Immune Regulation by Selective Estrogen Receptor Modulators

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ABSTRACT

At menopause, the levels of estrogen decline, leading to loss of estrogen-mediated protective effects on bone and an increased risk of osteoporosis. Hormone replacement therapy, containing estrogen, has been used for many years to prevent and treat osteoporosis in postmenopausal women. However, the estrogen receptor agonistic effects on the reproductive organs increases the risk of developing cancer. Therefore, selective estrogen receptor modulators (SERMs) have been developed, that can act as tissue-specific estrogen receptor agonists or antagonists. This enables SERMs to mediate the positive effects of estrogen on bone metabolism while avoiding side effects on the reproductive organs.

Estrogen has a number of effects on the immune system; it decreases B- and T lymphopoiesis and increases antibody production. In addition, estrogen potently inhibits T-cell dependent inflammation and suppresses synovitis and inflammation-mediated bone loss in arthritis. Similarly to estrogen, the second-generation SERM raloxifene suppresses B-cell development and ameliorates arthritis. However, raloxifene lacks effects on antibody production and T-cell dependent inflammation.

Lasofoxifene and bazedoxifene are third-generation SERMs, approved for treatment of postmenopausal osteoporosis. The bone-protective properties of these compounds are well documented; however the effects of lasofoxifene and bazedoxifene on the immune system have not earlier been assessed. Therefore, the aim of the studies included in this thesis was to investigate the immune-regulating effects of these third-generation SERMs. We found that lasofoxifene and bazedoxifene suppressed B-cell development in ovariectomized (ovx) mice, but lacked effects on antibody production and on T-cell development. Furthermore, lasofoxifene and bazedoxifene did not suppress T-cell dependent inflammation, but potently inhibited synovitis and bone loss in mice subjected to experimental postmenopausal arthritis. Phenotypic analysis of lymph nodes in arthritic mice showed that while estrogen increased a subpopulation of dendritic cells (DCs), as well as T helper 17 (Th17) cells, B cells and surface markers connected to antigen-presentation on B cells, the SERMs lacked these effects.

In conclusion, the third-generation SERMs lasofoxifene and bazedoxifene suppressed experimental arthritis and inhibited B-cell development in ovx mice, but lacked effects on T-cell development and T-cell dependent inflammation. SERMs also lacked effects on lymph node DCs, B cells and T cells in arthritic mice. Therefore, further investigation is needed to find the target for the suppressive effects of SERMs on arthritis. Nonetheless, the anti-arthritic effects of the third-generation SERMs suggest possibility for an extension of the clinical indications of these drugs to include also postmenopausal RA.

Keywords: Mice, lasofoxifene, bazedoxifene, raloxifene, estrogen, osteoporosis, B cells, T cells, rheumatoid arthritis
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POPULÄRVETENSKAPLIG SAMMANFATTNING

Vårt immunförsvar finns för att skydda oss från mikroorganismer, t.ex. bakterier och virus, som kan orsaka infektioner. När mikroorganismer försöker ta sig in i vår kropp och orsaka sjukdom aktiveras en rad olika celler i immunförsvaret (vita blodkroppar) som då börjar attackera och eliminera inkräktarna. Genom att kunna skilja mellan vad som är främmande och vad som tillhör vår kropp attackerar cellerna i immunförsvaret bara inkräktande mikroorganismer och inte kroppsegna strukturer. Immunförsvaret är uppdelat i två system; det första är det medfödda immunförsvaret som känner igen gemensamma strukturer som finns på mikroorganismer och då svarar snabbt genom att förstöra mikroorganismerna och förhindra deras förökning i kroppen. Det andra systemet är det förvärvade immunförsvaret som svarar långsammare men är noggrannare och känner igen mer specifika strukturer på inkräktarna. Cellerna i det förvärvade immunförsvaret utvecklar också ett minne för vad de tidigare stött på, vilket gör att de kan svara snabbare nästa gång de träffar på samma struktur. Det förvärvade immunförsvaret utgörs av så kallade B-celler och T-celler, där B-celler har som huvuduppgift att producera molekyler som kallas antikroppar. Antikroppar binder till ytan av mikroorganismer och signalerar till cellerna i det medfödda immunförsvaret att dessa inkräktare ska förstöras. T-celler har flera uppgifter men en av de viktigare är att producera särskilda signaleringsämnen som kallas cytokiner och är nödvändiga för aktivering av olika delar av immunförsvaret.

Om en bakterie eller ett virus börjat föröka sig svarar kroppen med att starta en inflammation. Inflammation karaktäriseras bland annat av svullnad och rodnad, på grund av en ökad blodtillströmning och ett ökat antal vita blodkroppar på platsen som försöker göra sig av med mikroorganismerna. Ibland när cellerna i immunförsvaret ska känna igen främmande ämnen blir det dock fel och de börjar istället attackera strukturer i vår egen kropp. När detta händer uppstår det som kallas autoimmunitet, vilket ordagrant betyder ”immunitet mot sig själv”. Autoimmuna sjukdomar karaktäriseras ofta av att man får en inflammation, men då till följd av att immunsystemet attackerar en kroppsegen struktur och inte en mikroorganism. Det finns många olika autoimmuna sjukdomar men i denna avhandling ligger fokus på ledgångsreumatism eller reumatoid artrit, förkortat RA. Patienter med RA har en kronisk inflammation i lederna och får även en förstörelse av skelettet som finns runt lederna. Ofta drabbas de också av allmän benskörhet, så kallad osteoporos.

Inflammation kan även uppstå om kroppen utsätts för främmande ämnen, såsom kemikalier, som immunförsvaret börjar reagera på. Vissa typer av sådana reaktioner är helt beroende av T-celler för att äga rum. Dessa kallas T-cellsberoende inflammationer.

