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ABSTRACT

Estrogen affects the development and regulation of the immune system. Treatment of gonadectomized mice with estrogen results in suppression of T and B lymphopoiesis, as well as decreased delayed type hypersensitivity reaction, granulocyte mediated inflammation and levels of IL-6 in serum. Conversely, immunoglobulin production is stimulated by estrogen. The effects of estrogen are mediated through the estrogen receptors (ER), ERα and ERβ, which are ligand activated transcription factors that induce expression of specific estrogen responsive genes. The aims of this thesis were to investigate the role of ERs on B lymphopoiesis and immunoglobulin production, as well as on the aged immune system. Furthermore, the ER specific effects of the synthetic molecule estren on T and B lymphopoiesis, T cell-mediated inflammation and submandibular glands were studied. ER knock-out mice lacking ERα, ERβ or both ERα and ERβ, were gonadectomized and treated with 17β-estradiol-3-benzoate (E2) or 4-estren-3α,17β-diol (estren).

We found that both ERα and ERβ are required for the estrogen-induced decreased frequency of B lymphopoietic cells in the bone marrow. ERα alone is necessary for the estrogen-mediated, as well as for the age-induced, increased frequency of immunoglobulin producing B cells. We could also show that estren inhibits inflammation through ER-mediated pathways, while the inhibitory effects on T and B lymphopoiesis are not dependent on ERs. Furthermore, estren promotes an androgen phenotype in submandibular glands that is independent of ERs.

In conclusion, our results show that the effects of estrogen on the immune system are mainly mediated via ERα, but signalling through ERβ is necessary for complete inhibitory effect on B lymphopoiesis. Furthermore, estren treatment induces effects on lymphopoiesis and submandibular glands that are not mediated through ERs, but instead possibly through the androgen receptor.

Key words: estrogen receptor knock-out mice, estrogen, estren, estrogen receptor, lymphopoiesis, T cells, B cells, immunoglobulin, inflammation

ORIGINAL PAPERS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals (I-IV):

I. **Malin C. Erlandsson, Charlotte A. Jonsson, Ulrika Islander, Claes Ohlsson and Hans Carlsten.** Oestrogen receptor specificity in oestradiol-mediated effects on B lymphopoiesis and immunoglobulin production in male mice.
Immunology 2003, 108:346-51.

II. **Ulrika Islander, Malin C. Erlandsson, Bengt Hasséus, Charlotte A. Jonsson, Claes Ohlsson, Jan-Åke Gustafsson, Ulf Dahlgren and Hans Carlsten.** Influence of oestrogen receptor alpha and beta on the immune system in aged female mice.
Immunology 2003, 110:149-57.

III. **Ulrika Islander, Malin C. Erlandsson, Tina Chavoshi, Caroline Jochems, Sofia Movérare, Stefan Nilsson, Claes Ohlsson, Jan-Åke Gustafsson and Hans Carlsten.** Estren-mediated inhibition of T lymphopoiesis is estrogen receptor-independent whereas its suppression of T cell-mediated inflammation is estrogen receptor-dependent.
Clinical and Experimental Immunology 2005, 139:210-215.

IV. **Ulrika Islander, Bengt Hasséus, Malin C. Erlandsson, Caroline Jochems, Sofia Movérare Skrtic, Marie Lindberg, Jan-Åke Gustafsson, Claes Ohlsson and Hans Carlsten.** Estren promotes androgen phenotypes in primary lymphoid organs and submandibular glands.
BMC Immunology 2005, 6:16.

CONTENTS

ABBREVIATIONS

AF	Transactivation function
APC	Antigen presenting cell
AR	Androgen receptor
BERKO	Estrogen receptor β knock-out
DC	Dendritic cell
DERKO	Double estrogen receptor knock-out
DHT	5α-dihydrotestosterone
DN	Double negative
DP	Double positive
DTH	Delayed type hypersensitivity
E2	17β-estradiol-3-benzoate
ER	Estrogen receptor
ERα	Estrogen receptor α
ERβ	Estrogen receptor β
ERE	Estrogen response element
ERKO	Estrogen receptor α knock-out
Estren	4-estren-3α,17β-diol
GCT	Granular convoluted tubular
Ig	Immunoglobulin
IL	Interleukin
MHC	Major histocompatibility complex
ORX	Orchidectomized
OVX	Ovariectomized
SFC	Spot forming cell
SMG	Submandibular gland
SP	Single positive
TF	Transcription factor
Th	T helper
WT	Wild type

INTRODUCTION

Estrogens exert several biological effects in both females and males, but their main function is to regulate development and growth of the female sexual organs and other tissues associated with the reproductive system. Besides having effects on reproduction, estrogens also affect the immune system. It has been suggested that the female susceptibility to autoimmune diseases could in part be due to effects mediated by sex hormones. For example, multiple sclerosis, rheumatoid arthritis and systemic lupus erythematosus are more common in females compared to males [1, 2]. In the following studies we have examined the estrogenic effects on the mouse immune system by using estrogen receptor knock-out mice and treatments with estrogen or estren.

The immune system

The major function of the immune system is to defend the host against invading pathogens. Cells of the innate immune system provide the initial defence against microbes in a non-antigen-specific way, but it is not always powerful enough to eliminate infectious organisms. The lymphocytes of the acquired immune system provide an adaptable defence through antigen-specific receptors that recognize the pathogen in question. In addition, the acquired immune system provides increased protection against re-infection with the same pathogen.

T lymphopoiesis

T cells derive from stem cells in the bone marrow but their main development and maturation takes place in the thymus. Progenitor T cells leave the bone marrow and arrive to the thymus cortex where they at first are double negative (DN) cells, expressing neither CD4 nor CD8 (fig. 1).

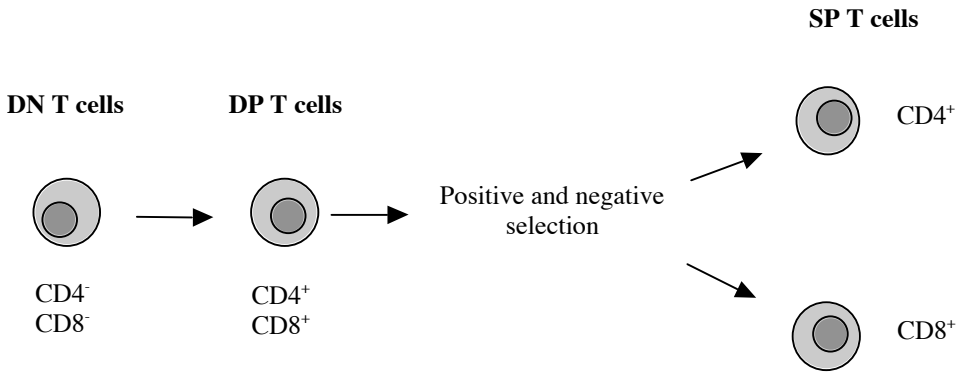


Figure 1: Schematic drawing of important steps in the T lymphopoiesis.

The cells start to rearrange their unique T cell receptor (TCR), and subsequently they become double positive (DP) cells, expressing both CD4 and CD8. At this stage the positive and negative selection of the T cells occur. Positive selection is the first step in the T cell selection process enabling the thymocytes to recognize MHC class I or class II. The T cells that bind to MHC class I become CD8 single positive cells, and the ones that bind to MHC class II become CD4 single positive T cells. Thus, positive selection leads to survival of T cells that are capable of recognising self-MHC. The T cells that do not recognize the MHC molecule will undergo apoptosis. After positive selection, the surviving T cells will migrate into the thymic medulla where the negative selection occurs. During this process the T cells that bind with a too high avidity to a MHC/peptide complex will selectively be eliminated and go through apoptosis. About 95% of the thymocytes will die in the thymus during the different stages of the T cell development. If the T cells survive positive and negative selection, they become naïve single positive (SP) T cells and will leave the thymus to circulate the body. In the thymus the DN T cells, DP T cells, CD4 SP T cells and CD8 SP T cells constitute approximately 5%, 80-85%, 10% and 5% of the total thymocytes respectively [3].

B lymphopoiesis

Like T cells, B cells develop from stem cells in the bone marrow. However a precursor B cell will not leave the bone marrow until it has differentiated into an immature B cell. Some of the markers expressed on the surface of the differentiating B cells are described schematically in figure 2 [4, 5].

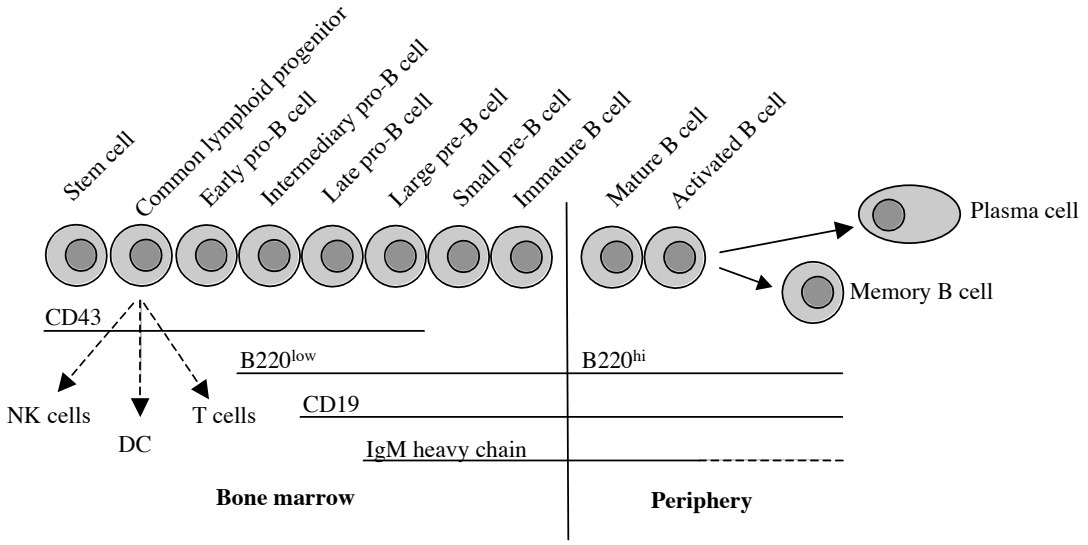


Figure 2: Schematic drawing of important stages in the murine B lymphopoiesis.

Bone marrow stem cells develop into common lymphoid progenitors (CLP), which can form NK cells, dendritic cells (DCs), T cells and B cells. From the intermediary pro-B cell stage, the B cells express B220/CD45R (B220), and from the late pro-B cell stage they express CD19. Both B220 and CD19 are surface markers associated with the B cell receptor. Successful rearrangement of the IgM heavy chain genes results in expression of μ on the surface of large pre-B cells. Once a light chain gene is assembled and a complete IgM molecule is expressed on the cell surface, the cell is defined as an immature B cell. Self-antigens are presented to the immature B cells in the bone marrow, and cross-linkage of auto-antibodies expressed on the surface leads to death of the B cell, or a rearrangement of the auto-reactive B cell receptor. As a result of the negative selection process, only ~10% of the produced immature B cells are recruited into the periphery and further developed into mature B cells expressing both IgM and IgD.

