Sex steroid hormones

– roles in adaptive immunity and vascular pathology

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"There is nothing like looking, if you want to find something. You certainly usually find something, if you look, but it is not always quite the something you were after."

J.R.R. Tolkien

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ABSTRACT

The prevalence of autoimmune diseases is higher in women than men, while for cardiovascular disease, there is a male predominance. The sexual dimorphism of autoimmune and cardiovascular diseases probably relates to a number of factors, e.g. difference in exposure to risk factors and response to therapy, together with the effects of sex steroid hormones on disease pathophysiology. The sex difference and the effect of sex steroid hormones sometimes coincide while sometimes not: male sex and testosterone protect from autoimmune disease while male sex is considered a risk factor for CVD although testosterone is atheroprotective. Owing to this, it is important to in detail understand the targets and mechanisms for the effects of sex steroid hormones in vascular pathology and adaptive immunity. This thesis aimed to 1) determine the role of catechol-*O*-methyltransferase (COMT) for the vasculo-protective actions of estradiol, 2) determine the role of the androgen receptor (AR) in the atheroprotection actions of testosterone, 3) investigate the role of the AR in neointimal hyperplasia, 4) determine the mechanisms and target cells for AR-mediated regulation of B cell homeostasis, and 5) determine the mechanisms and target cells for AR-mediated regulation of T cell homeostasis in mice. Concluding the results in this thesis, we found that testosterone exerts its inhibitory effect on B lymphopoiesis in males by targeting the AR in osteoblasts while the thymic epithelial cells are a target for AR-mediated inhibition of T lymphopoiesis. A distinct regulation of peripheral B and T cell homeostasis may involve non-hematopoietic spleen cells and inhibition of B cell activating factor (BAFF) production. Moreover, testosterone exerts atheroprotection through AR-dependent as well as AR-independent pathways. The AR also mediates protection from neointimal hyperplasia as a response to vascular injury, possibly through regulation of endothelial nitric oxide production leading to reduced proliferatory capacity of vascular smooth muscle cells. Lastly, the COMT enzyme is dispensable for vascular protection by estradiol *in vivo*. Although the conclusions in this thesis increase our understanding of the role of sex steroid hormones in adaptive immunity and vascular pathology, they also raise new questions that warrant further investigation.

Keywords: COMT, androgen receptor, testosterone **ISBN:** 978-91-628-8825-1

SAMMANFATTNING PÅ SVENSKA

Prevalensen, dvs. hur stor andel av en befolkning som är drabbade av sjukdom, för autoimmun sjukdom (t.ex. reumatiska sjukdomar) är högre hos kvinnor än hos män medan för kardiovaskulär sjukdom (t.ex. åderförkalkning och hjärtinfarkt) gäller mottsatsen, prevalensen är högre hos män. Skillnaden i utveckling och förlopp för autoimmun och kardiovaskulär sjukdom mellan män och kvinnor beror troligtvis inte enbart på effekten av könshormoner, utan en rad faktorer kan påverka sjukdomsuppkomst och förlopp, så som exponering för riskfaktorer och terapisvar, som också kan skilja mellan könen. Könsskillnaden i prevalens och effekten av könshormoner sammanfaller ibland men inte alltid; manligt kön och testosteron skyddar mot autoimmun sjukdom men för kardiovaskulär sjukdom anses manligt kön vara en riskfaktor till skillnad från testosteron som visats vara skyddande.

I denna avhandling har effekterna av testosteron och estradiol, det viktigaste ”manliga” respektive ”kvinnliga” könshormonet, undersökts för att öka förståelsen för hur dessa hormon påverkar uppkomsten av ateroskleros, dvs. åderförkalkning, och neointima bildning, dvs. den process där cellnybildning efter kärlskada ökar tjockleken på kärlet. Vi har också undersökt hur testosteron kan reglera det adaptiva (specifika) immunförsvarets celler, dvs. antalet B- och T-lymfocyter.

Först undersöktes om ett enzym kallat katekol-*O*-metyltransferas (COMT) påverkar effektiviteten av den kärlskyddande effekten av östrogen. COMT bidrar till nedbrytningen av estradiol i kroppen och bildar 2-metoxyestradiol, en estradiol-metabolit (nedbrytningsprodukt), som har visats ha kärlskyddande effekter i experimentella modeller för åderförkalkning och kärlskada. Vi kunde med hjälp av möss som saknar genen för COMT, och alltså inte kan bilda 2-metoxyestradiol, visa att denna metabolit inte är nödvändig för den skyddande effekten av estradiol på blodkärlen.

Sedan undersöktes hur androgenreceptorn (dvs. mottagarmolekylen för testosteron) påverkar utvecklingen av åderförkalkning och kärlskada. Vi kunde visa i möss som saknar genen för androgenreceptorn att den kärlskyddande effekten av testosteron delvis går via androgenreceptorn men också via andra vägar. Androgenreceptorn är också viktig för att skydda mot den cellnybildning som sker i kärlet efter kärlskada. Testosteron påverkar produktionen av ett enzym som är viktigt för att producera kväveoxid i endotelet, det innersta lagret i kärlväggen. Kväveoxid kan i sin tur minska delningskapaciteten i glatta muskelceller, de celler som utgör cell-nybildningen i kärlet.

Till sist så undersöktes hur testosteron, via androgenreceptorn, påverkar B- och T-cellantal. Med hjälp av möss som saknar androgenreceptorn enbart i en viss cell kunde vi visa att androgenreceptorn i osteoblaster (de celler som bildar ben) reglerar B-lymfopoes, dvs. bildningen av nya B-celler. Androgenreceptorn i tymus-epitelceller, celler som bygger upp tymus som är det organ där T-lymfopoes sker, reglerar nybildningen av T-celler. Trots stora effekter på antalet nybildade B-

respektive T-celler i dessa möss så påverkades inte de perifera B- och T-cellantalen, dvs. antalet B- och T-celler i resten av kroppen. Det perifera cellantalet tros i stället vara beroende av produktion av en överlevnadsfaktor, BAFF, i mjälten. BAFF ökar vid testosteronbrist samt om androgenreceptorn saknas. Framtida studier behövs för att visa att testosteron hämmar B- och T-cellantal via sänkt produktion av BAFF.

Forskning på underliggande mekanismer och målceller för effekten av könshormoner är viktig ur många aspekter. Det ökar vår förståelse för könshormonsbiologi ur ett grundforskningsperspektiv men det har också viktig klinisk betydelse:

1) Testosteronbehandling till äldre män har fått mycket uppmärksamhet de senaste åren och ökar stadigt. Behandlingsmöjligheter som minskar risken för biverkningar är mycket efterfrågade och SARMs, selektiva androgenreceptormodulerare, öppnar upp för en cellspecifik behandling, dvs. att åstadkomma de goda effekterna av androgener i t.ex. skelett medan man undviker de dåliga effekterna i t.ex. prostata. Att hitta målcellen som är viktig för effekten av testosteron i kärl och för adaptivt immunförsvar är ett viktigt steg i att utveckla SARMs som har en kärlskyddande respektive bromsande effekt på autoimmunitet. Vidare så visar vi med denna forskning att androgenreceptorn utgör en ny terapeutisk möjlighet att hämma restenos efter kranskärlsinterventioner.

2) Läkemedel som hämmar BAFF (Belimumab®) är en ny behandlingsmöjlighet för autoimmun sjukdom. Eftersom testosteronbrist kan öka risken för autoimmun sjukdom och BAFF-hämmare minskar autoimmunitet, öppnar vårt fynd att BAFF är reglerat av testosteron upp för att även behandla autoimmun sjukdom med en SARM riktad mot den cell i mjälten som producerar BAFF. Vidare så kan våra data tyda på att män med autoimmun sjukdom och låga testosteronnivåer skulle kunna ha särskild nytta av BAFF-hämmare.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals (I–V).

I: Anna S. Wilhelmson, Johan Bourghardt-Fagman, Joseph A. Gogos, Per Fogelstrand, and Åsa Tivesten
Catechol-O-Methyltransferase Is Dispensable for Vascular Protection by Estradiol in Mouse Models of Atherosclerosis and Neointima Formation
Endocrinology 152: 4683–4690, 2011

II: Johan Bourghardt, Anna S. Wilhelmson, Camilla Alexanderson, Karel De Gendt, Guido Verhoeven, Alexandra Krettek, Claes Ohlsson, and Åsa Tivesten
Androgen Receptor-Dependent and Independent Atheroprotection by Testosterone in Male Mice
Endocrinology 151: 5428–5437, 2010

III: Anna S. Wilhelmson, Johan Bourghardt-Fagman, Inger Johansson, Maria E. Johansson, Per Lindahl, Karel De Gendt, Guido Verhoeven, Per Fogelstrand, and Åsa Tivesten
Increased Neointimal Hyperplasia Following Vascular Injury in Male Androgen Receptor Knockout Mice
In manuscript

IV: Anna S. Wilhelmson, Alexandra Stubelius, Johan Bourghardt-Fagman, Anna Stern, Stephen Malin, Lill Mårtensson-Bopp, Mikael C. Karlsson, Hans Carlsten, and Åsa Tivesten
Testosterone Regulates B cell Homeostasis by Targeting Osteoblasts in Bone and the Survival Factor BAFF in Spleen
In manuscript

V: Anna S. Wilhelmson, Alexandra Stubelius, Johan Bourghardt-Fagman, Ulrika Islander, Hans Carlsten, and Åsa Tivesten
Increased T Lymphopoiesis but Unchanged Peripheral T cell Number Following Depletion of the Androgen Receptor in Thymus Epithelial Cells
In manuscript

CONTENT

ABBREVIATIONS

2-ME2	2-Methoxy-Estradiol
2-HE2	2-Hydroxy-Estradiol
APCs	Antigen-Presenting Cells
ApoE	Apolipoprotein E
AR	Androgen Receptor
ARKO	Androgen Receptor Knockout
BAFF	B cell Activation Factor
BCR	B Cell Receptor
COMT	Catechol-*O*-Methyltransferase
CVD	Cardiovascular Disease
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
eNOS	endothelial Nitric Oxide Synthase
ER	Estrogen Receptor
FDc	Follicular Dendritic cell
FO	Follicular
I/M	Intima to Media
IEL	Internal Elastic Lamina
IFNγ	Interferon gamma
HDL	High-Density Lipoprotein
HRT	Hormone Replacement Therapy
LDL	Low-Density Lipoprotein
LDLR	Low-Density Lipoprotein Receptor
LPS	Lipopolysaccharide
MHC	Major Histocompatibility Complex
MZ	Marginal Zone
NO	Nitric Oxide
ORX	Orchiectomy
OVX	Ovariectomy
RA	Rheumatoid Arthritis
SARMs	Selective Androgen Receptor Modulators
SHBG	Sex Hormone Binding Globulin
SLE	Systemic Lupus Erythematosus
TCR	T Cell Receptor
TECs	Thymic Epithelial Cells
Tfm	Testicular feminization
TNFα	Tumor Necrosis Factor alpha
VCAM-1	Vascular Cell Adhesion Molecule-1
VLDL	Very Low-Density Lipoprotein
VSMC	Vascular Smooth Muscle cell
WT	Wild-Type

1 INTRODUCTION

This thesis discusses the androgen receptor (AR)-mediated effects of androgens in adaptive immunity and vascular pathology as well as the catechol-*O*-methyltransferase (COMT)-mediated effects of estrogens in vascular pathology. Here is an introduction to the topics sex steroids, adaptive immunity, and cardiovascular disease, followed by a brief presentation of the gaps in knowledge which this thesis attempts to address.

1.1 Sex steroid hormones

Sex steroid hormones are produced in the gonads: the testes in men and the ovaries in women. In humans, as opposed to rodents (e.g. mice and rats), sex steroid hormones are also produced from sex steroid precursors which origin from the adrenal cortex[1]. Sex steroid hormones include androgens, estrogens, and progesterone. In this thesis the focus lies on the effects of androgens and estrogens.