Det är välkänt att hormoner kan reglera funktionen av immunförsvaret. Man har länge vetat att kvinnor drabbas oftare än män av autoimmuna sjukdomar, vilket har lett till ett stort intresse för vad det kvinnliga könshormonet östrogen har för effekter på vårt

immunförsvar. Genom att studera immunförsvaret i både djur och människor under graviditet, då östrogennivåerna är höga, samt under behandling med östrogen, har man kunnat kartlägga en rad effekter. Östrogen blockerar bildningen av både B-celler och T-celler, men kan samtidigt hämma inflammation som beror på T-celler och vissa autoimmuna sjukdomar. RA är en sådan sjukdom där östrogen har visat sig ha fördelaktiga effekter. Därför är det inte förvånande att fler kvinnor drabbas av RA efter klimakteriet, då östrogenproduktionen stannar av. Östrogen har även skyddande effekter på benomsättningen i kroppen vilket också förklarar varför många kvinnor drabbas av osteoporos efter klimakteriet. Under många år behandlade man kvinnor som genomgått klimakteriet med hormonersättningsterapi som innehöll östrogen. Då såg man att man kunde förhindra uppkomsten av osteoporos, men tyvärr fann man även att denna behandling ledde till en ökad risk att drabbas av cancer i livmodern, vilket gjorde att man till stor del slutade behandla kvinnor med hormonersättning. Istället började man utveckla syntetiska läkemedel som kan agera som östrogen i vissa delar av kroppen, t.ex. skelettet, men som saknar östrogeneffekter på livmodern, för att på så sätt undvika den ökade cancerrisken. Dessa läkemedel kallas selektiva östrogenreceptormodulerare (SERM). Lasofoxifen och bazedoxifen är två läkemedel som tillhör den tredje generationen av SERM. Lasofoxifen och bazedoxifen har fördelaktiga effekter på bentätheten i skelettet och bidrar inte till en ökad risk för livmodercancer, vilket gör dem lämpliga för behandling och förebyggande av benskörhet hos kvinnor efter klimakteriet. Då inga tidigare studier har tidigare visat hur dessa läkemedel påverkar immunförsvaret utgör denna fråga huvudmålet med arbetena i avhandlingen.

De tre arbeten som ingår i denna avhandling beskriver effekter av lasofoxifen och bazedoxifen: (I) på bildningen av B-celler och på antikroppsproduktion, (II) på bildningen av T-celler och på T-cellberoende inflammation och (III) på experimentell artrit och på generell benskörhet i samband med artrit.

I alla tre arbeten har vi använt oss av kastrerade honmöss som saknar produktion av kroppseget östrogen, för att efterlikna situationen efter klimakteriet. Förutom möss som behandlades med SERM inkluderade vi även möss som behandlades med östrogen eller med placebo som kontrollgrupper. För att titta på hur SERMen påverkar bildningen av B- och T-celler behandlade vi mössen och undersökte sedan hur cellerna utvecklats. För att undersöka SERMens effekt på T-cellsberoende inflammation användes en modell där en retande kemikalie penslades på mössen och svullnaden som uppstod var ett mått på inflammation. För att undersöka SERMens effekter på artrit användes en musmodell för artrit, där man ger mössen ett protein som finns i ledbrosk – kollagen, blandat med bakterier, vilket leder till att immunförsvaret aktiveras och börjar attackera kollagenet i lederna. Detta ger en sjukdom som liknar RA hos människor.

Vi fann att lasofoxifen och bazedoxifen skiljde sig från östrogen i vissa immunologiska avseenden, men hade liknande effekter i andra. Till skillnad från

östrogen kunde inget av dessa läkemedel varken hämma utvecklingen av T-celler eller den T-cellsberoende inflammation vi undersökte. De kunde heller inte öka produktionen av antikroppar från B-celler. Däremot kunde lasofoxifen och bazedoxifen, likt östrogen, hämma bildningen av B-celler och de kunde även minska både ledinflammation och benskörhet i möss med artrit. Exakt hur lasofoxifen och bazedoxifen påverkar immunförsvaret för att hämma artrit är dock ännu oklart.

Sammanfattningsvis har arbetena i denna avhandling bidragit till att klargöra vilka effekter lasofoxifen och bazedoxifen har på olika delar av immunförsvaret och på utveckling av inflammatoriska tillstånd. Vårt mål är att fortsätta undersöka hur dessa läkemedel även påverkar andra autoimmuna sjukdomar.

LIST OF PAPERS

This thesis is based on the following papers referred to in the text by their Roman numerals

I. Angelina I. Bernardi, Annica Andersson, Louise Grahnemo, Merja Nurkkala-Karlsson, Claes Ohlsson, Hans Carlsten and Ulrika Islander. **Effects of lasofoxifene and bazedoxifene on B cell development and function.**
Immunity, Inflammation and Disease. 2014 Dec;2(4):214-225.

II. Angelina I. Bernardi, Annica Andersson, Alexandra Stubelius, Louise Grahnemo, Hans Carlsten and Ulrika Islander. **Selective estrogen receptor modulators in T cell development and T-cell dependent inflammation.**
Accepted for publication in *Immunobiology*, February 2015.

III. Annica Andersson, Angelina I. Bernardi, Alexandra Stubelius, Merja Nurkkala-Karlsson, Claes Ohlsson, Hans Carlsten and Ulrika Islander. **Selective estrogen receptor modulators lasofoxifene and bazedoxifene inhibit joint inflammation and osteoporosis in experimental postmenopausal arthritis.**
Submitted Manuscript

OTHER PUBLICATIONS

Other publications not included in the thesis:

Ola Grimsholm*, Weicheng Ren*, Angelina I. Bernardi, Haixia Chen, Giljun Park, Alessandro Camponeschi, Dongfeng Chen, Berglind Bergmann, Nina Höök, Sofia Andersson, Anneli Strömberg, Inger Gjertsson, Susanna Cardell, Ulf Yrlid, Alessandra De Riva and Inga-Lill Mårtensson. **Absence of surrogate light chain results in spontaneous autoreactive germinal centres expanding VH81X expressing B cells.** Accepted for publication in *Nature Communication*, April 2015.