Acquired immunity

Acquired immunity is characterized by specificity and memory exerted by T and B lymphocytes. The activation of naïve T and B cells in response to a specific antigen, subsequently leads to proliferation and differentiation of these cells. In addition to providing effector cells, this response generates immunological memory that leads to protection from recurrent challenge by the same pathogen.

Using the T cell receptor, T cells recognize specific antigenic peptides presented on MHC molecules of antigen presenting cells (APCs), including DCs, macrophages and B cells. After activation, the T cells activate, recruit or kill other cells through expression of effector molecules that are either cell membrane associated or secreted into the extra cellular milieu. T cells can be divided into two main subsets based on the cell surface expression of CD4 and CD8, which are co-receptors for MHC class II and class I, respectively. The main function of activated $CD4^+$ T helper (Th) cells is to start, enhance or suppress other cells of both the innate and acquired immune system. On the other hand, the $CD8^+$ cytotoxic T cells are able to kill cells that are infected by virus or intracellular bacteria. Naïve $CD4^+$ T cells differentiate into Th1 or Th2 cells, characterized by different cytokine production patterns. Th1 cells typically secret IFN-γ, which stimulates phagocytosis and killing of intracellular microbes. The Th2 cells typically produce IL-4 and IL-10 and are involved in the activation of B cells and stimulation of immunoglobulin (Ig) production.

The activation of antigen specific B lymphocytes is initiated by binding of an antigen to membrane bound Ig molecules. The antigen is internalized and presented on cell surface MHC class II molecules for recognition by Th cells. The interaction between the activated Th cells and the B cells, stimulate B cell proliferation and differentiation into memory B cells and Ig secreting plasma cells. Cytokines produced by the Th cells will direct the B cells in switching into production of different Ig isotypes.

Inflammation

Inflammation is the local response to an injury or infection. An inflammatory response is characterized by local redness, swelling, heat and pain. Phagocytes that are resident in the tissue play an active part in the inflammatory response. Upon activation they can release vasodilatory molecules, chemokines and pro-inflammatory cytokines like TNF, IL-1 and IL-6. Blood vessels dilate and the vessel wall permeability is increased, followed by an increased migration of leukocytes into the tissue. Anti-inflammatory cytokines like TGF-β and IL-10 are released from T cells and other cell types to control the inflammation.

Inflammatory responses can be either pure innate responses or to a various degree dependent on the acquired immune system. In T cell driven inflammation, a sensitisation phase is needed before a second exposure to the antigen can result in an inflammatory response. The T cell-mediated delayed type hypersensitivity (DTH) reaction is elicited in two separate steps; a sensitisation phase, followed by a challenge phase, after which the DTH response is measured. Sensitisation of the DTH reaction requires the antigen to be processed by tissue resident APCs and presented to antigen-specific T cells. The T cells then become activated and proliferate within the draining lymph node. The proliferation leads to generation of short-lived effector T cells and long lived memory T cells. In the challenge phase, the same antigen is presented to antigen-specific memory T cells located at the site of elicitation. Once activated by the antigen, antigen-specific T cells start to proliferate and produce cytokines ultimately leading to an inflammatory response including vasodilatation and migration of leukocytes into the inflammatory site. The disappearance of the exogenous antigen and death of the effector T cells leads to resolution of the inflammatory response.

Ageing

Ageing affects the immune system by a general suppression of activity. One of the most well recognized age-related changes in the immune system is the thymic involution, characterized by a progressive reduction in size. Both T and B

lymphopoiesis occurs throughout life, but the number of lymphocytes produced by the thymus and bone marrow are substantially reduced in old individuals. This does not result in any significant changes in the total number of peripheral T and B cells, due to increased proliferation of these cells [6-8]. However, T cells from aged individuals have a more limited T cell receptor repertoire, and diminished capacity to proliferate to mitogens and other activation stimuli compared to young [8]. In B cells, there is a decrease in the quality of the antibody response in aged individuals. A shift from antibodies directed against foreign antigens, towards more auto-antibodies is noted, and also the affinity of antibodies produced by newly formed B cells is lower in aged individuals. However, this does not reflect a decrease in the quantity of Ig production [9, 10].

Estrogen and the estrogen receptors

Steroid hormones are a group of small lipophilic compounds produced by the adrenal cortex and the gonads (ovaries and testis), as well as by the placenta during pregnancy. Cholesterol is the precursor of all steroid hormones (fig. 3). A great part of the endocrine system is controlled by the central nervous system, which via the hypothalamus and the pituitary gland release hormones that act on the peripheral endocrine glands. Estrogen is the common name for the female sex steroids: estradiol, estrone and estriol. Estradiol is produced mainly in the ovaries through conversion from blood-derived cholesterol (fig. 3). In blood it binds reversibly to sex-hormone-binding globulin and albumin, leaving a free fraction of only 2-3% [11].

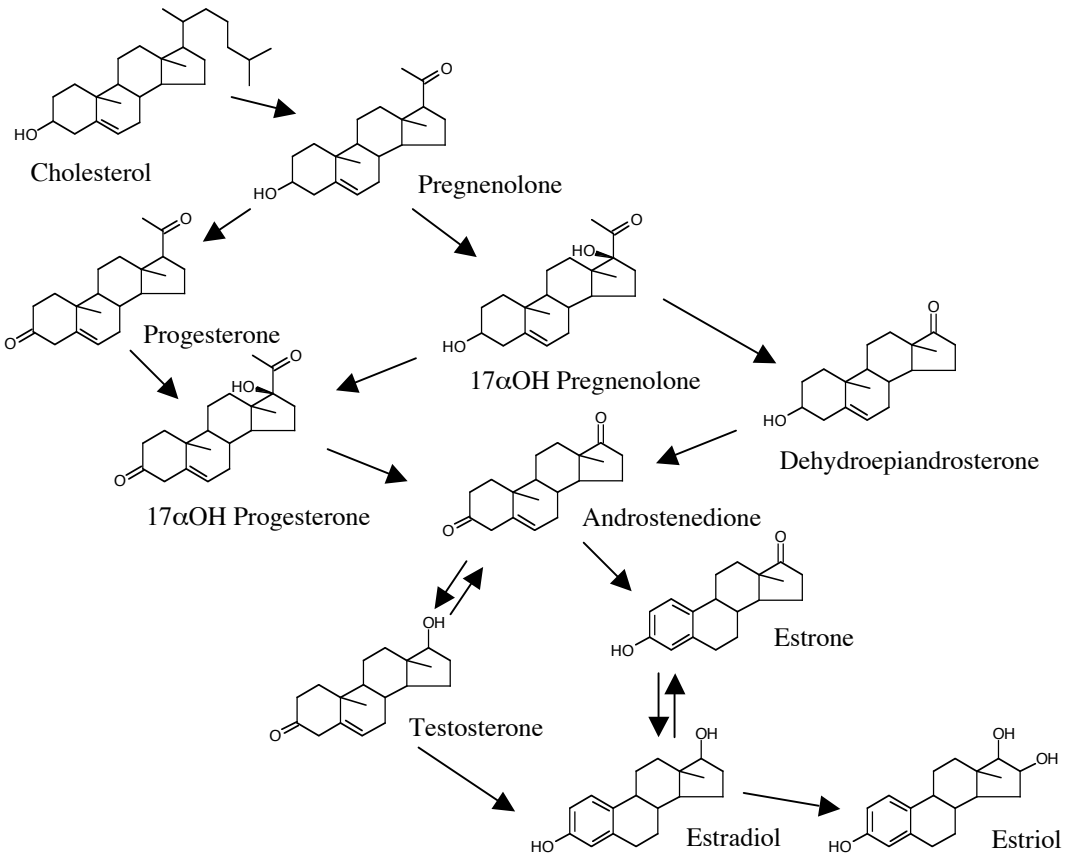


Figure 3: Sex steroid synthesis from cholesterol.

Estrogens are now known to influence the expression of a wide range of genes in the reproductive tract as well as in other areas [11]. For example, estrogens regulate the development and growth of the sexual organs and other tissues related to reproduction, including the mammary gland, uterus and ovaries in females, as well as testis and the prostate gland in males. Estrogens are also important to longitudinal bone growth. At puberty, it results in an increase of growth followed by the rapid cessation of longitudinal growth, in both males and females [12].

Structure of the estrogen receptors

The two estrogen receptors, ERα and ERβ, belong to the nuclear receptor super family, in which the members have structural and functional similarities. The ERα was cloned in 1986 [13] and the ERβ in 1996 [14]. Nuclear receptors are ligand-

activated transcription factors that consist of characteristic domains (fig. 4) harbouring the DNA-binding domain (DBD) and the ligand-binding domain (LBD). The N-terminal domain of the receptor contains the ligand-independent transactivation function 1 (AF-1), and within the LBD is the ligand-dependent transactivation function 2 (AF-2). The AF-1 and AF-2 are regions of the receptor involved in activation of gene transcription [15]. There is a high homology between ERα and ERβ in the DBD (97%), but a moderate homology (55%) in the LBD, and it has been shown that the two receptors exhibit similar but not identical ligand binding properties [14, 16]. ERβ appears to lack significant AF-1 activity, and thus depends entirely on AF-2 [17, 18].

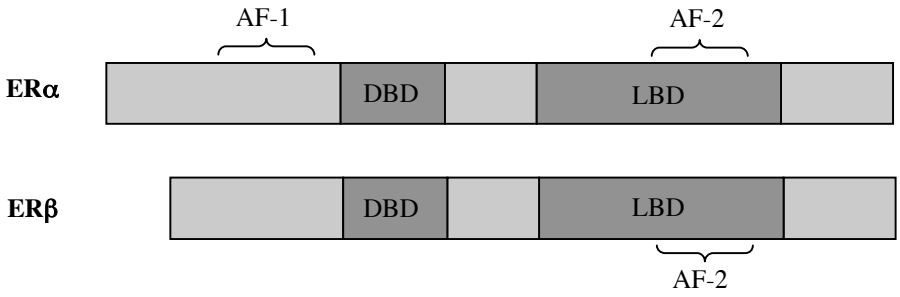


Figure 4: Schematic presentation of ERα and ERβ. Nuclear receptors consist of characteristic domains, harbouring the DNA-binding domain (DBD), the ligand-binding domain (LBD) and the transactivation functions 1 and 2 (AF-1, AF-2).

Classic transcriptional activity

ERs act as transcription factors in the nucleus when binding to a ligand. In classic genomic activity estrogen binds to the ERs, translocates to the nucleus and interacts with estrogen response elements (ERE) located in the promoter region of the responsive genes (fig. 5, pathway 1). Either homo- or heterodimerization can occur between ERα and ERβ [19, 20]. The promoter bound receptor dimer then forms a complex with co-regulatory proteins that influence transcription.

Non-classic transcriptional activity

ERs have also been shown to modulate gene expression at alternative regulatory DNA sequences, such as the AP-1 or SP-1 site [21, 22]. In these cases, the estrogen/ER complex alters transcription of genes through association with other DNA bound transcription factors, for example c-Jun and c-Fos (fig. 5, pathway 2).