1.1.1 Androgens and the androgen receptor

In males, testosterone, the main androgen, is mainly synthetized in the Leydig cells in testes. In the circulation, testosterone is to a large extent bound (≈98%) to albumin or sex hormone binding globulin (SHBG) with only a small fraction being free (≈2%). Testosterone levels in males are high during three phases of life; during fetal development, shortly after birth, and from puberty throughout adulthood. Testosterone is necessary to promote development of male reproductive organs and for reproduction. Testosterone levels in men peak at around twenty to thirty years of age and then begin to decline slowly with age[2], a phenomenon popularly referred to as the "andropause". In females, androgens are produced mainly by the ovaries and testosterone is the most important androgen also in females, although the levels are ≈10% of those in men[3].

Androgens mediate their effect mainly through the androgen receptor (AR). The AR can be stimulated either directly by testosterone or by the locally produced testosterone metabolite 5α-dihydrotestosterone via the enzyme 5α-reductase[4]. 5α-dihydrotestosterone is not present in high levels in circulation, but is in many tissues the main source of androgenic stimulation since it is

the most potent androgen, with two- to threefold higher affinity than testosterone for the AR. Testosterone can also be converted to estradiol (via the enzyme aromatase) that provides an alternative pathway for the effects of testosterone through activation of the estrogen receptors (ERs)[5]. Testosterone may also have effects independent of the classical sex steroid receptors[6] (Figure 1).

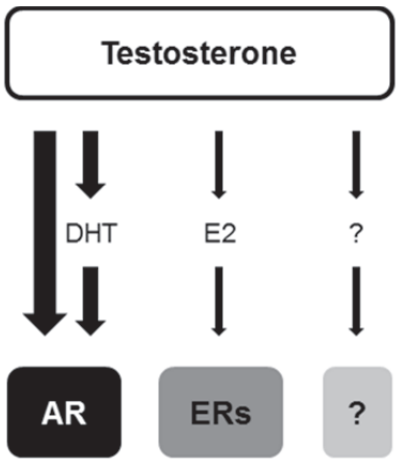

Figure 1. *Pathways for the actions of testosterone. DHT=dihydrotestosterone, E2=estradiol, AR=androgen receptor, ERs=estrogen receptors*

The AR is a 110 kDa nuclear protein consisting of a DNA-binding domain and a ligand-binding domain and belongs to the nuclear receptor super-family together with receptors for other steroid hormones. Androgen binding induces allosteric change, allowing the androgen/AR complex to enter into the nucleus and affect gene transcription.[7] In addition to genomic effects, sex steroids can also induce rapid non-genomic effects involving activation of signal cascades. Non-genomic effects of androgens are suggested to affect membrane flexibility, changes in intracellular calcium, or activation of second messengers either by membrane-bound AR or yet unidentified receptor(s)[8-10].

The AR is ubiquitously expressed and androgens affect most organs/tissues in the body[6,11]. Androgens have many physiological effects such as regulation of reproduction, muscle and bone mass, and distribution of body fat. Androgen deficiency results in reduced muscle and bone mass and sexual dysfunction etc. Androgen deficiency can be congenital (e.g. Klinefelter syndrome 47XXY), acquired (e.g. brain injury), or idiopathic. Further, low

androgen levels in men are associated with obesity, the metabolic syndrome, and cardiovascular disease (CVD), among others[12-15].

There is a polymorphic region in the AR gene where trinucleotide repeats (i.e. CAG- and GGN-repeats) can be of different length, which influences the transactivation function of the AR and/or the AR expression and the testosterone levels[16-23]. Studies have shown conflicting data on whether increased length is associated with lower AR activity or not[24], but an experimental mouse model with long CAG-repeat replicates the phenotype seen in humans and a very long sequence of CAG-repeats can lead to mild androgen insensitivity syndrome[25]. Other AR mutations can lead to androgen insensitivity syndrome which results in a partial or complete inability of the cells/tissues to respond to androgens via the AR, leading to impairment or prevention of development of male genitalia, as well as the development of male secondary sexual characteristics at puberty.[26,27]

Animal models of androgen insensitivity are useful tools for dissecting the role of AR in physiology and pathophysiology. The testicular feminization (Tfm) mouse[28] have a single nucleotide deletion in exon 2 of the AR gene leading to a truncated, non-functional AR protein[29]. The Tfm mice are infertile and their testes are small and located intra-abdominally. Besides the Tfm mouse, several AR knockout (ARKO) mouse models have been developed[30]. The phenotype of male ARKO mice is similar to the Tfm mice, with female-like external reproductive organs and small intra-abdominal testes. Further, these mice also have very low testosterone levels. The generation of ARKO mice uses Cre-loxP technology: Cre transgenic mice, expressing Cre recombinase either ubiquitously (for general (G)-ARKO) or in certain cell types (for cell-specific ARKO) are bred with mice where the AR is flanked by LoxP sites (Ar^{flox}). In the mice that inherit both the Cre construct and AR^{flox}, the Cre recombinase cuts out the sequence surrounded by LoxP sites, in our case exon 2 of the AR gene, generating a knockout of AR as a stop codon is introduced.[31] This technique enables the generation of not only G-ARKO mice but also cell-specific ARKO mice that can increase our understanding of the target cells for androgen/AR actions. This approach has also created the possibility to generate ARKO females (otherwise not possible due to male infertility in Tfm mice and G-ARKO mice).

1.1.2 Estrogens and estrogen metabolism

In women, estrogen levels vary greatly over the menstrual cycle until menopause when serum estrogen levels fall below those found in men[32] . Estrogens affect reproduction and many physiological/pathophysiological processes. Estrogens mainly exert their effects through the ERs α and β.

Estradiol, the most important circulating estrogen[3], is metabolized into compounds that are eliminated by the kidneys or the liver. Metabolism of estradiol includes glucuronidation, sulfation, esterification, or O-methylation of estradiol or its hydroxylated metabolites. The hydroxylation of estradiol is mediated by several of the CYP450 enzymes, mainly in the liver but also locally in the tissues. Through the enzymes CYP1A1 and CYP1B1, estradiol is metabolized to catechol-estradiols (i.e. 2-hydroxyestradiol (2-HE2) and 4-HE2). The catechol-estradiols can be further metabolized by the enzyme catechol-*O*-methyltransferase (COMT) to 2-methoxyestradiol (2-ME2) and 4-ME2, respectively (Figure 2). [33] Estradiol metabolites can act through ER-dependent and ER-independent mechanisms, exerting estrogen-like or other biological effects, however 2-ME2 has been suggested to have low or no binding affinity for the ERs.[34-36]

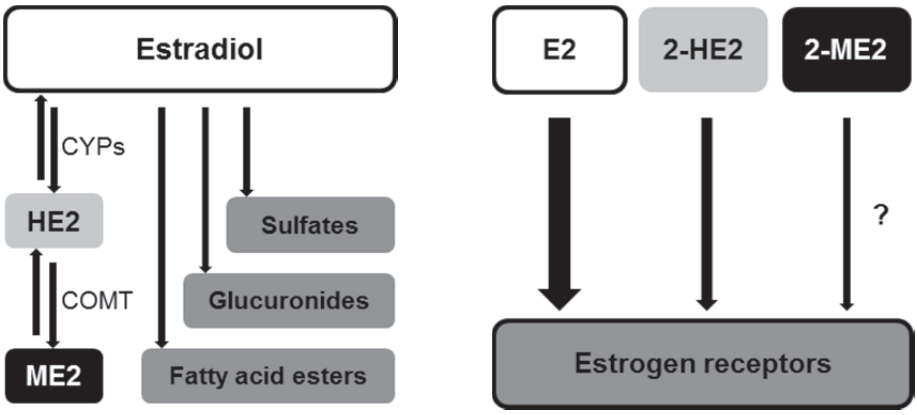

Figure 2. *Metabolism of estradiol and binding affinity of estradiol and its metabolites to the estrogen receptors. CYPs=cytochrome P450 enzymes, COMT=catechol-O-methyltransferase, HE2=hydroxyestradiol, ME2=methoxyestradiol, E2=estradiol, 2-HE2=2-hydroxyestradiol, 2-ME2=2-methoxyestradiol*

1.1.3 The mouse as an experimental model for human sex steroid biology

The mouse differs from a human with regard to sex steroid biology; firstly the mouse lacks the protein SHBG and sex hormones are bound only to albumin in plasma. This results in lower hormone levels but also a greater intra-individual variation compared to humans since SHBG prolongs the half-life of bound hormones. Secondly, the testosterone levels in males also depend on the rank in the hierarchy, where the dominant male has higher levels than the other males in a co-housed group[37]. Thirdly, the adult mouse does not produce the sex hormone precursor dehydroepiandrosterone (DHEA) from the adrenals. Consequently, removal of the gonads (i.e. testes in males and ovaries in females) renders the mouse completely androgen- and estrogen-deficient, and gonadectomy provides a simple tool for studies of the roles of endogenous sex steroids.[1]

1.2 The immune system

The immune system is the body's defense system; the cells of the immune system recognize non-self (i.e. pathogens, cancer cells, and altered molecules) and protects against infections, tumor development, and accumulation of potentially harmful substances. The immune system can be divided into innate (i.e. naive) and adaptive (i.e. acquired) immunity, where the innate immunity is traditionally viewed as the first line (hours) of defense against invading pathogens whereas the adaptive immunity is the second line (days) of defense with an action directed against a specific pathogen.[38]

The innate immune system includes phagocytes (e.g. dendritic cells and macrophages), the complement system, and natural killer cells which recognize structures that are shared by various classes of pathogens (i.e. pathogen-associated molecular patterns) for example lipopolysaccharide (LPS) or endotoxin present on bacteria and double-stranded RNA found in many viruses.[38]

The adaptive immune system includes B lymphocytes that produce antibodies and T lymphocytes that can be activated into effector T cells or helper T cells. The adaptive immune cells have a certain specificity generated during the lymphopoiesis due to rearrangement of the membrane-bound B cell receptors (BCRs, i.e. antibodies) on B cells, and the T cell receptors

(TCRs) on T cells. The antibodies recognize proteins, polysaccharides, lipids, and nucleic acids and the TCRs recognize small peptides displayed by major histocompatibility complex (MHC) on antigen presenting cells (APCs).[38]

1.2.1 T lymphopoiesis and T cells

T cells develop from lymphoid progenitors traveling from the bone marrow to the thymus, where the progenitors receive signals from surrounding cells, thymic epithelial cells (TECs) and APCs, which govern T lymphocyte development and survival. T lymphocytes develop through different precursor stages, double negative (DN, $CD4^-CD8^-$) 1 through 4, double positive (DP; $CD4^+CD8^+$), and then single positive (SP; $CD4^+$ or $CD8^+$) (Figure 3). In the periphery, e.g. spleen, lymph nodes, and circulation, T cells exists as SP cells: $CD4^+$ T cells, so called T helper cells, and $CD8^+$ T cells, so called cytotoxic T cells (Figure 3). The $CD4^+$ T helper cells can be further divided into Th1 cells that produce interferon-gamma (IFNγ) and thereby can activate macrophages, whereas Th2 cells secrete cytokines that stimulate B cells and their antibody production.[39-42]

In the thymus, two selection steps exist to ensure functional T cells; first, T cells are subjected to positive selection where recognition of the MHC molecules on APCs is tested. Second, auto-reactive T cells are negatively selected where APCs and TECs present self-antigens, and $CD4^+$ and $CD8^+$ T cells that recognize self-peptides displayed on MHC class II and MHC class I, respectively, become apoptotic and are sorted out. Positive selection occurs at the DP-stage and negative selection occurs during the transition between the DP- and SP-stage (Figure 3).

