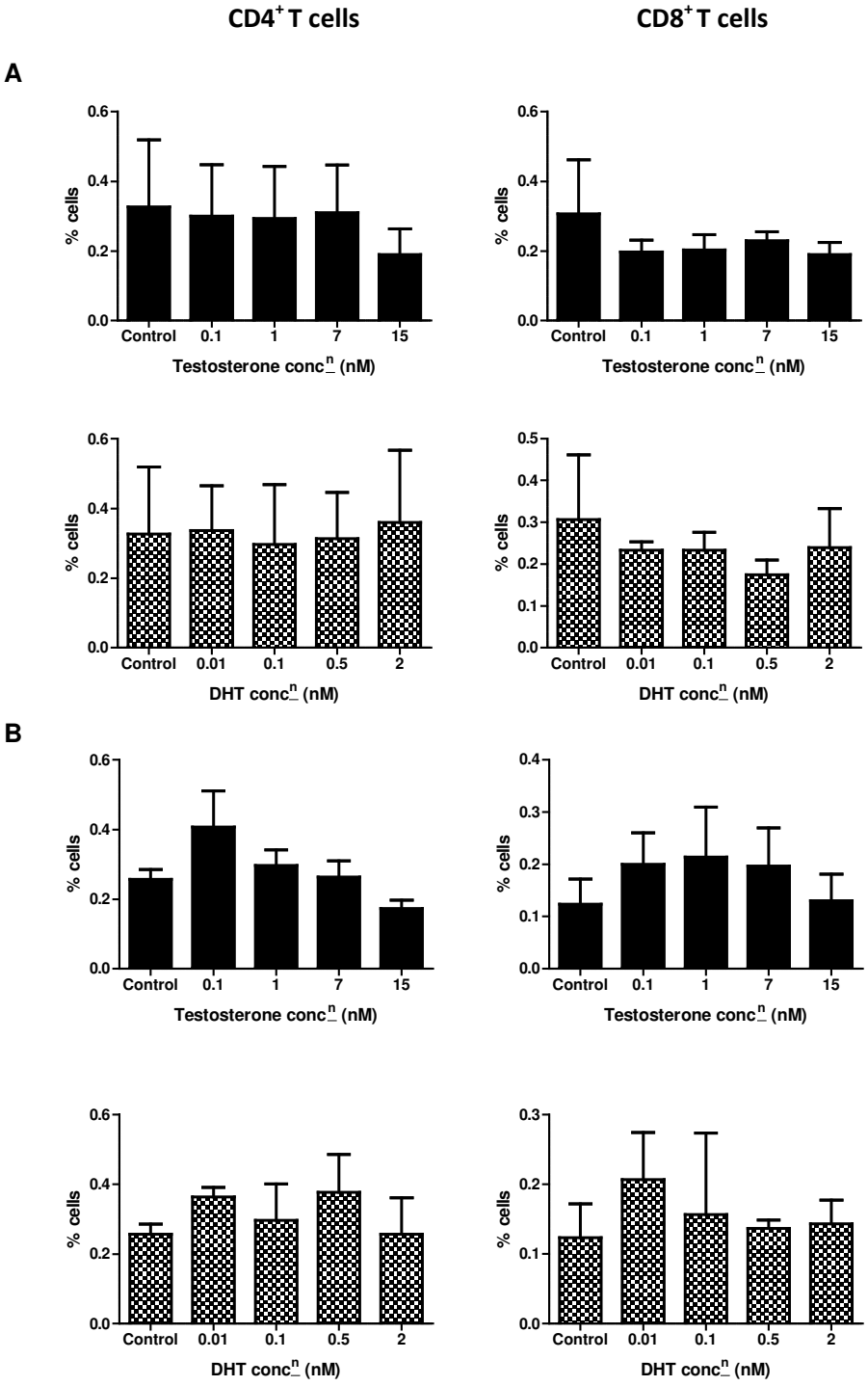
8.2 The proportion of IL-2 and IFNγ producing $CD4^+$ T cells after dutasteride treatment.



PBMCs were stimulated with anti CD3 and anti CD28 activation beads and cultured for 4 hours with different concentrations of dutasteride in the presence of brefeldin A to measure the proportion of **A**) IL-2 producing $CD4^+$ T cells and **B**) IL-2 producing $CD4^+$ T cells using flow cytometry (n=3). Error bars represent SEM.