Non-genomic activity

A variety of cell types respond rapidly to estrogen, making a non-genomic mechanism of action probable. These responses are likely to be mediated through different membrane-associated receptors. A membrane form of the ERα has been suggested [23-25], as well as a G protein-coupled receptor, GPR30 [26-28]. Binding of a ligand to a membrane-associated receptor, results in activation of different intracellular signal transduction pathways leading to rapid cellular and tissue responses (fig. 5, pathway 3). For example, estrogen is capable of modulating the physiology of nerve cells within seconds after application, and can stimulate rapid Ca^{2+} fluxes [25, 29, 30]. However membrane-initiated estrogen stimulation can also result in activation or deactivation of intracellular signalling pathways, which ultimately modulate the activity of transcription factors (TF) and thereby influence downstream gene transcription (fig. 5, pathway 4).

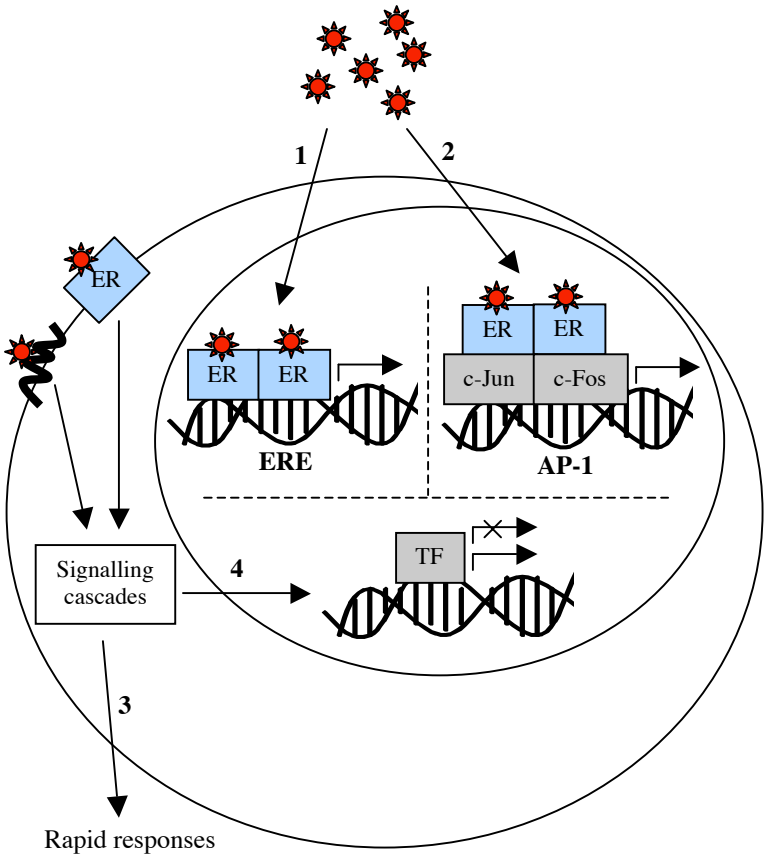
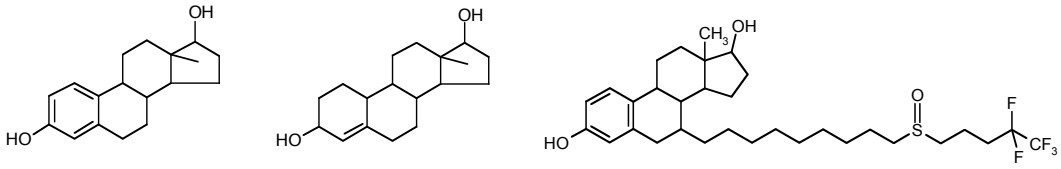


Figure 5: Mechanisms of estrogen signalling. The effects of estrogen can be mediated through several pathways; 1) Classic transcriptional activity, 2) Non-classic transcriptional activity, 3) Membrane-initiated activation resulting in a non-genomic response, 4) Membrane-initiated activation resulting in a transcriptional response.

Estren

A few years ago, a synthetic compound, estren-3α,17β-diol (estren) (fig. 6), was described by the group of Manolagas [31]. They proposed that estren is a mechanism-specific compound that reproduces only the non-genomic signalling pathway of estrogen, and is incapable of inducing the classical transcriptional pathway. Furthermore, they suggested that the effects of estren were mediated through both the ERs and the androgen receptor (AR) [31-35]. In contrast to Manolagas, we and others have found that estren has the capacity to induce genomic effects via both ERs and the AR [36-40].



17β-estradiol (E2) | 4-estren-3α,17β-diol (estren) | ICI 182,780

Figure 6: Molecular structures of 17β-estradiol, 4-estren-3α,17β-diol and ICI 182,780.

ICI 182,780

There are several ways to block the biological effects of estrogen. ICI 182,780 (fig. 6) is a potent anti-estrogen, which exerts its effects by binding to and blocking ERs. Binding of ICI 182,780 to the ERs results in a block of both the AF-1 and the AF-2 domains on the ER, impairs the ER dimerization, and disrupts the translocation of the ICI/ER complex into the nucleus. These effects result in complete abrogation of estrogen signalling through the ER [41]. Interestingly, recent studies have shown that ICI 182,780 may have an agonistic effect when bound to the membrane-associated receptor GPR30 [42].

Effects of estrogen on the immune system

Many autoimmune diseases are more common in females compared to males, and endogenous or added estrogen can have an impact on the severity of the disease. The mechanisms behind these clinical observations are not fully understood, however it is well established that estrogens affect the development and regulation of the immune system. Effects can be observed in both primary and secondary lymphoid organs, as well as in inflammatory responses.

T cells

Gonadectomy of mice leads to an increased thymic size and cellularity. In contrast, exposure to endogenous estrogen during pregnancy, or treatment of gonadectomized animals with estrogen, results in thymic atrophy, reduced thymic cellularity, reduced frequency of double positive (DP) T cells, but increased frequency of single positive (SP) T cells [43, 44]. The mechanisms behind the estrogen-induced thymic involution

is not clear, however ERα has been detected in both murine thymic stromal cells and thymocytes [45]. ERβ has not yet been found in mouse thymic tissue [46, 47], but at low levels in the rat thymus [16]. Staples *et al* showed that mice chimeric for $ER\alpha^{-/-}$ thymic stromal cells and $ER\alpha^{+/+}$ thymocytes, fail to undergo estrogen-induced thymic atrophy suggesting that stromal cells are of primary importance [48]. Furthermore, reduced proliferation of very early T cell precursors or apoptosis of DP T cells have been suggested as possible mechanisms [49, 50]. The biological function behind the thymic atrophy is not fully understood, but one possible reason could be maternal tolerance against an immunological foreign foetus, which requires alterations in thymocyte development.

By using ER knock-out mice, the impacts of estrogen signalling on the immune system have been studied. Staples *et al* showed that ERKO ($ER\alpha^{-}\beta^{+}$) mice had smaller thymi than WT mice, and that the ERKO mice displayed less thymic atrophy after exposure to estrogen compared to the WT mice [48]. In a previous study [44], we could confirm and expand these results, and showed that deletion of ERα in both ERKO and DERKO ($ER\alpha^{-}\beta^{-}$) mice resulted in hypoplasia of both thymus and spleen. Furthermore, a higher frequency of DP T cells but a lower frequency of SP T cells was found in $ER\alpha^{-}$ mice compared with $ER\alpha^{+}$ mice. Estrogen treatment of BERKO ($ER\alpha^{+}\beta^{-}$) mice resulted in a similar degree of thymic atrophy compared with WT mice, but displayed no alteration in the frequency of DP thymocytes [44].

B cells

Gonadectomy of mice increases the number of developing B cells in the bone marrow, while estrogen treatment results in a potent down-regulation of B lymphopoietic cells [51, 52]. Bone marrow stromal cells have been shown to express both ERα and ERβ [53, 54]. Furthermore, Smithson *et al* suggested that the estrogen-induced reduction of B lymphopoietic cells is mediated via effects on stromal cells, since exposure to estrogen reduce the B cell precursor expansion only when stromal cells are present in the cultures [55]. However, it has been shown that also small populations of very early B lineage cells are potential targets for the estrogen-induced reduction of B lymphopoietic cells [56, 57].

Despite the suppressing effect on early B cell development, estrogen treatment induces a shift towards increased differentiation of peripheral B cells. The frequency of B cells actively producing immunoglobulin (Ig) is elevated in estrogen treated mice [58], and long-term treatment with low doses of estrogen results in increased serum levels of Ig [59]. Furthermore, the serum titers of auto-antibodies are elevated in estrogen treated mice [60].

Immune responses

Estrogen has a dual effect on immune responses, being a suppressor of inflammation but a stimulator of antibody formation. *In vivo* experiments in mice have shown that estrogen down-regulates granulocyte-mediated inflammation [61], T cell dependent inflammation (DTH) [62, 63], NK cell activity [59] and levels of IL-6 in serum [64]. Even though estrogen has been shown to have fairly potent anti-inflammatory properties, some reports have shown that estrogen can enhance primary T cell responses and production of interferon-γ (IFN-γ) [65, 66]. It is possible that the down-regulatory effects of estrogen are exerted in later stages in the inflammation process. It has been shown that administration of estrogen after antigen sensitisation and close in time to antigen provocation is efficient to decrease inflammatory responses [63].

AIMS OF THE STUDY

The specific aims of this thesis were:

- To investigate the role of estrogen receptors on B lymphopoiesis and immunoglobulin production (paper I).

- To examine the role of estrogen receptors on the immune system in aged female mice (paper II).

- To study the estrogen receptor-specific effects of estren on T lymphopoiesis and T cell-mediated inflammation (paper III).

- To investigate the estrogenic and androgenic effects of estren on primary lymphoid organs and submandibular glands (paper IV).

METHODOLOGICAL CONSIDERATIONS

The purpose of this section is to give an overall view on materials and methods used in the work of this thesis. More detailed protocols are available in the publications included in the thesis.

Animals

Mice are widely used as an *in vivo* model to study the effects of sex steroids on the immune system. One advantage of using the mouse model is the possibility of performing transgenic modifications. Using transgenic techniques, the expression of genes of interest may be deleted (knocked out), or enhanced (over expression), and the effects on the immune system can be determined. In these studies, estrogen receptor knock-out mice and unmodified C57/BL6 mice have been used.

ER knock-out mice

The generation of DERKO ($ER\alpha^{-/-}\beta^{-/-}$) mice is somewhat complex, since both female and male ERKO ($ER\alpha^{-/-}\beta^{+/+}$) mice are infertile. Male double heterozygous ($ER\alpha^{+/-}\beta^{+/-}$) mice were mated with female double heterozygous mice on a mixed C57BL/6J/129 background resulting in WT ($ER\alpha^{+/+}\beta^{+/+}$), ERKO ($ER\alpha^{-/-}\beta^{+/+}$), BERKO ($ER\alpha^{+/+}\beta^{-/-}$) and DERKO ($ER\alpha^{-/-}\beta^{-/-}$) offspring (fig. 7) [67-69]. This breeding results in only one DERKO pup of the right sex out of 32 offspring. Genotyping of tail DNA was performed using polymerase chain reaction [70, 71].