The thymus is largest and most active during the neonatal and pre-adolescent periods. At puberty the thymus begins to involute and the thymic stroma is replaced by adipose tissue. Nevertheless, residual T lymphopoiesis does continue throughout adult life. Thymic hyperplasia (due to low hormonal levels or tumor growth) is associated with autoimmune disease, e.g. myasthenia gravis[43,44], whereas loss of the thymus at early age through genetic mutation (i.e. DiGeorge Syndrome) results in severe immune-deficiency and high susceptibility to infections[45].

1.2.2 B lymphopoiesis and B cells

B cells develop from lymphoid progenitors in bone marrow where the progenitors receive regulatory signals from stromal cells, such as endothelial cells, reticular cells, and osteoblasts. Different stromal cells are known to affect different stages in the B lymphopoiesis, e.g. osteoblasts support pre-pro- to pro-B cell transition in early B lymphocyte development[46]. The B cells develop through different precursor stages, first in bone marrow where the B lymphocytes develop through pre-pro B cells, pro-B, and pre-B into immature B cells that then leaves the bone marrow (Figure 3). The immature B cells home to the spleen where immature transitional T1 and T2 B cells develop into the mature B cell subsets divided into follicular (FO), marginal zone (MZ), and B1 B cells (Figure 3), and then further into plasma cells producing antibodies (IgG). A peritoneal subset of B cells exists; these B1 B cells produce so called natural antibodies (i.e. IgM). B1 cells are thought to originate from the fetal liver and not from the bone marrow[47]. B cells produce antigen-specific antibodies, but they are also APCs presenting antigen to T cells and can affect other inflammatory cells by producing cytokines. B cells can be divided into effector B cells producing pro-inflammatory cytokines and regulatory B cells producing anti-inflammatory cytokines.[47,48]

As for T lymphocytes, checkpoints exist to select functional B cells; first in bone marrow, B cells that interact with self-antigens on bone marrow stromal cells and either change their specificity (i.e. receptor editing) or if this fails, go into apoptosis (i.e. negative selection). Positive selection of B cells occurs in the spleen where B cells with a functional B cell receptor (BCR) receive survival signals.[49-53]

One such survival signal is B cell activation factor (BAFF), affecting survival/proliferation of B cells in spleen[54]. BAFF knockout mice have no peripheral B cells, showing the non-redundant action of BAFF[54], while BAFF transgenic mice have increased B cell subsets in the spleen (T1, T2, MZ, FO, and B1)[55,56]. Thus, the peripheral B cell homeostasis is dependent on BAFF.

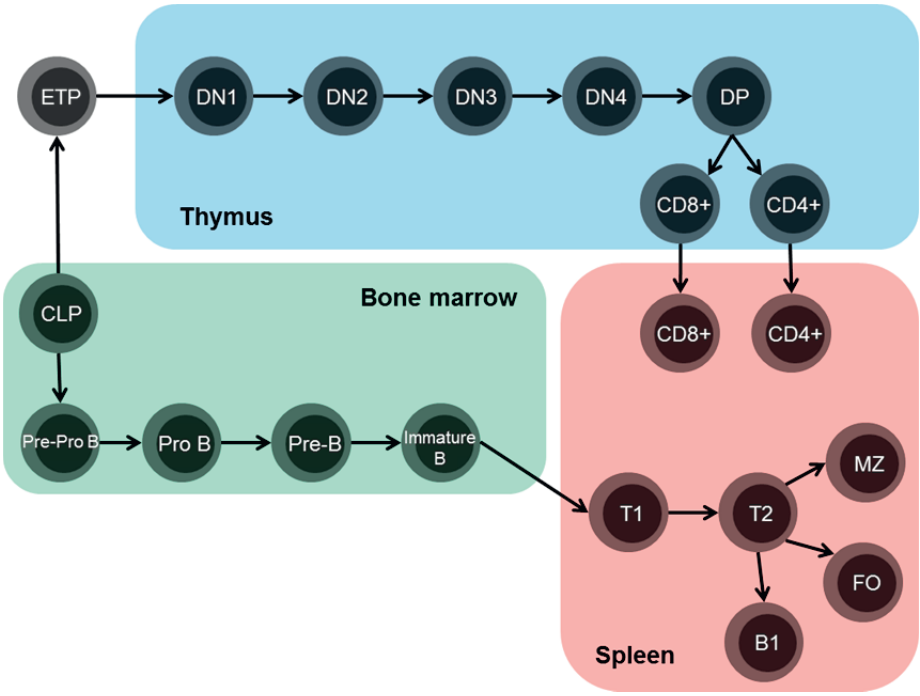

Figure 3. *Lymphopoiesis in bone marrow and thymus and mature B and T cells in spleen. CLP=common lymphoid progenitor, ETP=early thymic progenitor DN=double negative, DP=double positive, T=transitional, MZ=marginal zone, FO=follicular*

1.2.3 Tolerance and Autoimmunity

Autoimmune disease develops when the immune system starts to attack self-antigens and mounting an immune reaction against certain tissues/cells, i.e. a break in immunological tolerance when auto-reactive T or B lymphocytes escape negative selection. Immunological tolerance is divided into central or peripheral tolerance.[38] For T lymphocytes central tolerance is achieved in the thymus by negative selection (see section 1.2.1), but T cells are also subjected to peripheral tolerance; when the levels of co-stimulatory signals from other immune cells are low mature T cells that recognize antigens in peripheral tissues become anergic, leading to inactivation or apoptosis. Central tolerance for B cells is also achieved in the bone marrow (see section 1.2.2), and B cells are subjected to peripheral tolerance, where B cells that recognize self-antigens without T cell help, become anergic. Anergic B cells are subsequently excluded from the spleen follicles, thereby lack necessary survival signals, i.e. BAFF, and become apoptotic.[47,54,55,57-60]

BAFF is implicated in the development of autoimmune disease; excessive BAFF production in both humans and animal models has been associated with increased autoimmunity. A BAFF inhibitor (Belumimab®) is a newly approved drug for systemic lupus erythematosus (SLE) and is being tested in clinical trials for other autoimmune diseases.[55,59-62] High BAFF levels do not affect negative selection but can rescue anergic auto-reactive B cells and promote maturation into FO or MZ B cells. Conversely, BAFF inhibition preferentially depletes anergic auto-reactive compared to non-auto-reactive B cells.[47,57,58]

Defects in either checkpoint can lead to development of autoimmunity through improper survival of auto-reactive lymphocytes. These auto-reactive lymphocytes start to elicit an immune response to cells/tissues/molecules that are endogenous, i.e. collagen in rheumatoid arthritis (RA), acetylcholine receptors in myasthenia gravis, exocrine glands in Sjögren's syndrome, cell nuclei in scleroderma, and DNA in SLE, resulting in severe illness and disabilities.[38]

1.3 Cardiovascular disease

Cardiovascular disease (CVD) is an umbrella term for disorders of the heart and blood vessels including e.g. coronary heart disease, cerebrovascular disease, and peripheral arterial disease. CVD is the leading cause of death (≈30%) in the world and the main underlying cause of CVD is atherosclerosis[63], causing occlusion and thromboembolism. Risk factors for CVD can be divided into non-modifiable (i.e. age and genetic factors) and modifiable (i.e. smoking, diabetes, obesity, high serum cholesterol and/or triglyceride levels, high blood pressure, sedentary lifestyle, stress, and depression).

1.3.1 Atherosclerosis

Atherosclerosis is a chronic inflammatory disease[64-71], characterized by the formation of lesions/plaques in the arterial wall, which develop preferentially at sites with turbulent flow (i.e. branches, bifurcations, and curvatures)[72].

The initiation of atherosclerotic lesion formation is thought to involve retention of low-density lipoproteins (LDL) in the intima[73], the innermost layer of the vessel wall. The retained lipoproteins initiate an inflammatory response resulting in a vicious circle; in the inflamed intima, modification of LDL by oxidation[74] induces the endothelium to express adhesion molecules, causing leukocyte (e.g. monocyte and lymphocyte) infiltration[64,75]. Infiltrating monocytes are turned into macrophages by cytokines and growth factors produced in the inflamed intima and start to engulf oxidized LDL. These macrophages transform into foam cells and are now trapped inside the vessel wall. The macrophages/foam cells can activate T cells, continuing the inflammatory process as both macrophages and T cells produce pro-inflammatory cytokines such as IFNγ and tumor necrosis factor alpha (TNFα) leading to more inflammation and recruitment of more leukocytes. Together, the macrophages and T cells form fatty streaks, a precursor stage to more advanced lesions/plaques (Figure 4).[66,72]

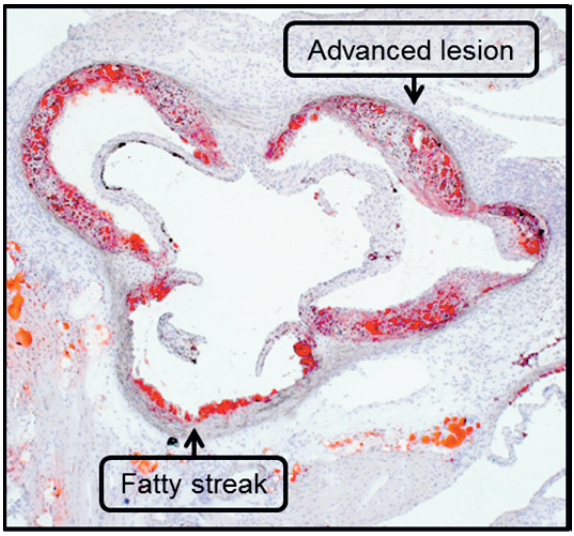

Figure 4. *Illustration of fatty streak vs. advanced lesion in the mouse aortic root. Red=Sudan IV staining of lipids.*

As fatty streaks grow into more advanced lesions, more and more lipids and inflammatory cells enter the vessel wall. Advanced lesions have a more complex composition; the core of the plaque consists of foam cells and extracellular lipid droplets covered with a fibrous cap of vascular smooth muscle cells (VSMCs) and a collagen-rich matrix.[76] Also other inflammatory cells, such as B cells, mast cells, and dendritic cells are present in the plaque.

Some plaques have a necrotic core and cholesterol may be deposited as cholesterol clefts.[72]

The human atherosclerotic lesions can be divided into stable and unstable plaques. Stable plaques are characterized by a thick fibrous cap, while in unstable plaques the inflammatory process has led to collagen degradation, thus weakening the fibrous cap and making it prone to rupture. As a plaque ruptures, the pro-thrombotic interior is exposed to the blood stream causing coagulation and thrombus formation.[72,77]

In the clinical setting, some atherosclerotic lesions cause stenosis of the vessels with ischemia as a result (i.e. angina pectoris), some plaques erode or rupture causing thrombus formation (i.e. myocardial infarction and stroke), whereas others remain non-symptomatic.

1.3.2 Adaptive immunity in atherosclerosis

Both T and B lymphocytes have been implicated in atherosclerosis development, with autoimmune-like responses playing a role in the progression of atherosclerotic lesions, independently of the serum lipid profile. The main auto-antigens that have been suggested as potential triggers of autoimmune responses in atherosclerosis are modified forms of LDL, heat shock proteins, and β_2-glycoprotein I (ApoH)[65,78,79]. T cells have long been known to support the inflammatory process in the lesions and help drive the plaque progression, while recent studies have shed new light on the role of B cells in atherosclerosis.[64-71,80]

T cells exist in plaques both in humans[81,82] and in mice[83] and depletion of T cells results in reduced lesion formation[84-87]. Published data strongly suggest that $CD4^+$ T cells aggravate atherosclerosis[88-90] while the role for $CD8^+$ T cells is less evident[91].

The role for B cells in atherogenesis is more complex; total B lymphocyte deficiency seems to aggravate atherosclerosis[92,93], while deletion of mature B cells[94] or adoptive transfer of different B cell subsets suggests that B1 B cell inhibit, whereas B2 B cells accelerate, disease progression[95]. This advises a different effect of B1 B cells producing natural antibodies compared to B2 (conventional B cells, i.e. mature B cells in spleen) B cells. Moreover, BAFF receptor deficiency, either general or B cell-specific, and BAFF depletion

both attenuate atherosclerosis[96-98]. The fact that BAFF supports survival of splenic but not peritoneal B1 cells[47] further strengthen the notion that B2 B cells are pro-atherogenic.