Ren W, Grimsholm O, Bernardi AI, Höök N, Stern A, Cavallini N, Mårtensson IL. **Surrogate light chain is required for central and peripheral B-cell tolerance and inhibits anti-DNA antibody production by marginal zone B cells.** *Eur J Immunol*. 2014 Dec 27. doi: 10.1002/eji.201444917. [Epub ahead of print].

Johansson ME, Ulleryd MA, Bernardi A, Lundberg AM, Andersson A, Folkersen L, Fogelstrand L, Islander U, Yan ZQ, Hansson GK**. a7 Nicotinic acetylcholine receptor is expressed in human atherosclerosis and inhibits disease in mice-brief report**. *Arterioscler Thromb Vasc Biol*. 2014 Dec;34(12):2632-6.

TABLE OF CONTENTS

ABBREVIATIONS

CD	Cluster of differentiation
TNF	Tumor necrosis factor
TGF	Transforming growth factor
IL	Interleukin
IFN	Interferon
HRT	Hormone replacement therapy
RA	Rheumatoid arthritis
SERM	Selective estrogen receptor modulators
ovx	Ovarectomized
E2	Estradiol
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
ERE	Estrogen response elements
BMD	Bone mineral density
APC	Antigen-presenting cell
DC	Dendritic cell
MHC	Major histocompatibility complex
NK	Natural killer
IgHC	Immunoglobulin heavy chain
IgLC	Immunoglobulin light chain
Pro-B	Progenitor B
Pre-B	Precursor B
BCR	B-cell receptor
T1	Transitional 1
T2	Transitional 2
FO	Follicular
MZ	Marginal zone
BAFF	B-cell activating factor
GC	Germinal center
AID	Activation-induced deaminase
Bcl-2	B cell lymphoma 2
TCR	T-cell receptor
DN	Double-negative
DP	Double-positive
SP	Single-positive
Th	Helper T
Treg	Regulatory T cell
FoxP3	Forkhead box P3
DTH	Delayed-type hypersensitivity
M-CSF	Macrophage colony stimulating factor
RANK	Receptor activator of NF-kB
RANKL	Receptor activator of NF-kB ligand
OPG	Osteoprotegerin
COMP	Cartilage-oligomeric matrix protein
RF	Rheumatoid Factor
ACPA	Anti-Citrullinated Protein Antibodies
CII	Collagen type II
CIA	Collagen-induced Arthritis
CAIA	Collagen-antibody induced arthritis
AIA	Antigen-Induced Arthritis
SLE	Systemic lupus erythematous

INTRODUCTION

The immune system functions to protect us from infections by discriminating between foreign and endogenous structures. It comprises the unspecific innate immune system, which mediates a rapid response to invading microbes, and the adaptive immune system, which provides a specific response and develops immunological memory. However, the immune system does not work as an isolated system, but is regulated by e.g. the central nervous system and the endocrine system. The female sex hormones estrogens (comprising estrone, estradiol and estriol) have well-documented effects on the immune system, both during homeostasis and in autoimmunity. Human and experimental studies of the immune system in pregnancy and during estrogen treatment have established that estrogen potently modulates both the formation and effector functions of cells in the adaptive immune system; B- and T cells. In addition, the increased prevalence of autoimmunity in women further stresses the immunological role of estrogen. The incidence of rheumatoid arthritis (RA), an autoimmune condition characterized by inflammation in the joints and bone destruction, increases at menopause when estrogen levels decline, suggesting a protective role for estrogen in this disease. In addition, both pregnancy and estrogen treatment suppress inflammation and prevent bone loss in arthritis.

As treatment of postmenopausal women with hormone replacement therapy (HRT), containing estrogen and progesterone, is connected with severe side effects, selective estrogen receptor modulators (SERMs) have been developed to achieve the beneficial effects of estrogen on bone metabolism while avoiding the estrogenic side effects. The studies included in this thesis focus on immune regulation by SERMs. We have investigated the effects of the third-generation SERMs lasofoxifene and bazedoxifene on cells of the adaptive immune system, on experimental arthritis and on inflammation-mediated bone loss. In order to avoid the influence of endogenous estrogen, ovariectomized (ovx) mice have been used. Thus, the frame of this thesis aims at reviewing the immunological effects of estrogen and the second-generation SERM raloxifene, as determined by others, together with the results from the studies of the third-generation SERMs lasofoxifene and bazedoxifene in papers I-III.

ESTROGEN AND SELECTIVE ESTROGEN RECEPTOR MODULATORS

Estrogen

Estrogen is the common name for the female sex hormones estrone (E1), estradiol (E2) and estriol (E3), where E2 is most potent. E2 is mainly produced by the ovaries and is the predominant form during the reproductive years. E1 is the major estrogen found after menopause, while E3 is only found in significant levels during pregnancy. The effects of estrogen are mainly mediated by the classical estrogen receptors ERα and ERβ, cloned in 1986 [1] and 1996 [2], respectively. These receptors belong to the nuclear receptor family of transcription factors and consist of a ligand-binding domain and a DNA-binding domain [3]. ERα and ERβ share approximately 97% sequence similarity in the DNA-binding domain and 55 % in the ligand-binding domain [4]. The classical transcription pathway of ER activation includes ligand binding followed by receptor dimerization and binding to estrogen response elements (EREs) located in the promoter regions of estrogen-regulated genes [5, 6]. When bound to EREs, the ER interacts with co-regulating proteins, leading to modulation of transcription [7, 8] (Fig. 1, pathway 1). Apart from this classical transcription pathway, estrogen can also signal through the non-classical transcription pathway, via alternative non-ERE binding transcription factors, such as the SP-1 and AP-1 transcription factors [9, 10] (Fig. 1, pathway 2). In addition, there are membrane-associated estrogen receptors, such as GPR30, through which estrogen can modulate intracellular signalling pathways and cause transcriptional activity (Fig. 1, pathway 3) or non-genomic response [11, 12] (Fig. 1, pathway 4). Non-genomic response can also be generated through association of ERα to the membrane [13] (Fig. 1, pathway 4).