	$ER\alpha^{+/+}$	**$ER\alpha^{-/-}$**
$ER\beta^{+/+}$	WT	ERKO
$ER\beta^{-/-}$	BERKO	DERKO

Figure 7: Estrogen receptor expression in ER knock-out mice.

The ERKO and DERKO mice used in this thesis were not completely ERα inactivated. The mice express N-terminally modified transcripts of the receptor, generating a truncated ERα with remaining AF-2 activity, while AF-1 is absent. However, presence of the truncated ERα has been associated with only minor effects on uterine weight [72, 73]. A second ERKO model has been generated, which have neither AF-1 nor AF-2 and therefore has no remaining ERα activity [74].

Gonadectomy and hormone treatments

Gonadectomy (ovariectomy, OVX / orchidectomy, ORX) was used for studying the effects of sex steroid deficiency. OVX was performed by removal of the ovaries after a flank incision, and testis were removed after a scrotal incision. The operation procedure was carried out under Ketalar/Domitor anaesthesia, and after surgery the mice were left to rest for two weeks before the start of experiments.

The doses of 17β-estradiol-3-benzoate (E2) used in the experiments were chosen to correspond to normal levels of 17β-estradiol in mouse serum. In paper I, treatment with 0.9 μg E2, 5 days per week for 2.5 weeks resulted in serum 17β-estradiol levels of ~60 pg/ml [75], and in paper II treatment with 3.2 μg E2, five days per week for three weeks resulted in serum 17β-estradiol levels of ~140 pg/ml [76]. In mice, normal levels of estradiol in serum vary between 25 and 50 pg/ml during diestrus, while it is between 150 and 200 pg/ml in the estrus phase [77]. The dose of estren in papers III and IV, 0.75 μg/mouse/day, was chosen as being slightly lower than that used by the group of Manolagas [31].

T cells in the thymus

Thymi were removed and weighed at the end of experiments. Single cell suspensions were prepared and the expression of surface molecules CD3, CD4 and CD8 were analyzed by flow cytometry. Early T lymphopoietic cells are double positive (DP) expressing both CD4 and CD8 on their surface while the naïve T cells are single positive (SP) expressing either CD4 or CD8.

T cell activity

Proliferative activity of T cells was examined by *in vitro* cultures of spleen cells with the T cell mitogen Concanavalin A (ConA). The T cell proliferation was analysed by addition of [^{3}H]-thymidine 24 h prior to counting using a β-counter. The Th2 cytokine IL-10 was measured in supernatants from the cell cultures using enzyme-linked immunosorbent assay (ELISA).

T cell-mediated inflammation

The T cell-mediated delayed type hypersensitivity (DTH) reaction was used to study the inflammatory response. Mice were sensitised by cutaneous application of 4–ethoxymethylene-2-phenyloxazolone (OXA) on the abdomen skin. Six days after sensitisation the mice were challenged by application OXA on both sides of the right ear, and the ear thickness was measured 24 hours after challenge. At the termination of experiments, serum was collected from the mice and the levels of the pro-inflammatory cytokine IL-6 was measured using a bioassay.

B cells in the bone marrow

Bone marrow cells were harvested from femur and tibia, by removing the proximal and distal ends and flushing the cells out of the bone cavity using PBS. The total number of cells was calculated, and the cells were analyzed for the expression of surface molecules B220/CD45R (B220), IgM heavy chain (μ) or CD19, by flow cytometry.

The surface molecule B220 and the μ chain are expressed with various intensities on different stages of B lymphopoietic cells. Accordingly, four fractions of B lymphopoietic cells were identified using anti-B220 and anti-μ antibodies (fig. 8). The first fraction is B220^{lo} and μ chain negative cells (fraction 1), which includes intermediary and late pro-B cells. Fraction 2 consists of B220^{lo} and μ chain positive cells, and includes large and small pre-B cells as well as immature B cells. The B220^{hi} and μ chain positive fraction (fraction 3), represents mature B cells. These three stages correspond to the late part of B lymphopoiesis, from the time of rearrangement of the heavy chain Ig genes to newly formed mature B cells. Finally, the B220^{hi} and μ chain

negative fraction (fraction 4), represents Ig switched activated B cells as well as memory B cells.

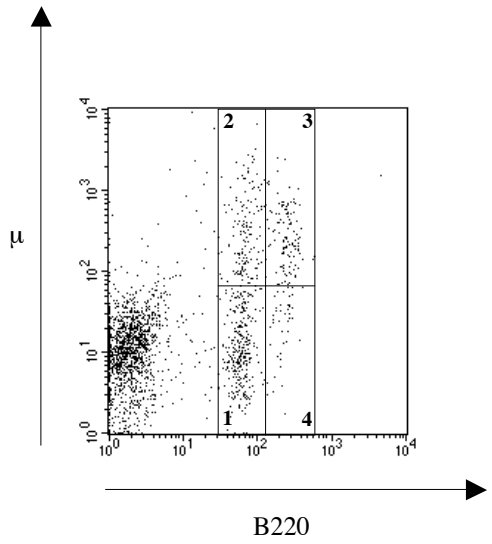


Figure 8: Representative flow cytometry plot of the four fractions of B lymphopoietic cells obtained by staining with B220 and μ.

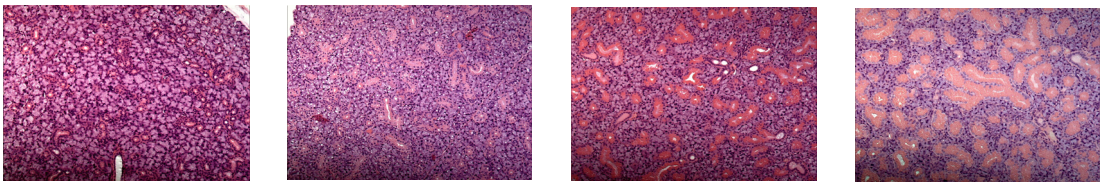
B cell activity

One important activity of B cells is the production of Ig. The enzyme-linked immunospot assay (ELISPOT) was used for enumeration of IgM, IgG, and IgA spot forming cells (SFC) in freshly isolated spleen and bone marrow cell suspensions.

Submandibular glands

Submandibular glands (SMGs) are sexually dimorphic in rodents. The secretory activity of these glands is mainly localized to the acinar cells and the granular convoluted tubular (GCT) cells. The GCT cells are under hormonal control involving androgens, resulting in larger GCT cells in males compared to females [78-80].

Submandibular glands (SMGs) were removed and weighed at the end of experiments. Histological examination of SMGs was performed in a light microscope after paraformaldehyde fixation and staining with hematoxylin and eosin. The degree of the androgen phenotype in the SMG sections were scored from 0 to 3, where 0 represents lack of androgen phenotype and 3 maximal score for androgen phenotype (fig. 9).



Score 0 Score 1 Score 2 Score 3

Figure 9: Representative pictures of the scoring points for androgen phenotype in SMGs.

RESULTS AND COMMENTS

Paper I

Gonadectomy of both male and female mice stimulates B lymphopoiesis, which is demonstrated by increased frequency of $B220^+$ cells in bone marrow [51, 81]. This effect is likely to depend on the removal of endogenous estrogen since it has been shown that in intact aged male rats the frequency of B lymphopoietic cells correlates to serum estradiol rather than serum testosterone levels [82]. Replacement of estrogen suppresses B lymphopoiesis in female as well as in male mice [83]. In paper I, 7 months old male mice of all four ER genotypes were orchidectomized (ORX) and treated with 0.9 μg E2/mouse, 5 days/week for 2.5 weeks. B lymphopoietic cell-phenotypes in bone marrow were analysed by flow cytometry. As expected in WT mice, the frequency of $B220^+$ cells decreased dramatically from more than 15% to less than 5% after exposure to E2. A decrease of smaller magnitude was found in both BERKO ($ER\alpha^+\beta^-$) and ERKO ($ER\alpha^-\beta^+$) mice whereas no decrease at all was seen in DERKO ($ER\alpha^-\beta^-$) mice. These results show that both ERα and ERβ contribute to the E2-induced inhibition of B lymphopoiesis in male mice (fig. 10). Notably, similar results were obtained in paper II using 4 months old ovariectomized female mice.

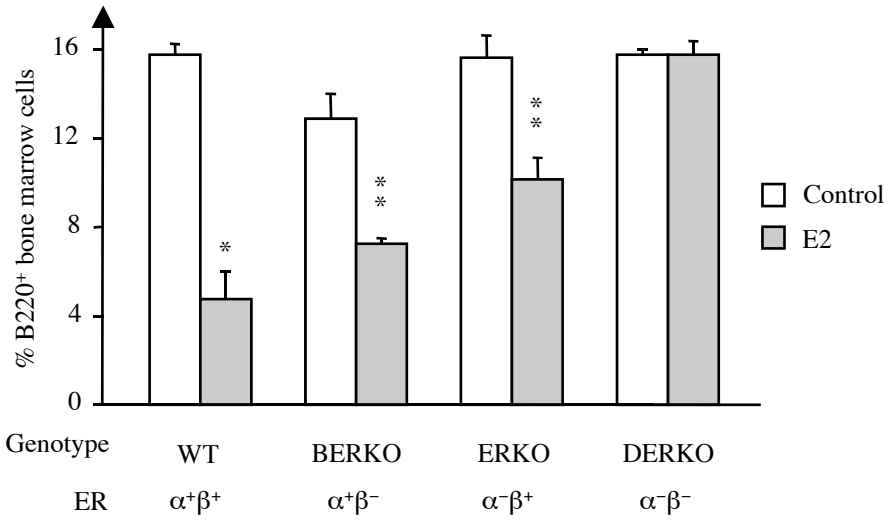


Figure 10: Both ERα and ERβ are needed for full estrogen-mediated inhibitory effect on B lymphopoiesis. ORX mice of all ER genotypes were treated with 0.9 μg E2/mouse 5 days/week for 2.5 weeks. Control mice received olive oil. A two-way ANOVA reveals that both ERα and ERβ contribute to achieve full effect. ERα: $P < 0.001$, ERβ: $P < 0.001$. Mann-Whitney U test: control vs. E2 treatment * $P < 0.05$, ** $P < 0.01$. Results are presented as mean ± SEM.

It is well known that treatment with estrogen increases Ig production. Bone marrow and spleen cells from ORX mice treated with E2 or olive oil as control, were subjected to an ELISPOT assay enumerating cells producing IgM, IgG and IgA. The frequencies of B cells actively producing Ig were increased in WT mice after E2 exposure. A similar effect was found in BERKO mice, but not in ERKO and DERKO mice (fig. 11A-B). These results show that ERα, but not ERβ, is involved in the estrogen-mediated increase in Ig production from B cells in both bone marrow and spleen.