1.3.3 Mouse models of atherosclerosis

To investigate the pathogenesis of atherosclerosis, animal models are a useful tool. The mouse is commonly used to study atherosclerosis, however, atherosclerosis do not develop spontaneously in mice. In order to generate atherogenesis in mice, the animals need to be manipulated either with an inflammatory diet containing cholate (Paigen diet[99,100]) or with genetic alterations, i.e. knockout of genes involved in lipid metabolism[101]. Two such knockout models have become widely used: the LDL receptor-deficient ($LDLR^{-/-}$) and the apolipoprotein E-deficient ($ApoE^{-/-}$) mice.

$LDLR^{-/-}$ mice[102] develop insufficient hypercholesterolemia to generate atherosclerosis unless fed high-fat diet. The lesions develop throughout the aorta, with large lesions in the aortic root and the coronaries, although features of advanced lesions only exist after prolonged high-fat feeding. The $LDLR^{-/-}$ mice have a human-like lipid profile with most plasma cholesterol carried in LDL.

$ApoE^{-/-}$ mice[103] spontaneously develop hypercholesterolemia and atherosclerosis, with lesions forming throughout the aorta, and the innominate and coronary arteries. Progression of the lesions can be greatly accelerated by high-fat diet and lesions become complex with foam cells, necrotic cores, and fibrous caps (Figure 4). In contrast to humans, $ApoE^{-/-}$ mice have most cholesterol in plasma carried in very low density lipoprotein (VLDL)[101].

Of note, plaque rupture seldom occurs in the mouse models of atherosclerosis[104].

1.3.4 Neointimal hyperplasia

In humans, in contrast to rodents, atherogenesis has been shown to be triggered by a process called intimal thickening or neointimal hyperplasia[105,106], where a thickening of the intima occurs before any lipid or macrophage infiltration. The neointima is composed of vascular smooth muscle cells (VSMCs) and extracellular matrix and can retain lipids on

proteoglycans on the VSMC surface. Neointimal hyperplasia can also form as a response to vascular injury where revascularization procedures (e.g. stenting and bypass surgeries) can induce the phenomenon leading to restenosis of the vessel.

The biology of VSMCs plays an important role in the development of neointimal hyperplasia. The response of normally quiescent VSMCs to various stimuli results in proliferation and migration to the intima, leading to the formation of a VSMC-rich layer localized between the endothelium and the internal elastic lamina (i.e. the neointima). It is well recognized that the endothelium provides protective signals that maintain medial VSMCs in a quiescent state, and that nitric oxide (NO) is central in this context[107]. In the endothelium, NO production is regulated by endothelial nitric oxide synthase (eNOS), an enzyme producing NO from the amino acid L-arginine. NO is secreted from the endothelium and exerts effects on the VSMC layer (e.g. regulates vascular tone by inducing relaxation of the VSMC). NO is also important in keeping the VSMC in a quiescent, non-proliferatory state[108], an effect which is dependent on induction of cell cycle inhibitors, such as p21, p27, and p57[109-114] .

1.3.5 Mouse models of neointimal hyperplasia

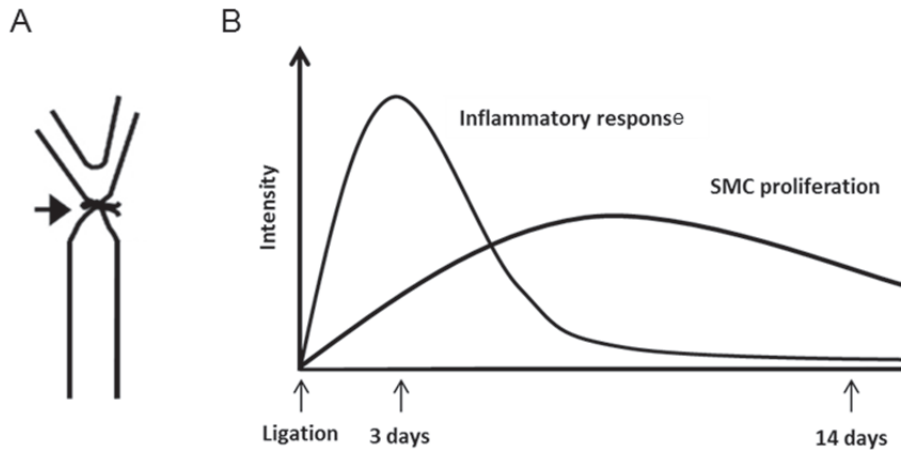

Figure 5. *A. Illustration of carotid ligation. B. Time course for neointimal hyperplasia in the model.*

Several mouse models have been described where either mechanical or electrical injury of a vessel, usually the carotid or femoral artery, is

conducted to induce an injury response in the vessel wall[115-119]. One such model that is commonly used is the carotid ligation model[120], where one of the common carotid arteries is ligated and as a response a neointima is formed. The ligation first induces a rapid inflammatory response peaking at around 3 days post injury followed by proliferation of VSMCs (Figure 5).

1.4 Sexual dimorphism in disease prevalence and actions of sex steroid hormones

The prevalence of autoimmune diseases is higher in women than men[121,122], with female sex being a risk factor for e.g. myasthenia gravis, RA, and SLE[121,122]. In RA for example, for every one man being affected, 2–3 women are disease-struck, while the numbers are 1:8–9 in SLE. On the other hand, for CVD there is a male predominance[6,11] at younger ages and women develop CVD approximately 10 years later in life compared to men.[123]

The discrepancy in the epidemiology, development, and outcomes of CVD and autoimmune disease between men and women suggests an intrinsic sexual dimorphism in susceptibility to the diseases. This dimorphism probably relates to a number of factors (e.g. differing exposure to risk factors and therapy etc.), together with the effects of sex steroid hormones. At a cellular level, there are fundamental differences between men and women that are a direct result of genetic differences due to the sexual genotype of each cell, either XX or XY.[122,124,125] Thus, it is important to separate the sex difference in prevalence/incidence of disease from the actions of the different hormones. The sex difference and hormonal effect sometimes coincide while sometimes not; male gender and testosterone protect from autoimmune disease while male gender is considered a risk factor for CVD but, nevertheless testosterone is atheroprotective in males.

1.4.1 Sex hormones and autoimmunity

Testosterone, has been suggested to protect against autoimmune disease, and androgen deficiency in men is associated with increased risk of autoimmunity[126,127]. Also in animal models of autoimmune disease, testosterone has been shown to have protective effects; orchiectomy (i.e. removal of testes and thereby all endogenous testosterone production in

rodents) exacerbates while testosterone treatment ameliorates disease[128,129]. In contrast to testosterone, estradiol has been ascribed an important role in accelerating autoimmune disease[130-132]. However, the mechanisms for the diverging effects of testosterone and estradiol in development and severity of autoimmune disease are not known. A plausible explanation for the protective effects of androgens on autoimmunity is the ability of androgens to lower B and T cell number. However, this warrants further investigation.

1.4.2 Sex hormones and CVD

The potential beneficial effects of estrogen on atherogenesis and CVD are controversial. Animal models of atherosclerosis have consistently reported an unequivocal atheroprotective effect in both males and females (via ERs)[133-143]. However, hormone replacement therapy (HRT) to women has shown both protective and adverse effects on CVD[144-150]. Revised studies show a protective effect in women with few menopausal years receiving HRT[151], giving rise to the "timing hypothesis" where estrogen is believed to exert protective effects in early disease progression but having adverse effects in advanced disease[152-154].

The vasculoprotective effects of estradiol have been extensively studied in animal models and ERα signaling is essential for the protective effect of estradiol on atherosclerosis and neointimal formation. Specifically, there is an important role for ERα in the endothelial cell in mediating these effects[140,141,143,152,155-160]. Further, estrogen metabolites, such as 2-ME2, have been suggested to mediate some of the effects of estradiol on the vasculature[161-163], e.g. through inhibition of VSMC proliferation, inhibition of angiogenesis, and inhibition of monocyte-adhesion[36,163-173]. These effects are thought to be mediated by the ability of 2-ME2 to inhibit hypoxia induced factor 1-alfa (HIF-1α)[36,165,172,174-176]. COMT-mediated production of 2-ME2 has been demonstrated to mediate the antimitogenic effect of estradiol *in vitro*[167,177]. However, whether 2-ME2 mediates protective actions of estradiol on vasculature *in vivo* is not known.

Despite a higher incidence of CVD in men compared to women, most evidence suggests that androgens protect from atherosclerotic disease in men[6,11]. Low serum testosterone generally associates with increased fat mass, an adverse metabolic risk profile, and increased atherosclerosis in men[2,12,178-180]. Furthermore, several prospective studies report associations between low

testosterone levels and cardiovascular events[13,14,181-183], a notion also supported by experimental data[135,184-189]. Hence, declining testosterone levels that accompany increasing age may adversely affect cardiovascular health[2].

Compared to estradiol, the role of testosterone in vasculoprotection has been less investigated. Two earlier studies addressed putative pathways for the atheroprotective effect of testosterone in male mice. One study found that an aromatase inhibitor blocked the effect of testosterone indirectly indicating that the AR pathway is of less importance[187]. A study of testosterone treatment to Tfm mice also indicated that the effects of testosterone on atherogenesis are independent of the AR[190]. However, since the latter study did not treat WT mice with testosterone, the relative importance of AR-dependent vs. AR-independent pathways could not be determined. Hence, no previous studies adequately address the role of the AR pathway in the effect of testosterone on atherosclerosis in mice. Furthermore, the importance of the AR in neointimal hyperplasia has not previously been evaluated, nor has any mechanisms for this effect of testosterone[191] been described.[192]

2 AIM

The general aim of this thesis was to evaluate the roles of sex steroid hormones in adaptive immunity and vascular pathology.

The specific aims of the five papers included in this thesis were:

I: To determine the role of COMT for the vasculoprotective actions of estradiol in male and female mice.

II: To determine the role of the AR in the atheroprotective actions of testosterone in male mice.

III: To investigate the role of the AR in neointimal hyperplasia in male mice.

IV: To determine the mechanisms and target cells for androgen/AR-mediated regulation of B cell homeostasis in male mice.

V: To determine the mechanisms and target cells for androgen/AR-mediated regulation of T cell homeostasis in male mice.

3 METHODOLOGICAL CONSIDERATIONS

The methods used in this thesis are described in detail in the Material and Methods sections of the individual papers, while a more general discussion of the methods included is presented here.

Animal models

Due to a resistance in wild-type (WT) mice to atherosclerosis development, mice were on ApoE$^{-/-}$ background (ApoE-M, C57/BL6, Taconic) for atherosclerosis evaluation (Papers I–III). Details about this model are presented in the introduction of this thesis (section 1.3.3).

In Paper I, we used COMTKO mice[193], in which the *COMT* gene is deleted. These mice lack the ability to perform *O*-methylation of catecholestrogens (and catecholamines) and therefore are 2-ME2-deficient.

In papers II–V, we used different ARKO mice models. We have generated general as well as cell-specific ARKO mice. ARKO mice were generated by breeding AR$^{+/flox}$ females with Cre^{+} males[31].