In addition to regulating female reproduction, estrogen has important bone-protective properties and affects the nervous system as well as the cardiovascular system. Furthermore, estrogen has various effects on the immune system; indeed, ERs are expressed on most cells of the innate and adaptive immune system [14, 15]. At menopause, the ovarian production of estrogen declines, which is associated with an increased risk of developing e.g. osteoporosis and vasomotor symptoms such as hot flushes. During the second half of the 20th century, postmenopausal women were frequently treated with HRT – containing estrogen and progesterone – a treatment that successfully decreased the symptoms arising from the loss of estrogen. However, clinical trials evaluating the long-term effects of HRT revealed that HRT increased the risk of coronary heart disease, stroke, deep venous thrombosis, breast cancer, and endometrial cancer [16, 17]. Consequently, the use of HRT drastically decreased, and the search for compounds with the ability to provide beneficial estrogenic effects while avoiding negative effects was initiated.

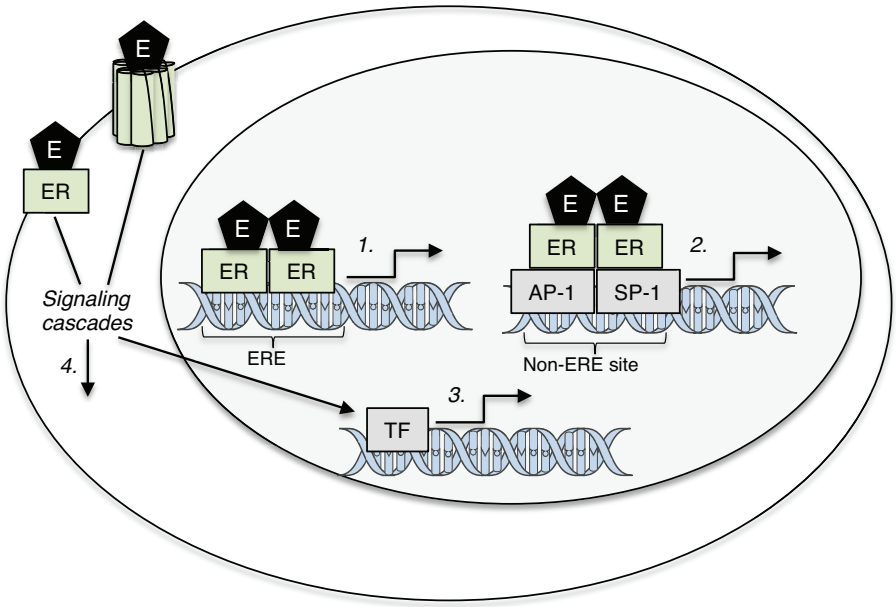

Figure 1. ER signalling pathways
1) The classical transcription pathway, 2) The non-classical transcription pathway, 3) Transcription by membrane-associated ER, 4) Non-genomic response by membrane-associated ER. E, estrogen; ER, estrogen receptor; ERE, estrogen-response element; TF, transcription factor.

Selective Estrogen Receptor Modulators

Selective estrogen receptor modulators (SERMs) are synthetic ER ligands able to exert ER agonistic effects in some tissues and ER neutral or antagonistic effects in other tissues. SERMs are primarily designed to mediate ER agonistic effects on bone, but have ER neutral or antagonistic effects on the breast and endometrium. Binding of SERMs to the ER induces a conformational change of the receptor followed by dimerization. This leads to either the recruitment of co-activators followed by activation of transcription, or the recruitment of co-repressors and inhibition of transcription (Fig. 2). Tissue selectivity of SERMs is determined by the distribution of ERα and ERβ and the availability of co-activators and co-repressors in the target tissue (Reviewed in [18]).

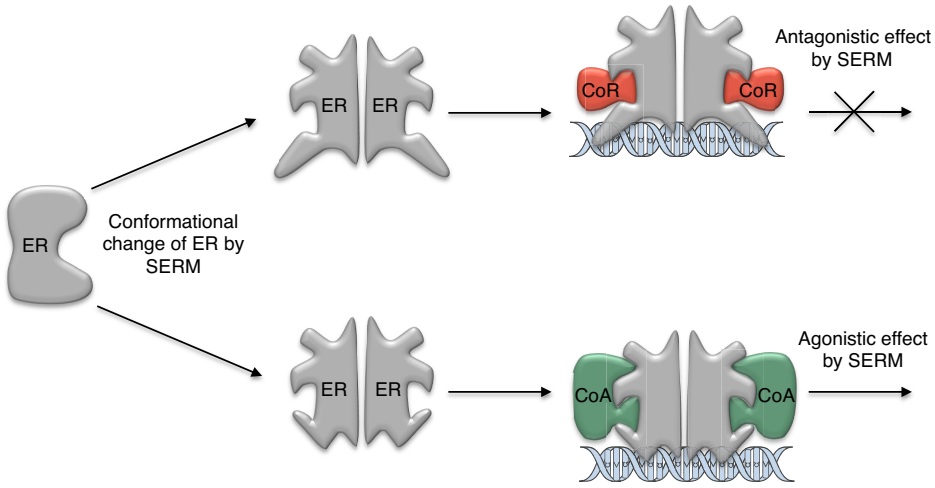

Figure 2. Mechanism of action of SERMs.
SERM binding to the ER leads to conformational changes of the receptor and recruitment of co-repressors and inhibition of transcription, or recruitment of co-activators and activation of transcription. ER, estrogen receptor; SERM, selective estrogen receptor modulator; CoR. co-repressor; CoA, co-activator.