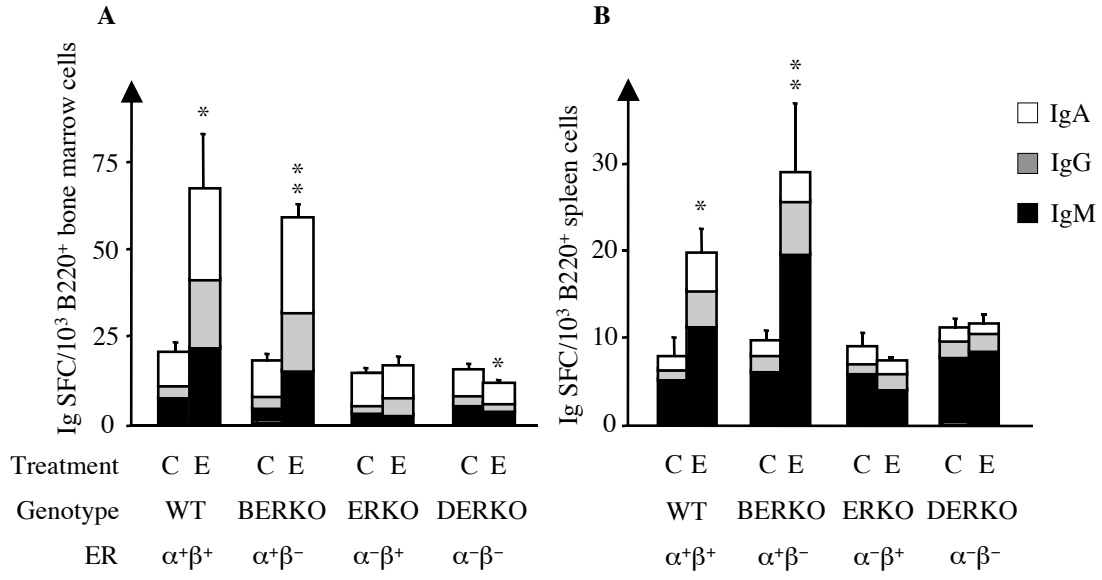


Figure 11: ERα, but not ERβ, is needed for the estrogen-induced increased frequency of Ig producing B cells in both bone marrow (A) and spleen (B). ORX mice of all ER genotypes were treated with 0.9 μg E2/mouse, 5 days/week for 2.5 weeks. Control mice received olive oil. Statistics are calculated on the summation of IgM, IgG and IgA. A two-way ANOVA reveals that ERα, but not ERβ, is required for full E2-mediated effect. **A**: ERα: $P < 0.001$, ERβ: ns; **B**: ERα: $P < 0.05$, ERβ: ns. Mann-Whitney U test: control vs. E2 treatment * $P < 0.05$, ** $P < 0.01$. Results are presented as mean ± SEM.

Paper II

The immune system is less effective at high ages. The production of B lymphocytes from the bone marrow is decreased, while the overall number of peripheral B cells remain constant during ageing [7]. However, serum concentrations of IgM, IgG and IgA have been reported to increase with age [10]. In paper II, untreated 4 months old female WT and 18 months old female WT, ERKO ($ER\alpha^{-}\beta^{+}$), BERKO ($ER\alpha^{+}\beta^{-}$) and DERKO ($ER\alpha^{-}\beta^{-}$) mice were compared in order to investigate the role of age and ER expression on Ig producing cells in the bone marrow (fig. 12). ELISPOT analysis showed that the frequency of IgM spot forming cells (SFC) clearly increased with age in WT mice, but no significant differences could be detected in IgG and IgA producing B cells. Furthermore, ERKO and DERKO mice had significantly lower frequencies of IgM SFC in the bone marrow compared to WT and BERKO mice. Interestingly, ERKO mice showed a significantly higher frequency of IgA producing bone marrow B cells compared to aged mice of the other genotypes.

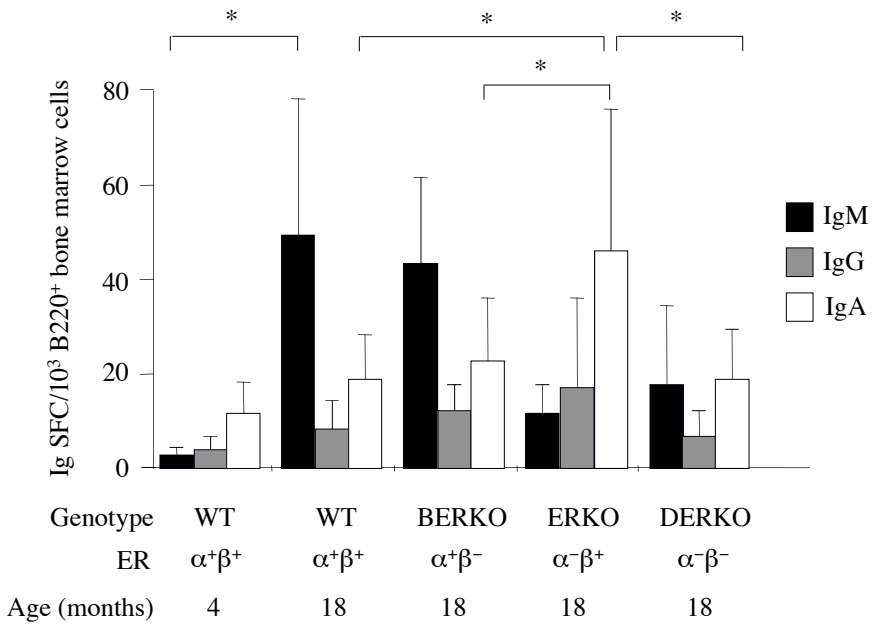


Figure 12: ERα is needed for the age-induced increased frequency of IgM producing B cells in the bone marrow. Aged WT mice display a significantly higher frequency of IgM SFC/1000 $B220^{+}$ cells compared to young WT mice, Students unpaired T test: * $P < 0.05$. A two-way ANOVA reveals that ERα is needed for the age-induced elevation of IgM SFC, ERα: $P < 0.001$, ERβ: P = ns. ERKO mice show significantly higher frequency of IgA producing bone marrow B cells compared to aged mice of the other genotypes, one-way ANOVA followed by Fisher's test: * $P < 0.05$. Results are presented as mean ± SD.

Age-induced involution of the thymus leads to decreased production of naïve T cells. In paper II we could confirm this and showed that aged WT mice had a lower frequency of double positive (DP) T lymphocytes compared to young WT mice (fig. 13). It has previously been shown that young ERKO and DERKO mice display higher frequencies of DP T cells compared to WT and BERKO mice [44]. We could now show that this effect is preserved also in very old female mice (fig. 13).

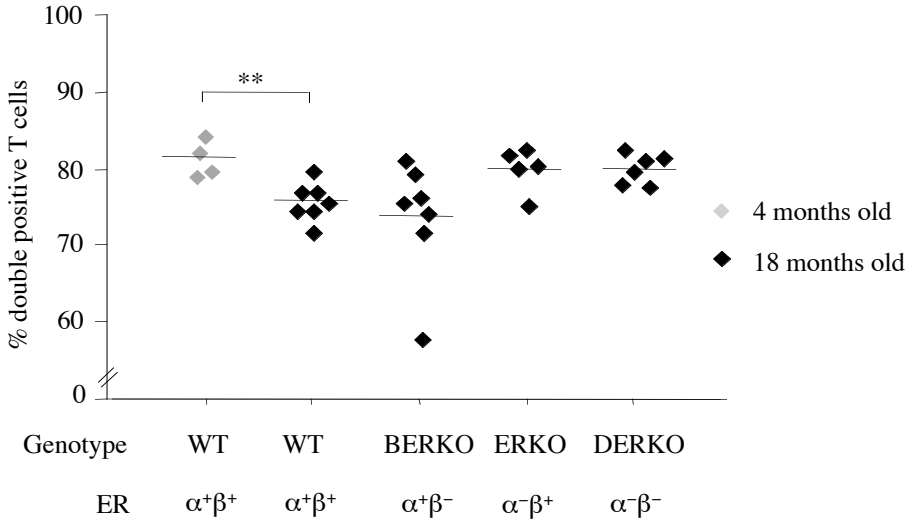


Figure 13: The ERα dependent decreased frequency of double positive T cells, is present also at a very high age. The frequency of DP T cells in the thymus is reduced in aged WT mice compared to young. Students unpaired T test: ** $P < 0.01$. A two-way ANOVA reveals that aged mice lacking ERα display a higher frequency of DP T cells compared to ERα$^+$ mice, ERα: $P < 0.01$, ERβ: P = ns. Horizontal lines represent mean values.

Paper III

4-estren-3α,17β-diol (estren) is a synthetic compound with structural similarities to E2. In paper III we compared the effects of treatment with E2 or estren on T lymphopoiesis and T cell-dependent inflammation. 11 months old female WT and DERKO ($ER\alpha^{-}\beta^{-}$) mice were ovariectomized (OVX) and treated with 0.7 μg E2/mouse/day or 75 μg estren/mouse/day during four weeks. Previous studies have shown that exposure to E2 results in reduced thymic weight and cellularity in both WT and BERKO ($ER\alpha^{+}\beta^{-}$) mice [44]. In paper III, we showed that DERKO mice lacked the E2-mediated reduction of thymic cellularity (fig. 14). In contrast, treatment of WT and DERKO mice with estren resulted in lower thymic cellularity in both genotypes (fig. 14), indicating that estren affects the thymus through ER independent pathways. Furthermore, similar results were obtained in paper IV using 3 months old OVX C57/BL6 mice co-treated with the ER antagonist ICI 182,780.

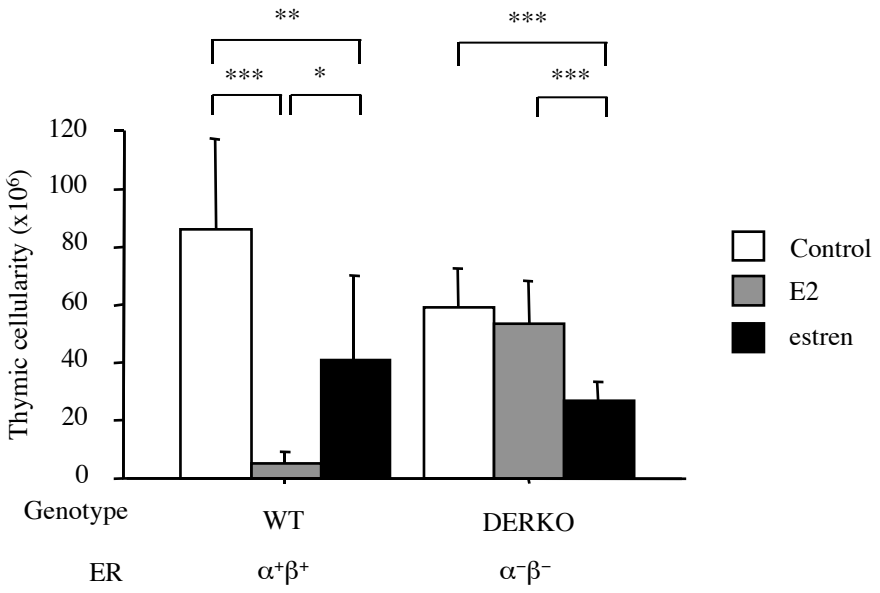


Figure 14: Estren affects thymic cellularity via ER independent pathways. 11 months old female OVX WT and DERKO mice were treated with 0.7 μg E2/mouse/day or 75 μg estren/mouse/day during four weeks. Control mice received olive oil. Treatment with E2 results in reduced thymic cellularity in WT mice but not in DERKO mice, while treatment with estren results in lower thymic cellularity also in DERKO mice. One-way ANOVA followed by Fisher's test was used to compare data from control mice with E2 or estren treated mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Results are presented as mean ± SD.