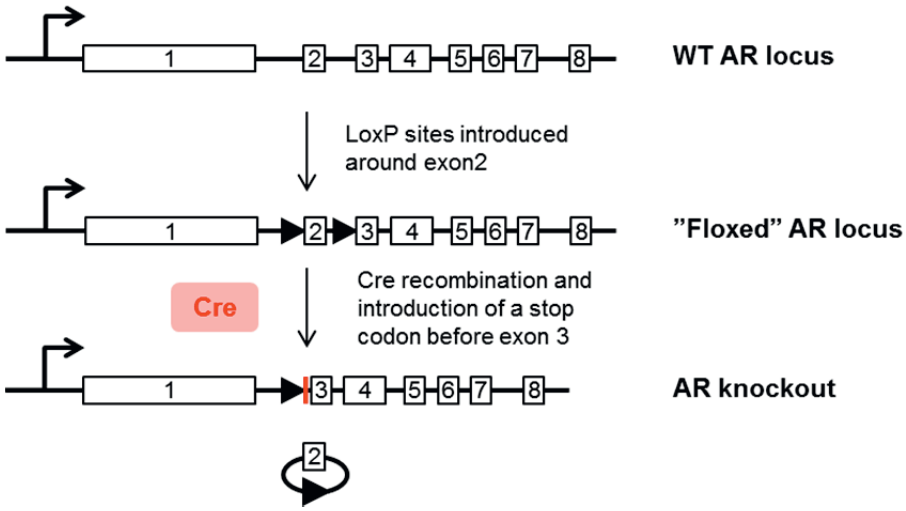

Figure 6. *Illustration of the androgen receptor gene and the generation of AR knockout alleles. WT=wild-type, AR=androgen receptor*

Different promoter sequences in the Cre constructs determine in which tissues/cells the Cre protein is expressed. The Pgk-Cre construct is expressed early during fetal development and generates a ubiquitous knockout of AR[31]. For the cell-specific ARKO we used mice with Cre under the control of different promoters; Osx-1-Cre (Jackson laboratory, Bar Harbor, Maine, USA) expressed in osteoprogenitors generating osteoblast-specific ARKO (O-ARKO), Mb1-Cre[194] expressed in B cells from the pro-B cell stage and CD19-Cre (Jackson laboratory) expressed from pre-B cell stage were used to generate B cell-specific ARKO (B-ARKO), LCK-Cre (Jackson laboratory) expressed in DN2 thymocytes was used to create T cell-specific ARKO (T-ARKO), and K5-Cre[195] expressed in keratinocytes was used to create epithelial-specific ARKO (E-ARKO). The efficacy and tissue/cell specificity of the cell-specific ARKO mice were assessed by quantifying exon 2, the floxed exon, compared to exon 3 in genomic (*g*)DNA (see RNA and DNA quantification).

To generate BM-derived cell-specific ARKO (BM-ARKO) in Papers IV and V, we used a transplantation approach where AR^- or AR^+ BM cells were transplanted into lethally irradiated WT mice. These mice were treated with broad-spectrum antibiotics to avoid infections, castrated and subsequently supplemented with testosterone (25 μg/day, see gonadectomy and hormonal treatment) to control for potential irradiation-induced androgen deficiency[196,197].

Diet

In order to avoid the potential effect of plant sterols, e.g. phytoestrogens, affecting the hormonal responses in the mice, all mice were fed a soy-free regular chow diet (R70; Lantmännen, Stockholm, Sweden or 2016; Harlan Teklad, Oxfordshire, UK) up to 8 weeks of age or until sacrifice. Mice in Papers I–II were fed an atherogenic diet (containing 21% fat from lard: 0.15% cholesterol - 821424; Special Diets Services, Essex, UK) from 8 weeks of age. The atherogenic diet increased serum total cholesterol, accelerating the atherosclerotic process and rendering the lesions more complex with necrotic cores, cholesterol clefts, and fibrous caps.

Gonadectomy and hormonal treatment

In all five papers gonadectomy, orchiectomy of males and ovariectomy of females, was used to eliminate endogenous sex steroid hormone production.

Gonadectomy renders the mice completely testosterone- and estrogen-deficient. For supplementation with testosterone or estradiol, the sex hormones were administered through subcutaneous slow-release pellets (Innovative Research of America, Sarasota, FL, USA) to assure steady testosterone or estradiol levels. The dosage of estradiol was based on previous published studies[140,162] to assure an atheroprotective level of the hormone; 6 μg/ day estradiol was used in Paper I. This dose is slightly supra-physiological in females, resulting in estradiol-related adverse effects such as uterine growth. In a pilot study, the dose of testosterone was evaluated; wet weights of prostate, seminal vesicles, and salivary glands in testosterone-treated orchiectomized males were assessed to evaluate a near-physiologic dose of testosterone where the weights were matched to sham-operated controls. A daily dose of 25 μg was chosen for the subsequent experiments.

Atherosclerosis evaluation

Atherosclerosis in mice is normally assessed *ex vivo* in aortas prepared *en face* or in sections of the aortic root. *En face* preparation of aortas includes fixation of the tissue in paraformaldehyde, dissection of adventitial fat and connective tissue, longitudinal incision from the aortic arch to the abdominal bifurcation, and pinning the vessel out flat. The lesion area is determined after Sudan IV staining of lipids and normalized to vessel size. This technique provides information about plaque burden and distribution throughout the aorta but does not allow determination of plaque composition. Cross-sections of the aortic root provide information on both lesion size and characteristics. Lipids can be quantified by Oil red O staining and plaque composition can be evaluated by chemical and immunohistochemical methods, e.g. Masson's trichrome staining for collagen content and presence of necrotic core and cholesterol clefts and Mac-2 immunostaining for macrophage content.

Neointimal hyperplasia evaluation

Neointimal hyperplasia was induced by carotid ligation and the vascular response was determined in cross-sections of the common carotid artery 3 days (Paper III), 2 weeks (Paper III), or 4 weeks (Paper I) after injury. ApoE-/- mice develop more severe neointimal hyperplasia after injury than WT mice[198,199]. Therefore, different time points were chosen for evaluating neointimal hyperplasia depending on ApoE-status (i.e. 2 weeks for $ApoE^{-/-}$

and 4 weeks for ApoE$^{+/+}$). Details about this model are presented in the introduction of this thesis (section 1.3.5).

The neointimal area was determined using autofluorescence of the elastic laminas. The area was normalized to the vessel size, i.e. to the length of the internal elastic lamina (IEL), or the media area where the intima–media (I/M) ratio was calculated. The composition of the neointima was evaluated by chemical and immunohistochemical methods, e.g. Masson's trichrome staining for collagen content as well as α-actin and Ki67 immunostaining, for VSMC content and for proliferating cells, respectively.

Evaluation of re-endothelialization

In paper III, the ability of the endothelium to regenerate was evaluated using an *in vivo* scraping injury model. In this model, the endothelial layer was removed in the common carotid artery using a thin wire, the blood flow was restored, and the endothelium was allowed to regenerate for 5 days. Unhealed vessel surface was stained using perfusion with Evan's blue, a dye which does not stain vessel segments with an intact endothelium.

Aortic explant culture

Ex vivo VSMC outgrowth from aortic tissue explants was used to study the proliferatory and/or migratory capacity of VSMC. VSMCs surrounding the tissue explant were counted after 9 days in culture using images captured in a phase-contrast microscope.

Phenotyping of the adaptive immune system

Flow cytometry was used to phenotype immune cells in Paper IV and V. The cells were labeled with fluorophore-conjugated antibodies and analyzed in a flow cytometer (FacsCantoII or Accuri, BD Bioscience). Cells from spleen, thymus, and BM were analyzed for their different lymphocyte subsets. The relative proportion of the different cells was determined using FlowJo software and the total numbers of the lymphocyte subsets were calculated from the total cellularity of the tissues/organs.

Serum measurements

The concentration of cytokines and growth factors in serum was quantified using commercially available enzyme-linked immune-sorbent assays

(ELISA). These were run either as single-analysis (i.e. BAFF) or as 7-plex panels (i.e. cytokines).

The serum concentrations of hormones were measured or assessed by different methods; testosterone and luteinizing hormone concentrations were determined using radioimmunoassays. Further, due to the low sensitivity of available mouse estradiol assays[200], the wet weight of the uterus was used as a sensitive marker of estradiol levels in mice[201].

Cholesterol and triglyceride concentrations were measured using chemo-luminescence and the distribution of lipids within the plasma lipoprotein fractions was assessed in pooled serum by fast-performance liquid chromatography gel filtration.

Lymphocyte proliferation

In Papers IV and V, proliferation of B and T cells was examined by *ex vivo* cultures of splenocytes stimulated with the lymphocyte mitogens LPS and concanavalin A, for B cell and T cell proliferation, respectively. Proliferation was measured by addition of ^{3}H-thymidine that was quantified in a β-counter and normalized to number of seeded T and B cells, respectively.

DNA and RNA quantification

Gene expression was evaluated in Papers II–V by real-time PCR (RT-PCR), which measures mRNA levels of certain gene transcripts. The method is based on the detection of *c*DNA sequences, generated from total RNA preparations by reverse transcription, using primers complementary to the cDNA sequence of interest. Amplification of a certain mRNA can then be correlated to an internal standard, i.e. a reference gene, giving an estimate of the relative expression of the gene of interest.

In this thesis we have developed a method to quantify the efficacy and specificity of the cell-specific knockouts. This was achieved by *g*DNA preparation from cells and tissues and quantification of exon 2 vs. exon 3 using primers specific for DNA sequences within the exons. The relative quantification was done using SYBR green, a molecule that fluoresces when bound to double stranded DNA.

Data generated using both methods were normalized to a reference gene/exon and were calculated using the $2^{-\Delta\Delta ct}$ method[202].

4 RESULTS AND CONCLUSIONS

Below is a brief description of the main results and conclusions of the five papers included in the thesis. For more details, see the full papers at the end of the thesis.

Paper I
Catechol-*O*-methyltransferase is dispensable for vascular protection by estradiol in mouse models of atherosclerosis and neointima formation

This study evaluated whether 2-ME2 mediates the vasculoprotective actions of estradiol *in vivo*. WT and COMTKO mice on an ApoE-deficient background were gonadectomized and treated with estradiol or placebo and atherosclerosis development was evaluated after 8 weeks of high-fat diet.

Exogenous estradiol reduced atherosclerotic lesion formation in both females (WT, -78%; COMTKO, -82%) and males (WT, -48%; COMTKO, -53%) and was equally effective in both genotypes. We further evaluated how exogenous estradiol affected neointima formation after ligation of the carotid artery in OVX female mice; estradiol reduced intimal hyperplasia to a similar extent in both WT (-80%) and COMTKO (-77%) mice. In ovarian intact female COMTKO mice, atherosclerosis was decreased (-25%) compared to WT controls.

We conclude that the COMT enzyme is dispensable for vascular protection by exogenous estradiol in experimental atherosclerosis and neointima formation *in vivo*. Instead, COMT deficiency in female mice with intact endogenous production of estradiol results in relative protection against atherosclerosis.

Paper II
Androgen receptor-dependent and independent atheroprotection by testosterone in male mice

In this study, we used ARKO mice on ApoE-deficient background to study the role of the AR in testosterone atheroprotection in male mice. Because ARKO mice are testosterone deficient, we sham-operated or orchiectomized the mice before puberty and orchiectomized mice were supplemented with placebo or a physiological testosterone dose.

In the aortic root, ARKO mice showed increased atherosclerotic lesion area (80%). Compared to placebo, testosterone reduced lesion area both in orchiectomized WT mice (50%) and ARKO mice (24%). However, lesion area was larger in testosterone-supplemented ARKO compared to testosterone-supplemented WT mice (57%). In WT mice, testosterone reduced the presence of a necrotic core in the plaque (80% among placebo-treated vs. 12% among testosterone-treated mice), whereas there was no significant effect in ARKO mice.

In conclusion, male ARKO mice on ApoE-deficient background display accelerated atherosclerosis. Testosterone treatment reduced atherosclerosis in both WT and ARKO mice. However, the effect on lesion area and complexity was more pronounced in WT than in ARKO mice, and the lesion area was larger in ARKO mice even after testosterone-supplementation. These results are consistent with an AR-dependent as well as an AR-independent component of testosterone atheroprotection in male mice.