There is currently a number of SERMs available and used for several indications, including prevention and treatment of breast cancer, osteoporosis and other postmenopausal symptoms. Tamoxifen was the first SERM to be approved by the FDA and was shown to prevent breast cancer in women at high risk [19], and to reduce mortality and prevent cancer recurrence in women with ER-positive breast cancer [20]. Furthermore, tamoxifen increases total bone mineral density (BMD) and reduces the overall risk of fractures in postmenopausal women with osteoporosis [21]. However, tamoxifen has ER-agonistic effects on the endometrium, leading to an increased risk of developing endometrial cancer [19]. Tamoxifen is currently used in the US and the EU for treatment of ER-positive breast cancer.

The second-generation SERM raloxifene was developed as an alternative to tamoxifene for breast cancer treatment. In addition to reducing the incidence of breast cancer [22], treatment with raloxifene also leads to an increase in lumbar spine BMD and a decreased risk of developing vertebral fractures [23, 24]. Raloxifene is currently approved for the prevention of breast cancer in the US and for the prevention and treatment of osteoporosis in the US and the EU [25]. Treatment with raloxifene causes a small increase in endometrial thickness, but no increased risk of endometrial hyperplasia or carcinoma [22]. In addition, treatment with raloxifene has been associated with a decrease in cardiovascular disease and serum lipids, but an increased

incidence of venous thromboembolism [24, 26]. We and others have reported that the effects of raloxifene in experimental animal studies are similar to those observed in clinical trials; decrease in the incidence of mammary tumours [27] and an increase in lumbar vertebral and total BMD ([28], Paper I), together with an increase in uterine wet weight ([29, 30], Paper I).

The third-generation SERMs lasofoxifene and bazedoxifene were recently approved for the prevention and treatment of postmenopausal osteoporosis. Lasofoxifene was approved in the EU 2009 but is not yet marketed while bazedoxifene is currently used in the EU and is under review for registration in the US. Lasofoxifene decreases the risk of both vertebral and non-vertebral fractures in postmenopausal women and improves lumbar spine BMD [31]. Lasofoxifene causes an increase in endometrial thickness, however, this is not accompanied by an increased risk of endometrial hyperplasia or carcinoma [32]. In addition, lasofoxifene has shown to lower serum lipids and reduce the risk of cardiovascular disease, but increase the risk of venous thromboembolism [31-33]. In experimental studies, lasofoxifene increases BMD in both male and female castrated animals and increases uterine wet weight in ovx mice ([34, 35], Paper I).

Bazedoxifene improves lumbar spine BMD, decreases the risk of vertebral fractures in postmenopausal women [36] and the risk of non-vertebral fractures in high-risk fracture patients [37]. Furthermore, bazedoxifene does not affect endometrial thickness [38]. Similarly to lasofoxifene, bazedoxifene causes a decrease in serum lipids and reduces the risk of cardiovascular disease, but increases the risk of venous thromboembolism [36, 39]. Bazedoxifene increases BMD in both male and female castrated animals, but does not influence uterine wet weight in ovx mice ([35, 40], Paper I).

In 2013, FDA approved a compound containing the combination of conjugated estrogens and bazedoxifene for the treatment of vasomotor symptoms and prevention of postmenopausal osteoporosis. By acting as an ER antagonist in the uterus, bazedoxifene blocks the ER-agonistic effects of estrogen on the endometrium, thereby reducing the risk of developing endometrial hyperplasia, while still obtaining the beneficial estrogenic vasomotor effects and bone-protective effects [41, 42].

THE IMMUNE SYSTEM

Introduction to the immune system

The immune system functions to protect the body from invading microbes such as bacteria and viruses that could cause infections. Upon pathogen encounter, the innate immune system is activated first, providing a quick, non-specific response – a response that is not modified upon repeated confrontations of a certain microbe. A number of cell types are included in the innate immune system. Neutrophils and macrophages are specialized in phagocytosis; i.e. they have the ability to ingest and eliminate pathogens and apoptotic cells through the production of toxic chemicals and degradative enzymes. Macrophages are also important as antigen-presenting cells (APCs), together with dendritic cells (DCs). APCs take up extracellular antigens and present them on major histocompatibility complex II (MHCII) molecules, thereby inducing adaptive immunity. In addition, macrophages produce cytokines and chemokines that attract neutrophils and lead to local inflammation. Also included in the innate immune system are natural killer (NK) cells, which are cytotoxic cells that kill tumour cells and cells infected with pathogens. In addition to these various cell types, innate immunity also includes the complement system, composed of proteins, which are activated by proteolytic cleavage and aid in elimination of microbes and production of inflammatory mediators.

The adaptive immune system is characterized by specificity. In contrast to the immediate response provided by the innate immune system, it takes several days for the adaptive immune system to be activated at the first confrontation of a pathogen. However, the adaptive immune system then provides a highly specific response and is able to recognize and remember the microbes, leading to an enhanced response upon repeated encounters. The adaptive immune system can be divided into humoral immunity and cell-mediated immunity, where humoral immunity includes protection against extracellular microbes while cell-mediated immunity mediates protection against phagocytosed and intracellular microbes. Humoral immunity is mediated by antibodies, which are produced by B cells. Antibodies are secreted into the circulation and help neutralize and eliminate microbes before they gain access to tissues, and also label the microbes for phagocytosis. In addition to its function in innate immunity, the complement system also helps B cells to mount proper immune responses. Cell-mediated immunity is provided by T cells; helper T cells are activated through antigen-presentation by APCs, resulting in the production of cytokines. Cytotoxic T cells are activated through antigen-presentation by infected cells, which results in the killing of the pathogen-infected cells.