7 days before termination of the experiment, the mice were sensitised by cutaneous application of 4-ethoxymethylene-2-phenyloxazolone (OXA) on the abdomen, and 6 days later challenged by administration of OXA on the right ear. The delayed type hypersensitivity (DTH) response was measured as swelling of the ear 24 hours later. Results showed that both treatment with E2 and estren inhibited the DTH response in WT mice, while this could not be seen in DERKO mice (fig. 15).

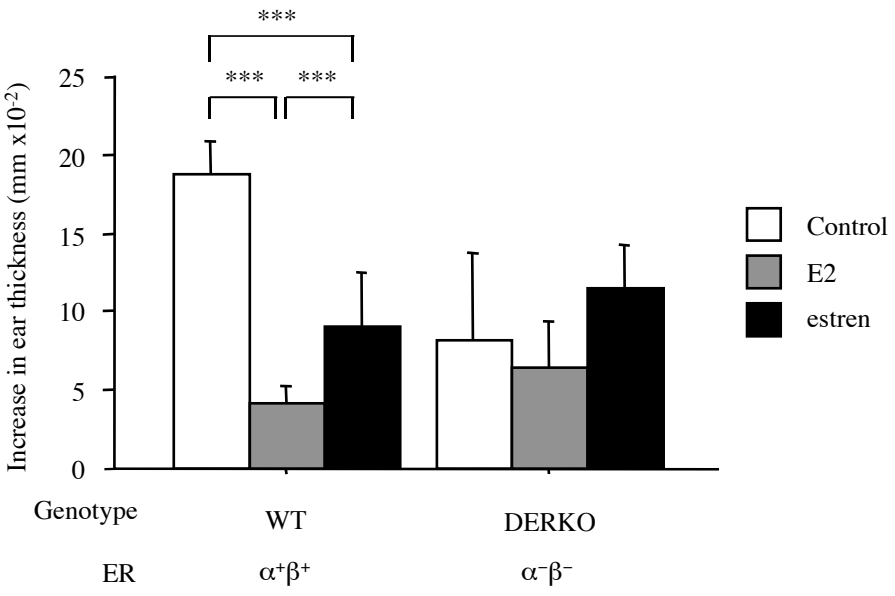


Figure 15: Estren inhibits the DTH response via ERs. 11 months old female OVX WT and DERKO mice were treated with 0.7 µg E2/mouse/day or 75 µg estren/mouse/day during four weeks. Control mice received olive oil. Treatment with both E2 and estren inhibited the DTH response in WT mice, while this could not be seen in DERKO mice. One-way ANOVA followed by Fisher's test was used to compare data from control mice with E2 or estren treated mice. *** $P < 0.001$. Results are presented as mean ± SD.

Paper IV

Several reports have shown that estren is a ligand for both the ERs and the AR [37-40]. In paper IV we investigated the estrogenic and possible androgenic effects of estren on primary lymphoid organs and submandibular glands (SMGs). The effects of estren were compared to those of 5α-dihydrotestosterone (DHT), a testosterone that can not be aromatized into estrogen. AR stimulation is known to down-regulate both T and B cell development [84-86], and accordingly we could show that treatment with both estren or DHT resulted in a lower frequency of $B220^+$ cells in bone marrow of both WT and DERKO ($ER\alpha^-\beta^-$) mice (fig. 16).

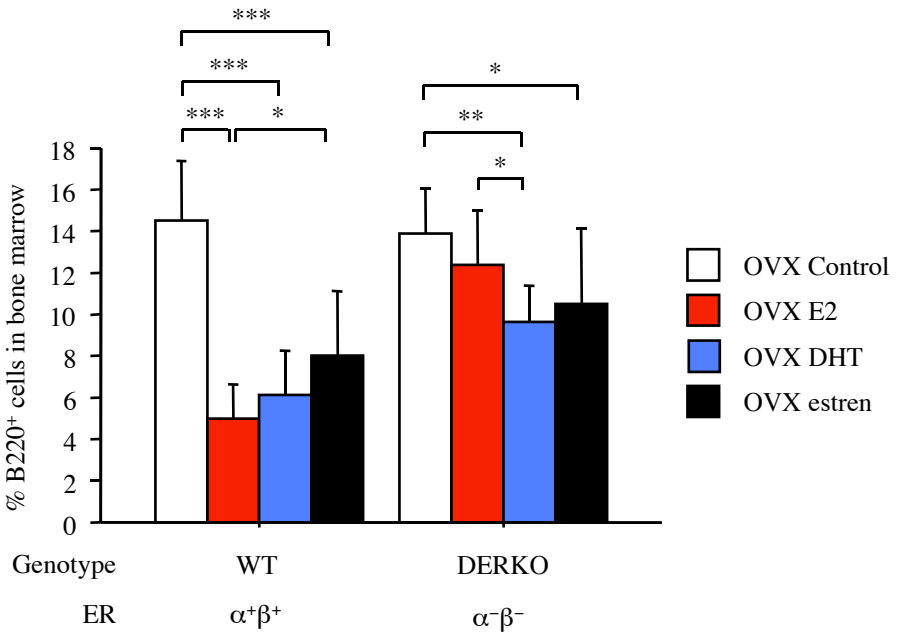


Figure 16: Both estren and DHT reduce the frequency of B lymphopoietic cells in the bone marrow, independent of ERs. 11-months-old female OVX WT and DERKO mice were treated with 0.7 μg E2/mouse/day, 120 μg DHT/mouse/day or 75μg estren/mouse/day during four weeks. Control mice received olive oil. As expected, the E2-mediated reduction of $B220^+$ cells in bone marrow of WT mice was lacking in DERKO mice. Treatment with estren or DHT resulted in a lower frequency of $B220^+$ cells in bone marrow of both WT and DERKO mice. One-way ANOVA followed by Fisher's test was used to compare data from mice in different treatment groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Results are presented as mean ± SD.

SMGs are sexually dimorphic in rodents, resulting in larger granular convoluted tubules in males compared to females [78-80]. In this study, the SMG sections were scored according to their androgen phenotype (fig. 9). Results showed that mice treated with either estren or DHT typically displayed an increased androgen phenotype compared to mice treated with E2 or control (fig. 17).

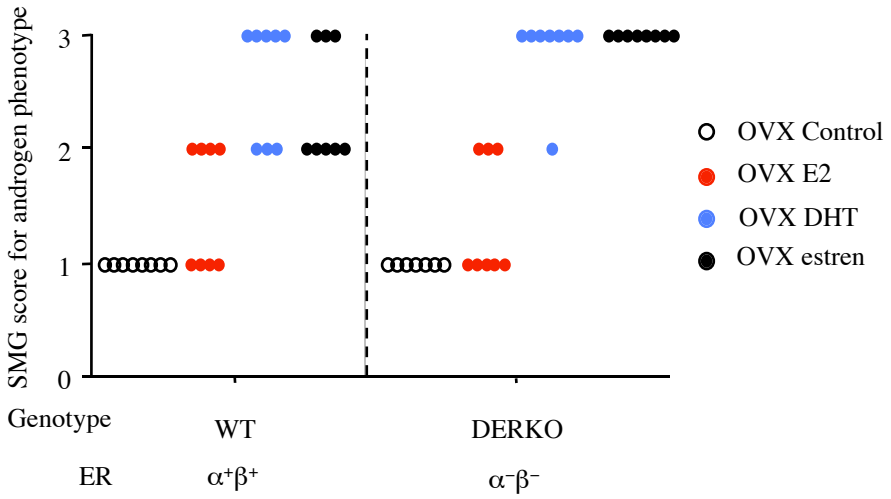


Figure 17: Both estren and DHT increase the androgen phenotype score in submandibular glands independent of ERs. 11-months-old female OVX WT and DERKO mice were treated with 0.7 μg E2/mouse/day, 120 μg DHT/mouse/day or 75μg estren/mouse/day during four weeks. Control mice received olive oil. E2 treatment did not dramatically affect the androgen phenotype of SMGs, in either WT or DERKO mice. The SMG score for androgen phenotype was high for both DHT and estren treated mice, of both WT and DERKO genotype, when compared to control mice.

SMGs in untreated 18-month-old WT, ERKO ($ER\alpha^{-}\beta^{+}$), BERKO ($ER\alpha^{+}\beta^{-}$), and DERKO ($ER\alpha^{-}\beta^{-}$) mice were investigated. We found that aged female ERKO mice displayed an increased SMG weight, size and androgen phenotype compared to aged mice of the other genotypes (fig. 18 A-C). We have previously shown that female ERKO mice have higher serum levels of testosterone compared to WT mice [87]. Therefore, high levels of testosterone in the ERKO mice could be responsible for the increased SMG weight, size and androgen phenotype shown in figure 18.