Paper III
Increased neointimal hyperplasia following vascular injury in male androgen receptor knockout mice

In this study we evaluated neointimal hyperplasia development in male ARKO mice using a vascular injury model.

Two weeks after ligation of the carotid artery, ARKO mice showed increased neointimal area (+104%) and mean intimal thickness (intimal area normalized to vessel size; +56%) compared to WT controls. Following endothelial denudation by an *in vivo* scraping injury, there was no difference in the re-endothelialization in ARKO compared to WT mice. *Ex vivo*, we observed increased outgrowth of VSMCs from ARKO compared to WT aortic tissue explants; the number of outgrown cells was almost doubled (+96%) in ARKO. Analyzing central regulators of the cell cycle, we found that mRNA levels of the cell cycle inhibitor *p27* were down-regulated in uninjured arteries from ARKO mice, while *p21* and *p57* levels were unchanged. Further, arterial *eNOS* mRNA expression was reduced in ARKO mice. In accordance, testosterone supplementation to orchiectomized male mice increased *p27* and *eNOS* mRNA in uninjured artery in an AR-dependent manner.

In conclusion, male ARKO mice display increased neointimal hyperplasia as a response to vascular injury. The mechanism likely involves decreased endothelial nitric oxide production, leading to a down-regulation of p27 in VSMCs and thereby increased proliferative capacity of VSMCs.

Paper IV
Testosterone regulates B cell homeostasis by targeting osteoblasts in bone and the survival factor BAFF in spleen

In this study we elucidated the mechanism and target cells for androgenic regulation of B cell homeostasis. We utilized the ARKO mouse model to investigate AR-mediated effects of androgens on BM B lymphopoiesis and the peripheral B cell pool in male mice. General (G-ARKO) as well as osteoblast- (O-ARKO), B cell- (B-ARKO), and BM derived cell-specific (BM-ARKO) knockout of the AR were studied.

We show that G-ARKO leads to increased BM B lymphopoiesis from the pro-B cell stage and that O-ARKO mimics the increased B lymphopoiesis observed in G-ARKO mice. Further, the number of peripheral B cells in spleen was increased in G-ARKO mice, but not regulated in O-ARKO, B-ARKO, or BM-ARKO. G-ARKO, but not BM-ARKO, displayed increased serum levels of BAFF, and androgens/AR regulated splenic expression of BAFF.

We conclude that testosterone exerts its inhibitory effect on B lymphopoiesis in males by targeting the AR in osteoblasts. A distinct regulation of peripheral B cell homeostasis may involve non-hematopoietic spleen cells and inhibition of the production of BAFF.

Paper V
Increased T lymphopoiesis but unchanged peripheral T cell number following depletion of the androgen receptor in thymus epithelial cells

In this study, we elucidated the mechanism and target cells for androgenic regulation of T cell homeostasis. We utilized the androgen receptor (AR) knockout (ARKO) mouse model to investigate how the AR mediates the effects of androgens on T lymphopoiesis and the peripheral T cell pool in spleen, using general- (G-ARKO) as well as T cell- (T-ARKO), bone marrow

derived cell- (BM-ARKO), and epithelial cell- (E-ARKO) specific knockout of the AR.

We found that G-ARKO mice had increased T lymphopoiesis in thymus and increased peripheral T cell number in spleen. These effects were neither T cell- nor hematopoietic cell-intrinsic, since T- and BM-ARKO mice had unaltered T lymphopoiesis and/or thymus weight and peripheral T cell number. Further, removal of endogenous androgens by orchiectomy increased thymic expression of *Ccl25* and *Dll4*, important factors for T lymphopoiesis secreted by thymic epithelial cells (TECs). In line with an important role for TECs, E-ARKO mice had increased T lymphopoiesis in thymus. However, there was no change in the peripheral T cell number in the spleen of E-ARKO mice.

In conclusion, the TECs are a target for androgen/AR-mediated inhibition of T lymphopoiesis, possibly by inhibition of Ccl25 and Dll4 expression. However, inactivation of the AR neither in TECs, nor in T or BM-derived cells, alters the splenic T cell pool, suggesting a different, non-hematopoietic, androgen/AR target cell for the regulation of peripheral T cell homeostasis.

5 DISCUSSION

5.1 Estradiol, COMT, and vascular pathology

Estrogens have consistently been shown to inhibit atherosclerosis progression in rodents[134-137,139], chiefly through the ERs (mainly ERα)[140,143,156,159]. This is an effect that we clearly could replicate in Paper I, where exogenous estradiol-treatment reduced atherosclerotic lesion development by ≈80% in female and ≈50% in male mice. Estrogen has also been consistently reported to protect from vascular injury[138,141,155,203-212]. We were also able to replicate this effect in female mice where estradiol-treatment lowered the intimal area by ≈80% and the I/M ratio by ≈70%.

2-ME2 has been suggested to mediate the atheroprotective effects of estradiol in VSMCs *in vitro* [164,167,177,213] and earlier studies have ascribed several cardiovascular protective actions to 2-ME2, including inhibition of VSMC proliferation and extracellular matrix deposition, improved endothelial function, and decreased cholesterol levels[161,166,214]. Further, previous studies have demonstrated that administration of 2-ME2 protects against atherosclerosis development[162] as well as neointima formation and vascular remodeling[168,215]. However, the results in Paper I show that the vasculoprotective actions of estradiol *in vivo* occur independently of COMT-mediated 2-ME2 production. This finding is in line with recent studies demonstrating that the vascular protective actions of exogenous estradiol on both atherosclerosis and neointima formation depend on the expression of ERα[141,143,156,159]. The latter findings support our conclusions given the low binding affinity of 2-ME2 for ERs[35,36]. Thus, while Zacharia et al.[167] found a COMT-dependent effect of estradiol on VSMC proliferation in COMTKO cells *in vitro*, we see no such effects on atherosclerosis and neointimal hyperplasia in COMTKO mice *in vivo*. Notably, atherosclerosis and neointima formation are complex processes, involving many different cell types such as endothelial and immune cells, in addition to smooth muscle cells. Hence, if COMT-dependent inhibition of VSMC proliferation does neither account for the atherosclerosis nor neointimal phenotype *in vivo*, this supports other target cells for vasculoprotection of estradiol e.g. immune cells

or endothelial cells[143,156,159], and/or other mechanisms, such as lowering of serum cholesterol and/or triglycerides (see Paper I).

While COMT deficiency did not affect estradiol atheroprotection, we found that COMT deficiency reduced atherosclerotic lesion development in ovarian-intact female mice. Judging by their increased uterine weight[201], COMTKO mice had slightly elevated estradiol levels which may result in a relative protection against atherosclerosis development. This finding is consistent with epidemiological data associating the low-activity COMT polymorphism with protection against CVD events[216,217].

5.2 Testosterone, AR, and atherosclerosis

Androgens have been consistently reported to inhibit atherosclerosis progression in rodents[135,184-186,189]. However, whether the effect of testosterone is AR-dependent or AR-independent have been unclear and not thoroughly addressed[187,188,190]. In Paper II, we stringently investigated the role of AR in protection from atherosclerosis by testosterone. We could clearly demonstrate that testosterone exerts its effect on atherosclerosis through both AR-dependent and AR-independent actions, with the AR-mediated protection being the major pathway.

Paper II is the first to specifically address the relative role of the AR in atheroprotection in mice. In a previous study published by Nettleship et al.[190], the authors showed that exogenous testosterone normalized fatty streak formation in AR-mutant Tfm mice fed a cholate-containing diet. They suggested that this finding indicated that most of the effect of testosterone is AR-independent. However, they only treated Tfm and not WT controls with testosterone, and thus could not determine the relative importance of AR-dependent vs. AR-independent pathways. Other studies have been published with results in line with ours; treatment with the AR blocker flutamide inhibited most of the protective effect of testosterone on atherosclerotic plaque area in cholesterol-fed rabbits[188]. Further, the non-aromatizable AR agonist dihydrotestosterone (DHT) reduced atherosclerotic plaque area in the brachiocephalic artery of male ApoE-deficient mice[189].

Although our results from Paper II indicate that a large part of the effect of testosterone is AR-dependent, testosterone reduced atherosclerotic lesion area

in ARKO mice, showing that atheroprotection by testosterone also has an AR-independent component. Aromatization of testosterone to estradiol may mediate an AR-independent action of testosterone. Nathan et al.[187] found that an aromatase inhibitor completely blocked the effect of testosterone on atherosclerosis in LDLR-deficient mice, indicating that conversion to estradiol exerted the entire effect of testosterone. In comparison, Nettleship et al.[190] found that an aromatase inhibitor blocked only 20% of the effect of testosterone on fatty streak formation in Tfm mice, consistent with a relatively low level of aromatase expression in extragonadal tissues of mice[218]. In addition, it is conceivable that the higher testosterone dose used by Nathan et al.[187] (i.e. 5.6 times higher than ours) might have affected the relative importance of the aromatization pathway. In the study by Nettleship et al.[190], administration of testosterone to Tfm mice was done by intramuscular injections every two weeks, and although the authors claimed a physiological testosterone treatment, serum testosterone measurements showed that the treatment was intermittently supraphysiological. Hence, this treatment regimen, combined with the absence of testosterone treated WT controls, may have contributed to an overestimation of the role of AR-independent pathways. In addition to the estradiol/ER pathway there may be other AR-independent actions of testosterone[219].

5.2.1 Mechanisms for androgenic regulation of atherosclerosis?

Our results from paper II show that part of the atheroprotective effects of testosterone is mediated by the actions of AR; however, the exact mechanisms remain to be determined.

Increased atherosclerosis in mouse models might depend on traditional risk factors, such as high blood lipids, obesity, or insulin resistance. However, the male ARKO mice did not have any alterations of their blood lipid profile (paper II). While the ARKO mice have been shown by others to have late onset obesity[220], we did not observe any effect on body weight gain during high-fat diet-feeding and ARKO mice had lower body weight compared to WT after 8 weeks on high-fat diet in paper II. In a separate experiment (unpublished data), we evaluated lean vs. fat mass in the ARKO mice using dual-energy x-ray absorptiometry and we saw changes in lean/fat mass ratio due to lower lean body mass and higher fat mass in ARKO mice already at

young ages. These changes in body composition could lead to increased insulin resistance, but surprisingly ARKO mice did not have an altered glucose or insulin tolerance despite the increased relative fat mass. Thus, although the AR deficiency influences body composition, the blood lipid levels and insulin sensitivity are not adversely altered, suggesting other mechanisms for the increased atherogenesis in ARKO mice.

Besides metabolic parameters, vessel-intrinsic properties might affect atherogenesis. In Paper III, altered proliferatory/migratory capacity of ARKO VSMCs, is a possible mechanism for increased neointimal hyperplasia in ARKO mice. Given the association between intimal hyperplasia and atherogenesis (section 1.3.4), it is conceivable that these two endpoints share mechanisms such as an impact of androgens/AR on the endothelium[221], in parallel with the actions of estradiol/ERs[143,157,222]. Further, differential AR-mediated regulation of lesion area in different vessel segments, support that AR might regulate atherogenesis in a vessel-intrinsic manner; however this notion requires further investigation.

The immunomodulating capacity of androgens/AR is another strong candidate for regulating atherogenesis in the male ARKO mice. Even though the early inflammatory response in neointimal hyperplasia was not altered, the regulatory effects of androgens/AR on both B and T cells constitute plausible mechanisms for atherogenesis. An elevated adaptive immune response to modified LDL or other epitopes in the lesions could increase the vicious circle in plaque progression. We have data showing that ARKO mice have decreased IgM/IgG ratio of antibodies against modified LDL (i.e. ox-LDL and MDA-LDL; unpublished data), indicating that the ARKO mice have an increased immune responsiveness against modified LDL.