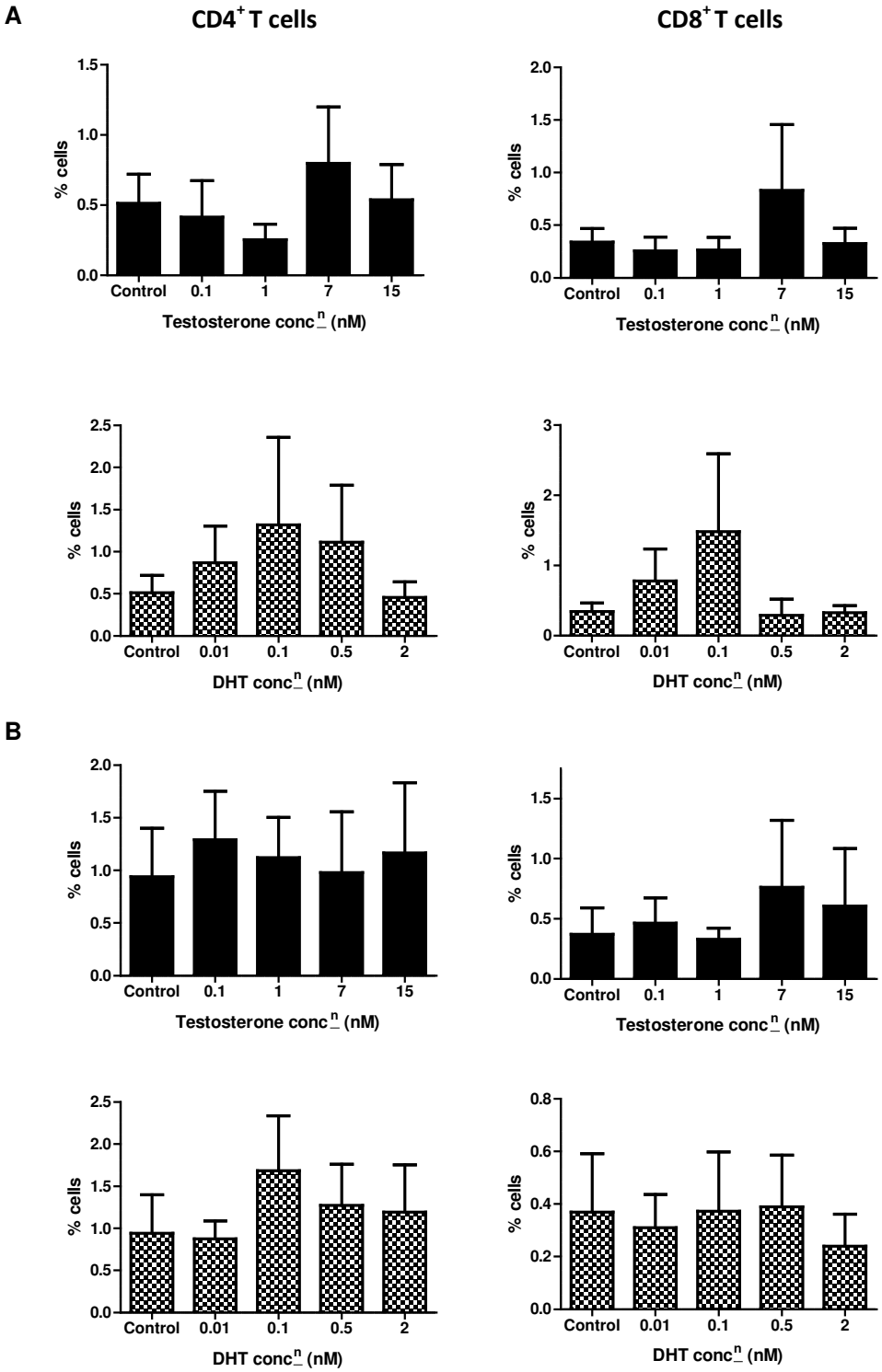
8.3 Cytokine production in T cells after testosterone and DHT treatment.

8.3. 1 IL-10 production by T cells after testosterone and DHT treatment.