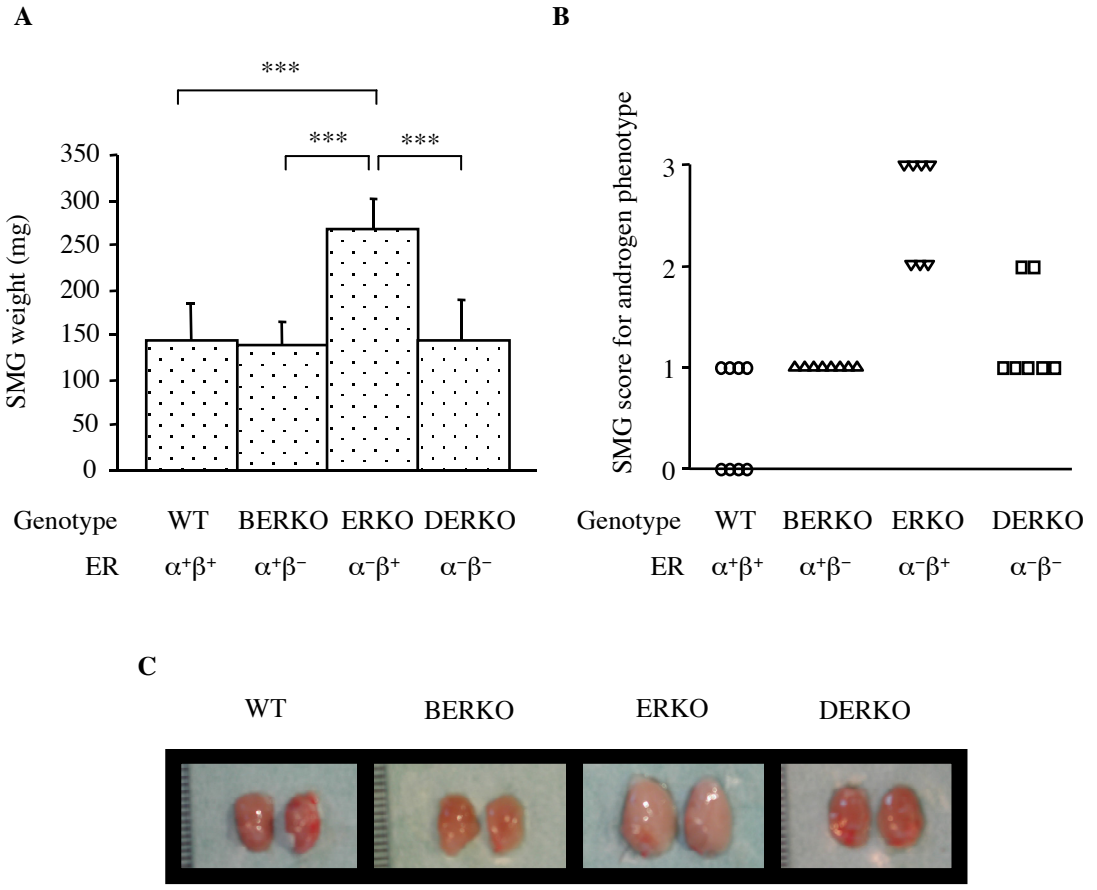


Figure 18: Aged female ERKO mice display an androgen phenotype of submandibular glands. Untreated 18-month-old WT, ERKO, BERKO, and DERKO mice were investigated. Aged female ERKO mice displayed an increased SMG weight **(A)**, androgen phenotype **(B)** and size **(C)**. One-way ANOVA followed by Fisher's test was used to compare data from mice of different genotypes in **(A)**. *** $P < 0.001$. Results are presented as mean ± SD.

GENERAL DISCUSSION

Estrogen is a steroid hormone that has an indispensable importance in female development and maturation, but also in a variety of other biological systems. Results form several research groups suggest that estrogen plays an active role in, among others: bone metabolic, immunological, neurological and rheumatic conditions. For example, it is well known that estrogen replacement has positive effects on postmenopausal osteoporosis. Furthermore, estrogen ameliorates the course of experimental autoimmune encephalomyelitis (EAE) [88] and collagen type II induced arthritis (CIA) [89], as well as their human counterparts multiple sclerosis (MS) [90] and rheumatoid arthritis (RA) [91]. The biological mechanisms responsible for the influence of estrogen in these conditions are largely unknown; therefore research in this field is of great importance. In this thesis we have focused on studying the effects of estrogen on the immune system, using estrogen receptor knock-out mice.

Estrogen receptor knock-out mice have proven to be valuable tools in defining the mechanisms by which estrogen exerts its effects in various systems. Some of the early reports using ERα and ERβ knock-out mice, revealed that intact female ERKO ($ER\alpha^{-}\beta^{+}$) and DERKO ($ER\alpha^{-}\beta^{-}$) mice have highly elevated serum levels of estradiol, while the levels in female BERKO ($ER\alpha^{+}\beta^{-}$) mice are normal [87, 92]. This suggests that the negative feedback system of estradiol is mediated via ERα. Furthermore, ERKO mice of both sexes are infertile while female BERKO mice have reduced fertility.

It is well known that gonadectomy stimulates, and estrogen treatment potently down-regulates B lymphopoiesis in the bone marrow of both female and male mice [51, 81, 83]. However, the mechanisms behind this are not fully understood. Direct action of estrogen on small populations of very early B lineage cells is one possible pathway [56, 57]. Furthermore, IL-7 is a B cell maturation factor produced by stromal cells, and lack of IL-7 signalling results in a decrease of B lymphopoietic cells [93, 94]. Therefore, another possibility for the estrogen-mediated suppression of B lymphopoiesis could be indirect, through impaired release of IL-7 from bone marrow stromal cells expressing both ERα and ERβ. Accordingly, in paper I, we show that both ERα and ERβ are required for the E2-induced decreased frequency of B

lymphopoietic cells in the pro-B and mature B cell fraction in the bone marrow (fig. 19). Estrogen is known to increase Ig production from B cells [59], and in paper I we show that ERα alone is required for the E2-mediated increased frequency of Ig producing cells in both bone marrow and spleen (fig. 19). It has been shown that ERs are present in peripheral B cells [55], however there are conflicting data whether only ERα or both receptors are transcribed [86, 95].

The immune responsiveness declines with age and it becomes a critical issue when the host is required to mount an immune response to a novel pathogen or a vaccination. Increased age results in thymic atrophy that involves loss of thymic epithelial cells and a decrease in thymopoiesis. Several hypotheses have been proposed to explain the mechanisms underlying the age-associated thymic involution, including a decline in the supply of T cell progenitors from the bone marrow, alterations in the productive rearrangement of the TCR, loss of cells within the thymic environment, or alterations in the levels of hormones, cytokines and growth factors [8]. Still, the specific mechanisms regarding age-induced thymic involution remains poorly understood. At puberty the thymic weight decrease rapidly. The increased levels of sex steroids, and decreased levels of growth hormone and insulin-like growth-factor-1, are believed to play a role. Furthermore, receptors for these hormones are widely distributed in thymocytes and thymic stromal cells [96]. IL-7 is a growth factor for T lymphopoietic cells and has been extensively studied as a potential key-factor for regulation of the age-induced changes in the thymic microenvironment. Some reports show that the production of IL-7 declines in old mice, however not all researchers agree with this observation [8, 96]. It has been claimed that there are no significant differences in the frequencies of SP and DP T cell subsets between young and aged animals, but significant differences in the frequencies of certain DN T cell subsets in the old thymus has been shown [8, 97]. Accordingly we found no differences in the frequencies of SP T cells between young and aged animals (Islander U, unpublished results), but in paper II we show an age-induced decreased frequency of DP T cells. Furthermore, we also show that the previously reported higher frequencies of DP T cells in $ER\alpha^+$ mice compared to $ER\alpha^-$ [44], is preserved also in very old mice (fig. 19).

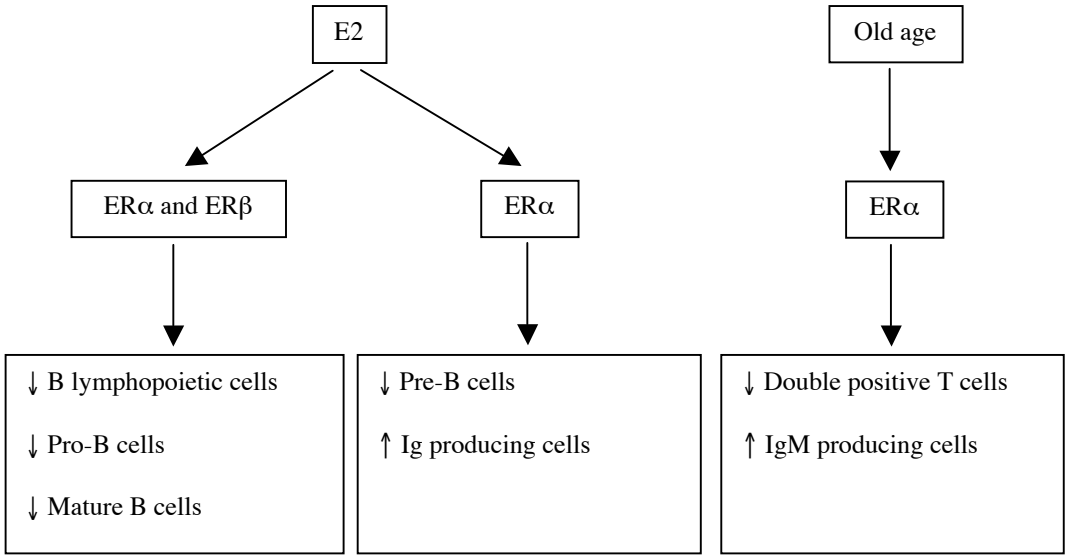


Figure 19: Schematic summary of ERα and ERβ mediated effects on lymphopoiesis and Ig producing cells after E2 treatment, or in aged female mice.

It has been suggested that the age-induced thymic involution indirectly leads to decreased production of B cells from the bone marrow [10]. Supporting this, it has been shown that the number of B lineage cells in the bone marrow decreases during ageing at a rate similar to, but somewhat later than the thymic involution [98]. Furthermore, Szabo *et al* detected IL-16 in supernatants from activated T cell cultures and showed that administration of IL-16 to thymic-deprived nude and old mice, partly reversed the impaired B cell development [99]. In accordance with our results published in paper II, it has been reported that the absolute numbers of B cells in both the pro-B and the pre-B cell fractions are reduced in aged individuals [100]. Also, it has been suggested that this reduction may be attributable to impaired release of IL-7 from bone marrow stromal cells or impaired signal transduction via the IL-7 receptor [100-102]. The serum concentrations of IgM, IgG and IgA increase with age, and the frequency of Ig secreting B cells increase two- to ten-fold during ageing. The basis for this B cell activation is not clear but increased production of IL-4 and IL-6 by T cells in old mice has been claimed to play a role [10]. Furthermore, the affinity of the antibodies are lower and the levels of auto-antibodies increase in aged individuals [9]. In paper II we show that the frequency of IgM producing cells in bone marrow increases with age in WT mice, but no significant differences was detected in IgG and

IgA producing B cells. Furthermore, ERα alone is required for the age-induced increased frequency of IgM producing B cells (fig. 19).

There has been a dispute for decades whether or not replacement of estrogen after menopause is beneficial for women. Hormone replacement therapy (HRT) is highly effective in relieving climacteric symptoms and preventing osteoporosis, and during the 1980s and 1990s the advantages seemed to outweigh the disadvantages. However, in the last few years, the risks have been considered significantly higher than the benefits. This is due to data from large clinical trials, such as the Women's Health Initiative (WHI), demonstrating increased risk of breast cancer, stroke and coronary heart disease after HRT [103]. There is much more to learn about the molecular actions of estrogen in each biological system, and also in understanding the interactions between different organ systems when estrogen levels decline and after HRT.

Selective ER modulators (SERMs) are synthetic ligands with tissue-specific agonistic or antagonistic properties. Tamoxifen is an ER antagonist used for treatment of estrogen-responsive breast cancer, but it has been recognized to have agonistic effects on bone and uterus. Raloxifene (Evista) has been shown to have antagonistic effects in breast tissue and agonistic effects on bone, making it approved for treatment of postmenopausal osteoporosis. Finally, ICI 182,780 (Faslodex) acts as a peripheral ER antagonist lacking all agonistic effects, and is used for treatment of estrogen-responsive breast cancer.

The selectivity of SERMs is a result of receptor conformational changes in response to binding of different ligands. The binding of estrogen to the ligand-binding domain (LBD) of the ERs induces a conformational change that facilitates binding of the ligand/receptor complex to DNA, associating with co-regulatory proteins, and start of transcription. Binding of Tamoxifen or Raloxifene to the LBD induces a conformational change that block the AF-2 domain on the receptor and consequently the association with co-regulators, resulting in a disability to start transcription. However the AF-1 domain is unblocked and still able to initiate gene expression. Different cell types harbour different co-regulatory proteins that are selective in

binding to ERα and/or ERβ. Therefore, depending on the cell type and the co-regulators present, Tamoxifen and Raloxifene have agonistic or antagonistic properties in different tissues. In contrast, the pure ER antagonist ICI 182,780 blocks both AF-1 and AF-2 which results in a complete inhibition of ERα and ERβ transcriptional activity [41, 104].