5.3 Androgens/AR and neointimal hyperplasia

In paper III, we evaluated the role of AR in neointimal formation, showing an important effect of AR in lowering neointimal hyperplasia to dimidiate levels. This result is consistent with previously published papers showing a protective effect of androgens on intimal hyperplasia[191]. However, we are the first to report increased neointimal hyperplasia in male ARKO mice. In accordance with our findings, Ikeda et al.[223] noted greater medial thickness

following angiotensin II administration to male ARKO mice, showing that the androgen/AR system exerts protective effects against angiotensin II-induced vascular remodeling. Tharp et al.[224] found that castration preceding balloon injury in male swine increased neointimal hyperplasia, suggesting that endogenous testosterone attenuates neointima formation. Thus, our results support and extend the results of previous *in vivo* studies suggesting an important modulatory role of androgens/AR on neointimal hyperplasia.

Furthermore, we evaluated the role of AR in re-endothelialization; we showed that AR does not affect the re-endothelialization in injured carotid arteries in male mice. Thus, in comparison with the increased re-endothelialization by estradiol/ERs[157,222,225,226], androgens/AR do not seem to affect the re-growth of the endothelium, but possibly affect the function of the endothelial cell.

In paper III, we showed that testosterone regulates *eNOS* expression in an AR-dependent manner, in line with previously published *in vitro* results on testosterone and nitric oxide (NO) production in endothelial cells[227]. Consistent with results in our study, testosterone treatment has been shown to ameliorate endothelial function[221], increase NO production and/or bioavailability partly through increased expression of NOS[228-234], both in hypogonadal men and in experimental models of androgen deficiency. In this regard, estradiol and testosterone seem to exert similar actions with both genomic (i.e. increase *eNOS* transcription) and non-genomic rapid effects (i.e. on NO production)[156,227,235].

In paper III, we found a reduction in the expression of the cell cycle inhibitor *p27* in uninjured arteries from ARKO mice, and testosterone increased vascular expression of *p27* in an AR-dependent manner. This result is in line with previous studies showing that p27 regulation by testosterone in VSMCs is associated with reduced proliferation *in vitro*[236] and p27 has been shown to reduce neointima formation *in vivo*[224,237]. p27 has a necessary and non-redundant role for the proliferative response of VSMCs [109,238,239] and studies show that endothelial NO production directly regulates *p27* expression in VSMCs[107,240]. Thus, an effect of testosterone on endothelial *eNOS* expression and thereby NO production would increase *p27* expression in VSMCs. This is a plausible mechanism for the findings in Paper III.

5.4 Sex steroid hormones in adaptive immunity

5.4.1 Androgen/AR targets for the regulation of B lymphopoiesis

In paper IV, we show that G-ARKO mice have increased B lymphopoiesis. This is consistent with previously published data that androgen/AR-deficiency increase BM B lymphopoiesis[241-246]. However, there has been conflicting data published regarding the target cell(s) for androgenic inhibition of B lymphopoiesis[245,246]. We could specify that AR in osteoblasts, i.e. in a non-hematopoietic cell, regulates B lymphopoiesis. The effect of O-ARKO on the number of immature B cells in BM was equivalent to that of G-ARKO, and these data are in line with studies demonstrating that osteoblasts support B lymphopoiesis from the pro-B cell stage[46,51-53,247,248]. However, despite increased B lymphopoiesis, AR-deficiency in osteoblasts did not alter the total peripheral B cell number. Our results in paper IV are in accordance with a paper in which targeting the chemokine CXCL12 in osteoblasts led to severely altered B lymphopoiesis in BM but nevertheless; peripheral B cell number remained unaffected[53]. Thus, we suggest that additional target cells and/or mechanism support the increased number of peripheral B cells in androgen/AR-deficient states.

5.4.2 Androgens/AR targets for the regulation of T lymphopoiesis

In paper V, we show that in both G- and E-ARKO mice all thymic T lymphocyte stages from DN through SP were increased, albeit with a different effect size. The smaller effect in E-ARKO vs. G-ARKO may be a result of an incomplete knockout of AR in E-ARKO mice or that additional AR target cells are important. Our data regarding the target cell for AR actions on T lymphopoiesis are in line previously published papers[249,250], showing a hematopoietic cell-extrinsic rather than cell-intrinsic AR-dependent inhibition of T lymphopoiesis. Further, we identified *Ccl25* and *Dll4* as androgen-regulated factors in the thymus. Both Ccl25 and Dll4 have been shown to have important functions in supporting T lymphopoiesis[39,42,251,252], indicating that an increase in these factors could increase T lymphopoiesis during androgen/AR-deficient states. Despite

increased thymic T lymphopoiesis in our E-ARKO mice, the peripheral T cell pool was unaltered. This suggests that other target cell(s) are important for androgenic regulation of the peripheral T cell pool size.

5.4.3 Androgen/AR target cells for the regulation of peripheral B and T cell number

In paper IV and V, we showed that G-ARKO mice have increased B and T cell number in spleen, consistent with previously published data[244,246,249]. Further, our results show that the inhibitory effect of testosterone on peripheral B and T cell number in male mice is AR-dependent.

Neither the peripheral B nor T cell pools were proportionally affected by AR depletion in osteoblasts and TECs, respectively. Since a previous study[246] proposed a cell-intrinsic effect of AR in regulating lymphocyte number, the peripheral B and T cell number was evaluated in B- and T-ARKO, respectively. However, in our studies we did not observe any cell-intrinsic effect of ARKO. We used both two different B cell-specific Cre-construct (Mb1-Cre and CD19-Cre expressed in early pro-B cells[194] and pre-B cells[253], respectively) and a T cell-specific Cre-construct (LCK-Cre expressed in DN2 T cell progenitors[254]) as well as BM-transplantation of AR-negative BM into WT recipients. All approaches lead to an efficient AR-knockout in B, T, and blood cells, respectively, but nevertheless we did not observe any change in peripheral lymphocyte number. Thus, androgens/AR regulate the mature B and T cell number by targeting a non-hematopoietic cell.

BAFF is a vital survival factor for peripheral B cells[54,56,255,256]. In Paper IV, we show that the circulating BAFF levels were increased and *Baff* mRNA was regulated in spleen in G-ARKO. Serum BAFF was not increased in BM-ARKO, in line with BAFF being regulated in non-hematopoietic, radiation-resistant, cells[255]. G-ARKO mice had increased splenic B cell subsets while peritoneal B1 B cells were not elevated, consistent with BAFF increasing survival of B cells in spleen while not affecting B1 B cells in peritoneum[47]. Further, our data also concur with the splenic pattern of B cell subsets in BAFF-overexpressing mice[47,54,56]. Thus, a change in BAFF homeostasis is a plausible explanation for the elevated number of peripheral B cells in G-ARKO.

BAFF also increases survival and/or proliferation of T cells[55,61,256-258]. Accordingly, BAFFKO mice have an altered peripheral T cell pool with lower numbers of T cells in spleen[61,259,260]. Further, BAFF transgenic[55] and BAFF-treated[258] mice have elevated $CD4^+$ T cell number in spleen, leading to a skewed CD4/CD8 ratio. Thus, the increased $CD4^+$ T cell number and skewed CD4/CD8 ratio of G-ARKO indicate that elevated BAFF levels might be a potential mechanism for both their increased peripheral T cell number, as well as B cell number.

5.4.4 Testosterone, estradiol, and BAFF

Estradiol suppresses B lymphopoiesis from early pre-B cell stage[261], thus, estradiol and testosterone both inhibit B lymphopoiesis. By contrast, estradiol has been shown to have a pro-survival effect on mature B cells by up-regulation of BAFF leading to increased relative number of mature B cells in spleen[130,131]. In accordance with the sex-biased prevalence of autoimmune diseases, estradiol has been proposed to accelerate and testosterone to inhibit autoimmunity[132]. Thus, estradiol elevates BAFF production, increases mature B cells, and accelerates autoimmune disease [126-132,241-246] while testosterone has the opposite actions[126-129,241-246]. Hence, it may be speculated that differential regulation of BAFF by estradiol and testosterone might partially explain the sex difference in autoimmune disease.

5.5 Indirect androgen/AR actions in adaptive immunity and vascular pathology

In papers III–V, we suggest the target cells for AR actions in adaptive immunity (i.e. osteoblasts, TECs, and non-hematopoietic spleen cells) and vascular pathology (i.e. endothelial cells) are affecting the phenotype of other cells (i.e. B and T lymphocytes and VSCMs, respectively). Thus, androgens/AR seems to affects the phenotypes in an indirect manner.

It is likely that these indirect actions of androgens/AR are mediated by secreted factors. For the indirect effect of endothelial cells on VSMCs the mechanisms may involve NO produced in endothelial cells. For lymphopoiesis the effect on B lymphopoiesis likely involves cytokines such as IL-7, SCF, or CXCL12[50,52,53] secreted form osteoblasts. The effect on T

lymphopoiesis may involve Dll4 and CCL25[42,252] production in TECs. For the splenic subsets of B and T cells, BAFF secreted by radiation-resistant, non-hematopoietic, spleen cells[255] potentially increases the number of peripheral B and T cells.

Thus, there is a striking concordance in the way androgens/AR regulate cell survival/proliferation of VSMCs and B and T cells, through inhibiting or increasing production of factors that in turn influence the effector cells. This aspect of androgenic activity has also been seen in other tissues/organs, such as prostate stromal cells – tumor cells[262], prostate stromal cells/smooth muscle cells – prostate epithelium[263-265], myeloid cells/sertoli cells – germ cells[31,266-268]. On the other hand, other effects of androgens/AR appear to be direct , such as the effects on adipocytes[269] and hepatocytes[270].

5.6 AR-dependent and AR–independent effects of testosterone

Testosterone can have both AR-dependent and AR-independent effects. In the papers included in this thesis (II–V) we have evaluated the relative importance of AR-dependent vs. AR-independent actions of testosterone in adaptive immunity and vascular pathology.

The effects of testosterone in regulation of peripheral B and T cell number are fully AR-dependent (Papers IV–V); testosterone lowers splenic B cells as well as CD4+ and $CD8^+$ T cells in WT mice, while testosterone did not affect the cell numbers in ARKO mice. The skewed CD4/CD8 ratio displayed the same pattern. Further, testosterone lowered *Baff* expression in spleen of WT but not ARKO mice. The relative importance of AR-dependent vs. AR-independent effect of testosterone on B and T lymphopoiesis was not determined in our studies; however, an AR-dependent thymic involution suggests that at least the effect on T lymphopoiesis would be AR-dependent. Thus, the effects of testosterone in adaptive immunity seem to be completely AR-dependent (Figure 6).

The relative importance of AR-dependent vs. AR-independent effects of testosterone in vascular pathology is more diverse. The effect on atherosclerosis (Paper II) was shown to be both AR-dependent as well as AR-independent. However, the AR-independent effect size varied in different

vessel segments. The relative AR-dependent and AR-independent effects of testosterone for inhibition of neointimal hyperplasia were not determined, but the putative mediators of the decreased proliferatory/migratory capacity of VSMCs (i.e. *eNOS* and *p27*) were inhibited by testosterone in an AR-dependent manner (Figure 6).

Taken together, the pattern of AR dependency indicate that the inhibitory effect of testosterone on B and T cell homeostasis may not be the sole mechanism for the atheroprotective effect, suggesting that additional mechanisms underlie the atheroprotective effects of testosterone. In accordance, the relative effect size of AR-dependent vs. AR-independent effects of testosterone on atherosclerosis in different vessel segments would suggest an important vessel-intrinsic, rather than a systemic, mechanism.