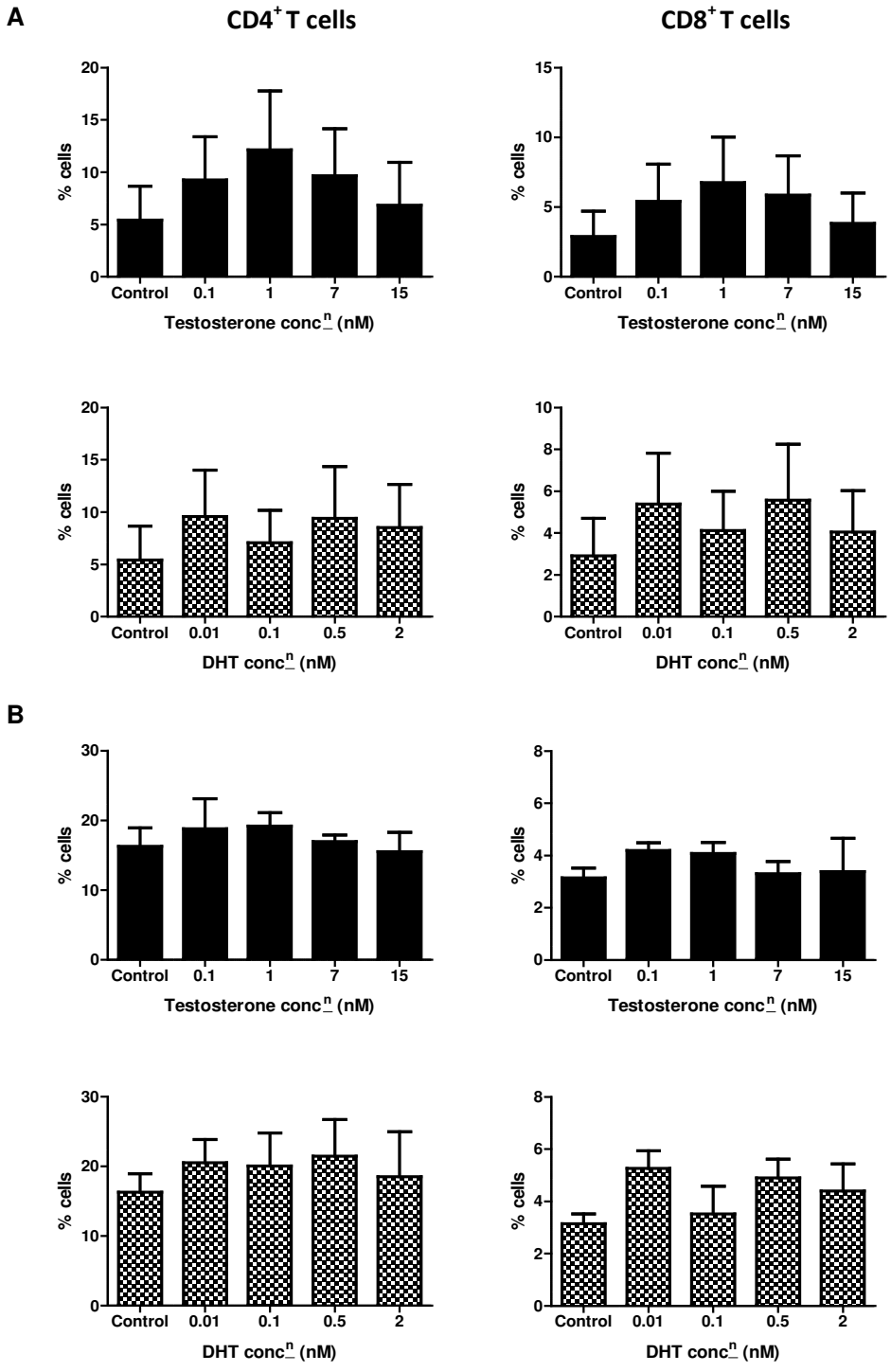
PBMCs were stimulated with anti CD3 and anti CD28 activation beads and cultured for 4 hours with different concentrations of testosterone (black bars) and DHT (checkered bars) in the presence of brefeldin A to measure the proportion of IL-10 producing T cells from **A**) females (n=3) and **B**) males (n=3) using flow cytometry. Error bars represent SEM.

8.3. 2 IL-17 production by T cells after testosterone and DHT treatment.



PBMCs were stimulated with anti CD3 and anti CD28 activation beads and cultured for 4 hours with different concentrations of testosterone (black bars) and DHT (checkered bars) in the presence of brefeldin A to measure the proportion of IL-17 producing T cells from **A**) females (n=3) and **B**) males (n=3) using flow cytometry. Error bars represent SEM.

8.3. 3 TNF-α production by T cells after testosterone and DHT treatment.



PBMCs were stimulated with anti CD3 and anti CD28 activation beads and cultured for 4 hours with different concentrations of testosterone (black bars) and DHT (checkered bars) in the presence of brefeldin A to measure the proportion of TNF-α producing T cells from **A**) females (n=3) and **B**) males (n=3) using flow cytometry. Error bars represent SEM