B cells – mediators of humoral immunity

B cells are key components of the adaptive immune system through their unique capacity to produce antibodies against a large number of foreign antigens. An antibody consists of two immunoglobulin heavy chains (IgHCs) linked together with two immunoglobulin light chains (IgLCs) where the upper parts of both chains are variable and constitute the antigen-binding region, referred to as the fragment antigen-binding region (Fab fragment). The diversity in antigen specificity is achieved through stepwise gene recombination of the IgHC and IgLC loci during bone marrow B-cell development [43]. The constant part of the antibody – the fragment crystallisable region (Fc region) – determines the effector function of the antibody; the five antibody isotypes are IgM, IgD, IgG, IgA, and IgE, where IgM and IgD are expressed on naïve B cells. After antigen-priming, class switch recombination of the Fc part can occur, resulting in the changing of the antibody isotype into IgG, IgA or IgE. When Fc receptor-expressing cells bind to the Fc-part of the antibodies, effector mechanisms are activated. Briefly, IgM participates in complement activation and IgG mainly functions to promote phagocytosis by macrophages and DCs. IgA mediates mucosal immunity and IgE is involved in allergic responses.

B-cell development and maturation

B cells develop in the bone marrow from hematopoietic stem cells. Surface marker patterns together with rearrangement status of the IgHC and IgLC are used to define the different stages of bone marrow B-cell development (Fig. 3). B220 is the pan-B cell marker used to define all B cell stages during B-cell development and maturation. Using the Basel nomenclature [44], the first stage, termed the progenitor B (pro-B) cell stage, is defined by expression of surface markers c-kit, but not yet CD19. Here, the transcription factor paired box 5 (Pax5) is induced, which is crucial for B-lineage commitment [45]. Thereafter, CD19 is expressed, together with c-kit, defining the precursor B (pre-B) I cell stage. At the pre-BI stage, IgHC rearrangement is initiated and the surrogate light chain is expressed [46, 47]. If the rearrangement of IgHC is productive, the IgHC can pair with the surrogate light chain to form a pre-B cell receptor (pre-BCR), which is expressed on the surface of these cells. At this stage, the cells have lost the expression of c-kit and gained expression of CD25 and are termed large pre-BII cells [48]. The development from pro-B cells to large pre-BII cells is dependent on IL-7 secretion from stromal cells [49, 50]. Pro-B, pre-BI and large pre-BII cells all express IL-7 receptors that signal survival and proliferation [51]. After the pre-BII cells have left the cell cycle they enter the small pre-BII stage where the IgLC is rearranged [52]. IgLC is then expressed together with the IgHC as a membrane-bound antibody of IgM subclass and expression of CD25 is lost, defining immature B cells. Cells that express a BCR consisting of IgHCs and IgLCs that pair well together receive strong BCR signalling and are positively selected. Cells that express a BCR where the pairing is weak, and cells that express an autoreactive BCR, will undergo clonal deletion or can be rescued by a process termed receptor editing [53, 54].

This process involves a secondary IgLC rearrangement and if the new IgLC can pair well with the existing IgHC, the cells will receive a level of BCR signalling high enough to mediate positive selection.

Positively selected immature B cells then translocate to the spleen where they as newly immigrants are termed transitional B cells (Fig. 3). These cells can in turn be divided into transitional 1 (T1) and transitional 2 (T2) B cells, where T1 B cells express CD93 and IgM, but not CD23, while T2 B cells express CD93, IgM and CD23. Transitional B cells are short-lived and sensitive to IgM-induced apoptosis [55]. In order to target cells that have escaped tolerance mechanisms in the bone marrow, transitional B cells are selected against autoreactivity [56] and studies have shown that receptor editing also can occur at this stage [57]. Positively selected transitional B cells then differentiate into follicular (FO) B cells or marginal zone (MZ) B cells. A strong BCR signal leads to differentiation into FO B cells and weak BCR signalling leads to commitment to the MZ B cell fate (Fig. 3). Signalling through the receptor for B-cell activating factor (BAFF) is not required for commitment to the FO B cell fate, but for differentiation to MZ B cells (reviewed in [58]). In the spleen, cells that are unable to respond to antigen are rendered silent, or anergic, which in addition to clonal deletion and receptor editing constitutes a third B-cell tolerance mechanism [59]

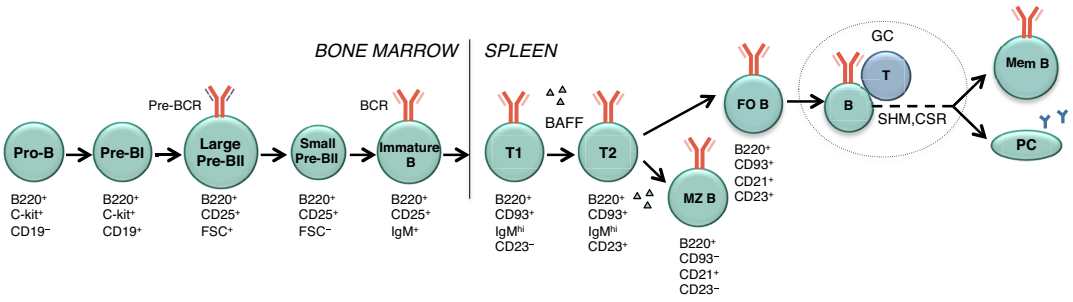

Figure 3. Schematic overview of B-cell development and maturation.
B lymphopoiesis occurs in the bone marrow and immature B cells migrate to the spleen for final maturation into MZ B cells or FO B cells. FO B cells can then enter GCs and differentiate into plasma cells or memory B cells. Pro-B, progenitor B cell; pre-B, precursor B cell; Pre-BCR, Pre-B cell receptor; BCR, B cell receptor; T1, transitional 1 B cell; T2, transitional 2 B cell; BAFF, B cell activating factor; MZ B, marginal zone B cell; FO B, follicular B cell; GC, germinal center; SHM, somatic hypermutation; CSR, class switch recombination; Mem B, memory B cell; PC, plasma cell.