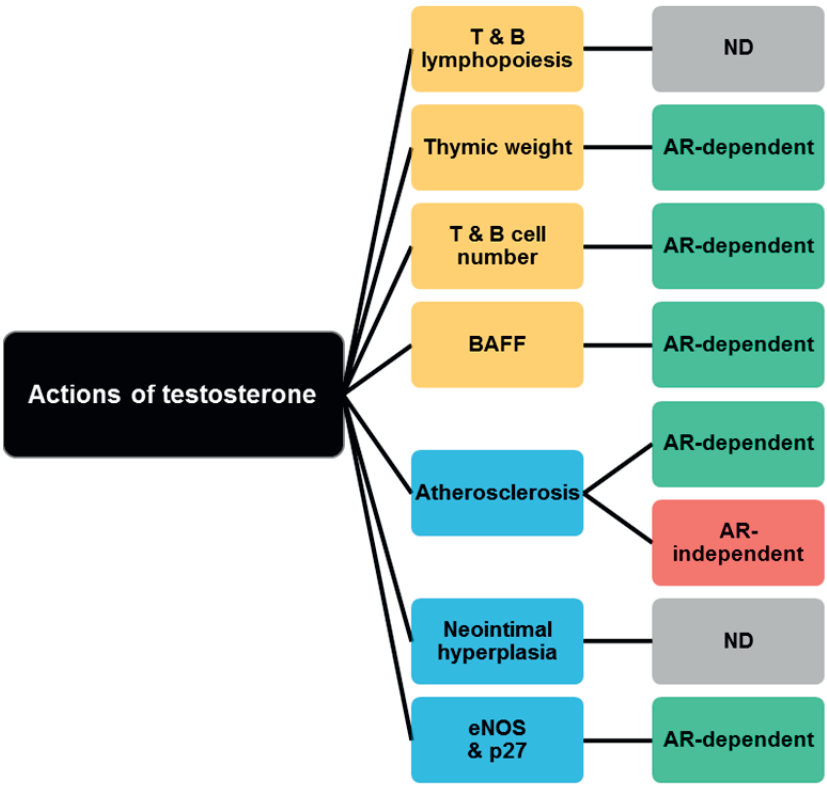

Figure 7. *AR-dependent vs. AR-independent effect of testosterone in adaptive immunity and vascular pathology. ND=not determined*

5.7 Clinical relevance

Gene knockout is an extreme manipulation of physiology where a gene is completely missing as compared with e.g. reduced function of a gene/protein, which would result in a less severe phenotype. In knockout mice the deletion of the gene is often produced early in development, which may cause adaptive responses. Further the expression of the Cre-recombinase construct both in general- or cell-specific knockouts may cause toxicity[271,272]. In this thesis results for general and cell-specific ARKO mice are presented, where one might argue that the ARKO mice may be an imperfect model due to both mentioned caveats. However the results are strengthened by data from orchiectomy, and thus removal of endogenous testosterone, experiments in WT mice that induce the same response/phenotype. In addition, we have thoroughly evaluated our control groups; e.g. both the LCK- and Mb1-Cre construct alone affected the lymphocyte number significantly compared to Cre-negative littermates, possibly due to toxicity of Cre or in Mb1-Cre^+ mice, a hemizygote knockout of the *CD79a* gene. Moreover, Osx1-cre^+ mice have a severe growth retardation phenotype[273] compared to Cre-negative littermates, in part due to malocclusion, an effect that we also clearly observed in our mice. Therefore having Cre^+ littermates as controls to cell-specific ARKO, was of great importance. Further, we see no effects of the AR^{flox} construct alone.

To further consolidate our findings, mutations in AR (i.e. CAG-repeat length or others) affecting AR functionality would then have consequences for autoimmune disease or CVD. Although conflicting data exist and few studies have examined autoimmune disease in relation to CAG-repeats in men, the length of CAG-repeats is associated with autoimmune diseases, such as RA[274-276] and SLE[277,278]. Moreover, chromosomal defects (i.e. Klinefelter syndrome 47XXY), affecting testosterone levels, have also been associated with autoimmunity, in particular with SLE but also with RA and myasthenia gravis[279-284]. Of note however, having an extra X chromosome might also influence autoimmunity since several risk genes[285] is located on the X chromosome and skewed X-inactivation is associated with autoimmune disease[286,287].

The length of the CAG-repeat polymorphism is inversely associated with CVD; long CAG-repeats are associated with increased risk for coronary

artery disease[288] and/or risk factors for CVD such as high cholesterol and the metabolic syndrome[289-292]. The Klinefelter syndrome is also associated with increased risk for CVD e.g. ischemic heart disease[293,294] and CVD risk factors such as insulin resistance, the metabolic syndrome, and increased carotid intima–media thickness[295].

Lastly, androgen deprivation therapy in treatment of prostate cancer is associated with an increased risk of autoimmunity[296,297] and CVD[298-300].

5.8 Clinical implications

The experimental results from this thesis may have consequences for future treatment options of autoimmune disease and CVD in the clinical setting.

Although the results in Paper I do not support endogenous 2-ME2 formation as a pathway for vascular protection by estradiol, exogenous 2-ME2 still may represent an effective and promising vascular protective agent at pharmacological doses[162,164,173,215].

Much interest has focused on testosterone supplementation to elderly men and the use of testosterone supplementation increases[6]. Compounds that activate or inhibit the AR in a tissue-specific way (selective androgen receptor modulators; SARMs) are receiving interest with the goal to achieve beneficial effects (e.g. on bone) and with no adverse effects (e.g. on the prostate)[301]. A crucial step for the design of SARMs with a beneficial cardiovascular and/or immunological profile will be the identification of the target cell(s) for the cardiovascular and immunological actions of androgens. From results in paper IV and V, we can identify important target cells for regulating central lymphopoiesis, i.e. osteoblasts and TECs, while we can narrow the search for a target cell regulating peripheral B and T cell homeostasis: i.e. a non-hematopoietic splenic stromal cell. Further research is needed to clarify which splenic cell subset that exerts the actions of androgens/AR, putatively, via lowered BAFF production. Also for the vasculoprotective effect of androgen/AR, future studies in which the AR is inactivated specifically in endothelial cells and VSMCs will be highly informative.

Our results suggest that the AR may represent a potential therapeutic target to limit neointimal hyperplasia and possibly clinical cardiovascular disease in

men. Since testosterone levels in men decline with age, and low testosterone levels have been implicated in CVD risk[14], androgen-deficient men might benefit from testosterone treatment. If testosterone supplementation is contraindicated, androgen-deficient (age-associate or induced, i.e. anti-androgen treatment in prostatic cancer patients) men might benefit from more aggressive treatment to reduce other CVD risk factors, such as to lower high blood pressure and serum cholesterol levels, and be encouraged to reduce lifestyle-associated risk factors, such as smoking, inactivity, unhealthy diet, etc.

From the results in paper IV, we can conclude that androgens/AR regulate BAFF, a pivotal survival factor for B cells and newly identified treatment target for autoimmune disease. BAFF is implicated in the development of autoimmunity where excessive BAFF production misrepresents B cell tolerance leading to increased survival of autoreactive B cells[57,58]. BAFF inhibition (Belimumab®) is a newly approved treatment for SLE and is in phase III clinical trials for other autoimmune diseases, such as RA and Sjögren's syndrome[59,60,302,303]. In line with our experimental data, BAFF has been shown to be negatively correlated to testosterone levels in psoriatic arthritis patients[304]. As BAFF is a successful therapeutic target for autoimmune disease, our findings open up a new potentially druggable mechanism for autoimmune disease where a SARM[305] directed to the spleen stromal cell compartment could lower BAFF levels with minimal adverse androgenic effects in other tissues. Further, as androgen deficiency leads to increased BAFF production, men with autoimmune disease and subnormal testosterone levels might particularly benefit from BAFF-inhibition.

6 FUTURE PERSPECTIVES

Although the conclusions from the papers included in this thesis increase our understanding of the role of sex steroid hormones in adaptive immunity and vascular pathology, they also raise new questions:

6.1 Target cells for the effects on peripheral B and T cell number?

For the immunological phenotype in androgen/AR deficiency, data from Paper IV and V show that the target cells for AR in lymphopoiesis include osteoblasts and TECs, while the target cell(s) responsible for the peripheral homeostasis of B and T cells remain(s) to be identified. We hypothesize that these target cells could be follicular dendritic cells (FDc) in spleen and therefore would be the target for androgens/AR responsible for BAFF production[255,306]. To test this hypothesis we have generated FDc-specific ARKO (FDc-ARKO) mice and the splenic B and T cell pool will shortly be analyzed in these mice.

6.2 Autoimmune disease in our models?

An important question to investigate is whether any of these cell-specific ARKO mice would develop autoimmune disease, since androgen/AR deficiency is known to increase autoimmune susceptibility[127-129,246,279,307-311]. For autoimmunity driven by B cells, O-ARKO would probably not increase autoimmune disease susceptibility as positive selection of B cells is achieved in the spleen. If FDc-ARKO will lead to increased BAFF production and, hence, an increased peripheral B cell pool, these mice would potentially be more prone to autoimmune disease. For T cell-driven autoimmunity, on the other hand, E-ARKO might be more susceptible to autoimmune disease development since both positive and negative selection occur within the thymus. Future studies using these cell-specific ARKO mice in models of RA, SLE, or other autoimmune diseases will be informative in finding the target cells for the protective effect of androgen/AR in autoimmune disease.

6.3 Atherosclerosis in our models?

Atherosclerosis is a chronic inflammatory disease and autoimmune disease is associated with increased risk of CVD events[65,312-316]. Therefore, it is not unlikely that the mechanism behind increased atherosclerosis development in ARKO partly depends on the heightened adaptive immune system in androgen/AR-deficient states. This notion warrants further investigation and studies are ongoing in our lab on atherosclerosis prone O- and E-ARKO mice. Also FDc-ARKO on an $ApoE^{-/-}$ background will be tested if this model shows elevated peripheral B and/or T homeostasis.

6.4 AR target cells in vasculature?

Additional experiments on both atherosclerosis and vascular injury in endothelial cell- and VSMC-specific ARKO mice would clarify the vessel-intrinsic mechanisms and target cells for the vasculoprotective effects of androgens/AR. In addition, co-culture of endothelial cells and VSMCs *in vitro* might provide mechanistic insight in the cross-talk between these cell types influenced by androgens. Ongoing studies in our lab are addressing both these hypotheses.

6.5 Increased atherosclerosis following preeclampsia in COMTKO females?

From the results in Paper I, we can conclude that endogenous production of 2-ME2 does not mediate the atheroprotective effects of estradiol in virgin mice. Yet, 2-ME2 seems important during pregnancy when levels are much increased due to placental induction of COMT[317-319]. Pregnant COMTKO females develop a preeclampsia-like state[318]; the mice display vascular defects in the placenta/decidua, with hyaline-like deposits containing foam cells in the vessel wall and thrombosis in the lumen as well as signs of endothelial damage. COMTKO mice receiving 2-ME2 showed restored placental health and reduced symptoms of preeclampsia. Thus, endogenous production of 2-ME2 via COMT likely plays an important role in maintaining vascular integrity during pregnancy. Notably, because only nulliparous/virgin female mice were included in Paper I, it cannot be excluded that COMTKO females would be prone to develop increased atherosclerosis and/or intimal hyperplasia after pregnancy, an important

notion to test since preeclampsia is associated with CVD events later in life[320,321]. Studies on atherogenesis in COMTKO female mice post pregnancy, and thus preeclampsia, would be informative in showing whether the association between preeclampsia and CVD is causative or solely a result of existence of common risk factors in these women[320].

6.6 The effects of sex vs. the effects of sex steroid hormones?

CVD and autoimmune disease both have a clear sexual dimorphism in spite of the fact that they are associated with each other and share many features (see section 1.3.2); male sex is a risk factor for CVD while female sex strongly associates with autoimmune disease. The long-term goal of the research on the effects of sex steroid hormones are not only to better understand the mechanisms by which sex steroid hormones exert their effects, but importantly also to understand the sex difference in disease prevalence and/or incidence. Future studies considering the match/mismatch between sex and sex steroid hormone effects on atherosclerosis and autoimmune disease will provide further mechanistic insight into underlying mechanisms and will likely identify novel therapeutic targets both within and outside the sex hormone system.

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