There is an ongoing search in finding new synthetic estrogen-like substances that reproduce only the beneficial effects of estrogen. Estren is a synthetic compound with structural similarities to estrogen, that was first described by Manolagas and colleagues [31]. They proposed that sex steroids affect reproductive tissues by classical genomic signalling, while the bone sparing effect of sex steroids is mediated through a non-genomic pathway. It was also suggested that ERα, ERβ or the androgen receptor (AR) can transmit the non-genomic signalling pathway irrespective of whether the ligand is an estrogen or an androgen. Furthermore, they showed that treatment with estren increases bone mass in OVX mice without affecting the reproductive organs, suggesting that estren is a mechanism-specific compound that only reproduces the non-genomic signalling of estrogen and thus can affect target cells also through the AR [31-35] (fig. 20). Due to these mechanism-specific properties, estren was presented as a potential future drug for treatment of postmenopausal osteoporosis.

However using ERα and ERβ knock-out mice, we recently demonstrated that estren has a moderate uteri proliferative effect, and that the trabecular bone sparing effect of E2 *in vivo* is mediated only via ERs and not via the AR (fig. 20) [36]. Using ERα and ERβ expressing reporter cell lines, we also showed that estren has the capacity to exert genomic effects via both ERα and ERβ. In conclusion, results from that study demonstrate that estren has the capacity to affect both bone and reproductive organs through classic genomic signalling via ERs [36].

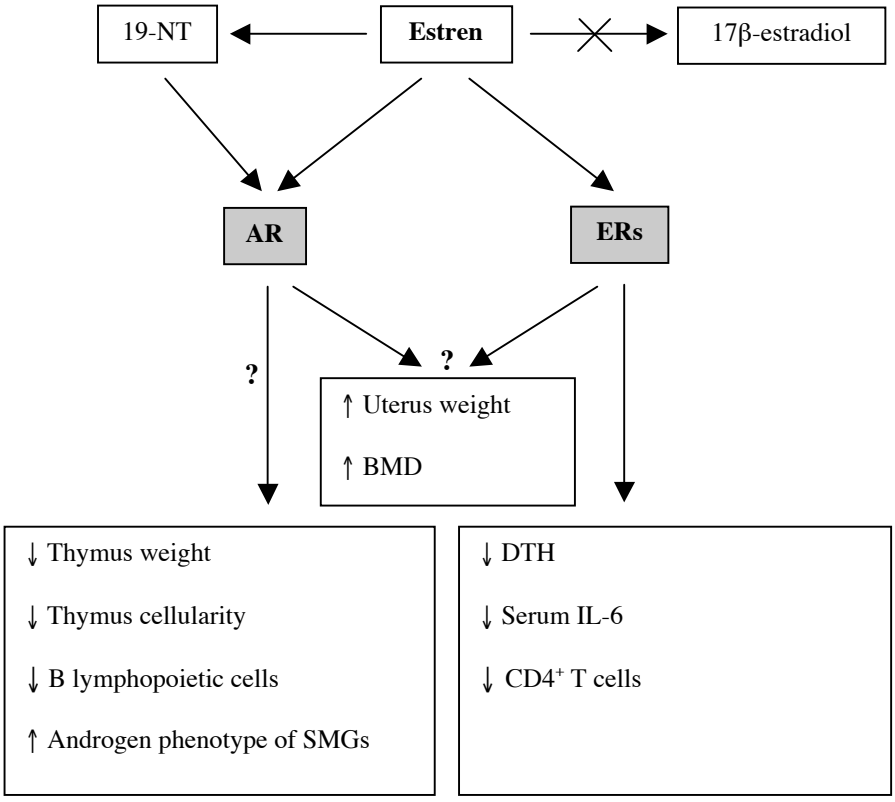


Figure 20: Schematic summary of steroid receptor signalling pathways for the effects of 4-estren-3α, 17β-diol (estren) on uterus, bone mineral density (BMD), immune responses and phenotype of submandibular glands (SMGs). Estren-mediated stimulation of uterus weight and BMD is mediated through activation of the ERs [36], the AR, or both receptors [31]. Treatment with estren inhibits lymphopoiesis and induces an increased androgen phenotype of SMGs, which are not mediated through ERs, but instead possibly through the AR (papers III and IV). Supporting this, it has been shown that estren can be metabolized into 19-nortestosterone (19-NT), a testosterone that is known to bind to the AR [37]. In contrast, the suppressive effects of estren on delayed type hypersensitivity (DTH) reaction, levels of the pro-inflammatory cytokine IL-6 in serum and the frequency of $CD4^+$ T cells in spleen, are dependent on ERs (paper III). These effects are not due to conversion of estren into 17β-estradiol, since treatment with estren does not result in increased serum levels of 17β-estradiol.

A number of reports have shown that estren can induce genomic effects via both the ERs and the AR [37-40]. Interestingly, Centrella *et al* also showed that estren can be metabolized into 19-nortestosterone (19-NT) (fig. 20), and that 19-NT binds to the AR with an affinity that is approximately 40% of that of dihydrotestosterone [37]. We have studied the effects of estren treatment on T and B lymphopoiesis and inflammatory responses (fig. 20). In papers III and IV we show that treatment with

estren reduces the thymus weight, cellularity and frequency of B lymphopoietic cells in bone marrow through ER-independent pathways. Furthermore, we also show that estren treatment induces an androgen phenotype of SMGs, and that the effects of estren are similar to those of 5α-dihydrotestosterone (DHT). It is well known that AR stimulation inhibits B and T lymphopoiesis [84, 85, 105], and results in increased androgen phenotype of SMGs [78-80]. Therefore the non-ER-mediated effects of estren could be mediated through the AR. However, our results do not directly prove any AR mediated effects of estren. In order to do that, AR knock-out mice or AR antagonists could be used. In contrast, the suppressive effects of estren on DTH reaction, levels of the pro-inflammatory cytokine IL-6 in serum and the frequency of $CD4^+$ T cells in spleen, are dependent on ERs. These effects are not due to conversion of estren into estradiol, since treatment with estren does not result in increased serum levels of 17β-estradiol (fig. 20).

Studies of how estrogen affects the immune system is important in order to reveal the mechanisms behind estrogen related conditions, for example the strong female susceptibility to autoimmune diseases. In this thesis we have focused on studying the effects of estrogen signalling through ERα and ERβ, and our data have added to the knowledge about the effects and molecular actions of estrogen. The search for new potential drugs that mimic the beneficial effects of estrogen, but lack the negative, is ongoing. Treatment with estren resulted in effects similar to those found after exposure to estrogen, however with the ability to use alternative signalling pathways. Although estren no longer is regarded as a promising candidate for treatment of postmenopausal osteoporosis, due to the reported proliferative effects on reproductive organs as well as its androgenic properties, our studies have contributed with valuable information in the future search for new potential hormone therapies.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Könshormonet östrogen spelar en viktig roll i utvecklingen av den kvinnliga kroppen, men det kan också påverka många andra biologiska system och funktioner, till exempel immunförsvaret. Autoimmuna sjukdomar är tillstånd där kroppens immunförsvar attackerar den kroppsegna vävnaden och därmed orsakar skada. Kvinnor drabbas i större utsträckning än män av autoimmuna sjukdomar, och man tror att östrogen här kan spela en roll. Exempel på autoimmuna sjukdomar är Systemisk Lupus Erythematosus (SLE) och ledgångsreumatism (RA). Studier har visat att om kvinnor med RA behandlats med östrogen så blir de bättre i sin sjukdom. Däremot blir kvinnor med SLE ofta sämre vid graviditet då östrogennivåerna i kroppen är höga. Mekanismerna för hur östrogen påverkar immunsystemet är inte fullständigt klarlagda och mer forskning inom området är nödvändig.

Det har debatterats i årtionden om hormonbehandling efter klimakteriet är fördelaktigt för kvinnor. Under 1980- och 1990-talet ansågs de positiva effekterna överväga, men under de senaste åren har riskerna bedömts vara större än fördelarna. Detta beror till stor del på en stor klinisk studie i USA som visade att hormonbehandling till äldre kvinnor kan öka risken för bröstcancer, hjärnblödning och hjärt- kärlsjukdom. Detta har lett till att sökandet har intensifierats efter syntetiska östrogenliknande molekyler, som har östrogenets positiva effekter men saknar de negativa. Vi har studerat molekylen estren, som är lik östrogen i strukturen, och som presenterades för några år sedan av en grupp forskare i USA.

Immunsystemet består av olika vita blodkroppar som skall skydda oss mot bakterier, virus och andra mikroorganismer. Exempel på vita blodkroppar är T celler och B celler. T celler utvecklas i tymus (brässen) och deltar bland annat vid inflammation. B celler utvecklas i benmärgen och har bland annat till uppgift att producera antikroppar. Östrogenbehandling medför en minskad produktion av T celler från tymus och B celler från benmärg, och det medför även minskad så kallad T cellmedierad inflammation hos möss. Däremot ger östrogenbehandling en ökad produktion av antikroppar ifrån B celler.

Östrogen påverkar cellerna i kroppen genom att binda till två olika östrogenreceptorer, ERα och ERβ. Genom att skapa möss som saknar östrogenreceptorerna och jämföra dessa med vanliga möss, så kan man undersöka vilka effekter på immunsystemet som härstammar från östrogenstimulering via dessa receptorer. Till studierna i avhandlingen använde vi oss av möss som saknar ERα, ERβ, eller både ERα och ERβ, och jämförde dessa med normala möss. Målet var att undersöka östrogenreceptorernas betydelse vid B cellsutveckling i benmärg, samt deras antikroppsproduktion. Dessutom studerade vi östrogenreceptorernas betydelse i det åldrade immunsystemet genom att jämföra 18 månader och 4 månader gamla möss. Vi undersökte även östrogenreceptorernas betydelse för estrens effekter på T och B cellsutveckling, och vid T cellmedierad inflammation.

Vi fann att både ERα och ERβ måste finnas för att östrogenbehandling skall leda till minskad B cellsutveckling, men endast ERα krävs för att östrogenbehandling skall ge ökad antikroppsproduktion. Hög ålder medför att fler B celler producerar antikroppar, och vi kunde visa att endast ERα behövs för att uppnå den effekten. Vi kunde visa att behandling av mössen med estren gav minskad inflammation och att detta medierades via östrogenreceptorer. Behandling med estren medförde också en minskning av både T och B cellsutvecklingen men detta skedde helt oberoende av östrogenreceptorerna. Studierna kunde inte bevisa men antydde att de estreninducerade effekter som inte medierades via östrogenreceptorer, troligtvis uppstod på grund av signalering genom receptorn för det manliga könshormonet testosteron.

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