B-cell effector functions

T-cell dependent immune response

B-cell response to T-cell dependent antigens involves the formation of germinal centers (GCs); specialized microstructures composed of separate B- and T-cell zones in secondary lymphoid organs (Fig. 3). In the GC reaction, the affinity and effector functions of antibodies are modified to optimize response to the antigen. Upon antigen encounter, B cells and T cells specific for the antigen accumulate at the border between the B- and T-cell zones and cognate B-T-cell interaction involving CD40-CD40L binding leads to proliferative expansion of B cells [60]. These expanded cells can then either assume an early memory phenotype, become short-lived plasma cells or initiate a GC reaction [61, 62]. During the GC reaction, rapidly proliferating B cells first undergo somatic hypermutation where the variable region of the BCR is modified which results in affinity maturation, i.e. increased binding affinity of the antibody. The B cells are then selected for survival and expansion based on the capacity of the antibodies to bind to antigens presented by follicular DCs. In addition, survival is also dependent on signals from follicular T cells (Tfh), including the production of IL-4, IL-21 and CD40-CD40L interaction (Reviewed in[63]). Cells that are not positively selected will undergo apoptosis followed by phagocytosis by macrophages, while cells that are selected will undergo class switch recombination. Class switch recombination involves changing of the constant part of the IgHC from IgM isotype to IgG, IgE, or IgA isotypes, thus altering the effector function of the antibody. Both somatic hypermutation and class switch recombination involve DNA strand breaks that require the enzyme activation-induced cytidine deaminase (AID) [64]. Before exiting the GC, the B cells will acquire plasma cell or memory B-cell phenotype (Fig. 3). Differentiation into plasma cells is initiated by down-regulation of the B cell gene expression program, and up-regulation of plasma cell genes, which is mainly achieved by the transcription factor B lymphocyte-induced maturation protein 1 (Blimp-1) [65, 66]. Regulation of differentiation into memory B cells is not fully understood; however, the transcription factor activated B cell factor 1 (ABF-1) is implicated in the decision to acquire memory B phenotype through suppression of plasma cell differentiation [67]. After antigen-encounter, antigen-specific plasma cells and memory B cells survive during an extended period of time; plasma cells are found in certain survival niches in the bone marrow where they produce of antibodies [68]. Upon repeated encounter of the antigen, this continuous production of antibodies enables instant response. Memory B cells do not secrete antibodies, but instead primarily circulate in the blood.

T-cell independent immune response

MZ B cells and B1 B cells constitute two B-cell subsets important for response to T-cell independent antigens such as microbial carbohydrates. MZ B cells reside in the MZ of the spleen at the border of circulation, where they function as sentinels [69],

while B1 cells are mainly found in the peritoneal and pleural cavity [70]. MZ B cells and B1 B cells together provide a rapid, but rather unspecific, innate-like response through the production of low affinity poly-reactive antibodies of IgM isotype [71]. These antibodies are termed natural antibodies as they have shown to be present also in the absence of pathogens [72].

Antigen presentation

B cells are able to act as APCs and activate naïve $CD4^+$ T cells [73]. Antigen-presentation by B cells comprises the binding of the antigen to the BCR and BCR-ligation, which induces internalization through receptor-mediated endocytosis. The antigen is then processed in endosomal vesicles into peptides, which are bound to and presented on MHCII molecules on the B cells (Reviewed in [74]). However, the significance of antigen-presentation by B cells in the activation of T cells has been debated. The finding that $CD4^+$ T-cell priming was not compromised when MHC molecules were lacking only on B cells [75], suggested that DCs as potent activators of naïve T cells were ultimately responsible for T-cell priming. However later studies have shown that some protein antigens are preferentially presented by B cells [76], leading to the conclusion that, in certain circumstances, B cells indeed contribute significantly as APCs.

B-cell mediated immune regulation

In addition to acting as positive regulators of the immune system, B cells can also mediate negative regulation of immune responses. A regulatory B-cell subset has been described in mice, identified by their ability to produce and secrete IL-10 [77] and a similar IL-10-producing B-cell population has also been found in humans [78]. IL-10 down-regulates the production of pro-inflammatory cytokines, such as IFNγ [79] and is also important for maintaining the immune-suppressive function of regulatory T cells (Tregs) [80]. Although a regulatory B-cell population can be found in a naïve setting, these cells are mostly implicated in autoimmunity.

Estrogen, SERMs and B cells

Estrogen deficiency caused by ovariectomy leads to an increase in B-cell development in the bone marrow [81]. On the contrary, increased levels of estrogen due to pregnancy or estrogen treatment cause a reduction in B lymphocytes in the bone marrow [82-84]. Studies have determined that the inhibitory effect occurs at the IL-7 sensitive differentiation stage of pro-B cells to pre-B cells [83, 85]. Without stromal cells that produce IL-7, the estrogen-mediated inhibition of the transition from pro-B to pre-B cells does not occur, indicating that estrogen inhibits B-cell development indirectly through stromal cells [83]. Estrogen also alters splenic B-cell populations; there is a prominent decrease in the T1 B-cell population as well as an increase in MZ B-cell population in estrogen-treated ovx mice[86]. The expansion of MZ B cells can be connected to a reduction in BCR signalling; estrogen up-regulates CD22 and SHP-1

in B cells, two negative regulators of BCR signalling, and also reduces the phosphorylation of extracellular-signal regulated kinases 1/2 (Erk1/2) after BCR activation in transitional B cells [87-89]. In addition, transitional B cells in estrogen-treated mice show an increased resistance to BCR-mediated apoptosis, due to an up-regulation of the anti-apoptotic protein B cell lymphoma 2 (Bcl-2), which also contributes to the increase in MZ B cells [87, 89]. In addition, the levels of the B-cell trophic factor BAFF are increased by estrogen, both in the spleen [86] and in serum (Paper I). Interestingly, it has also been shown that estrogen can break B-cell tolerance. When mice transgenic for a pathogenic antibody were treated with estrogen, these mice had increased serum titers of the pathogenic antibodies compared with untreated transgenic mice. This suggests that estrogen led to the escape from tolerance mechanisms of B cells carrying the pathogenic antibodies [90]. Moreover, addition of estrogen leads to elevated numbers of antibody-secreting cells in bone marrow and spleen in both ovx and intact animals [91, 92] and up-regulates the expression of AID in spleen, leading to increased somatic hypermutation and class switch recombination of the Ig locus [93].

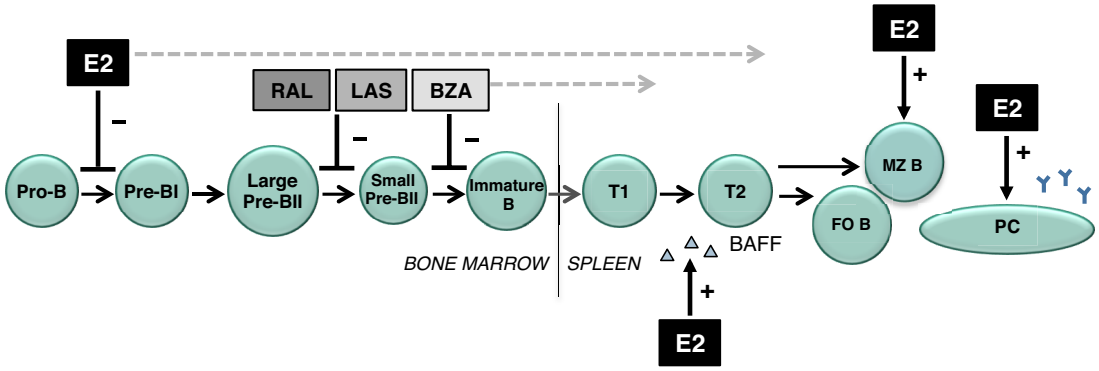

Figure 4. Effects of estrogen and SERMs on B-cell development and maturation.
Estrogen inhibits B-cell development at the pro-B to pre-BI stage and increases levels of BAFF, MZ B cells and antibody-secretion. SERMs suppress B-cell development at a later stage than estrogen and lack effects on BAFF, MZ B cells and antibody-secretion. E2, estradiol; Pro-B, progenitor B cell; pre-B, precursor B cell; T1, transitional 1 B cell; T2, transitional 2 B cell; BAFF, B cell activating factor; MZ B, marginal zone B cell; FO B, follicular B cell.

Bone marrow B cells decrease in ovx and sham-operated mice after administration of raloxifene [92], lasofoxifene, or bazedoxifene (Paper I). However, while estrogen decreases all populations from the pre-BI cells to immature B cells in the bone marrow, raloxifene and lasofoxifene retain normal numbers of pre-BI cells and large pre-BII cells, while significantly decreasing small pre-BII cells and immature B cells.

Bazedoxifene only decreases the immature B-cell population (Paper I) (Fig. 4). All three SERMs decrease the number of T1 cells, but do not alter the MZ population in the spleen (Paper I) (Fig. 4).
In addition, no increase in antibody-secreting cells ([92], Paper I) or serum levels of BAFF was noted in mice treated with SERMs (Paper I). In conclusion, SERMs suppress B lymphopoiesis later in development than estrogen, thus affecting fewer populations, and also lack increasing effects on antibody production (Fig. 4). The effects of SERMs on BCR signalling components and splenic expression of Bcl-2 and AID remain to be clarified, as well as their effects on B-cell tolerance.

T cells – mediators of cellular immunity

T cells provide cell-mediated immune responses. Similarly to B cells, T cells also carry an antigen-specific receptor, termed the T-cell receptor (TCR). The antigen specificities of TCRs are, as for BCRs, achieved through gene recombination during lymphopoiesis; however, the TCRs are not secreted like most BCRs, but rather participate in immune responses through mediation of cytokine production and cytotoxicity.

T-cell development

T-cell development occurs in the thymus; a primary lymphoid organ consisting of a cortex and a medulla separated by the vascularized corticomedullary junction[94]. Classically, T-cell development is dependent on the constant migration of multipotent lymphoid progenitors from the bone marrow to the thymus (Reviewed in [95]); however, more recent studies have established that thymopoiesis also can occur from intrathymic T-cell precursors, independent of immigrating stem cells [96]. The earliest T-cell progenitors lack expression of the TCR and the TCR co-receptors CD4 and CD8, and are termed double-negative (DN) T cells. During T-cell development, DN cells migrate through the thymus and can be divided into four stages based on the expression of CD25 and CD44 [97] (Fig. 5). The earliest T-cell precursors, DN1 cells, express CD44 but lack the expression of CD25. These cells are found in the inner cortex and move outwards through the cortex to enter the DN2 stage, now expressing both CD25 and CD44. Both the DN1 cells and a subpopulation of the DN2 population show broad lineage plasticity by retaining myeloid and NK-cell potential [98]. Notch-signalling has been defined as the crucial factor for maintaining T-cell commitment [99], and this first part of T-cell development is therefore Notch-dependent. The next developmental stage is the DN3 population, which expresses CD25, but low levels of CD44. Here, successful rearrangement of the TCR β locus leads to the expression of a pre-TCR, while the rearrangement of γ and δ segments leads to the expression of a γδTCR and commitment to the γδT lineage. When the cells lose both CD25 and CD44 they are termed DN4 cells and can be found in the outer cortex. Here, rearrangement of the TCRα gene segments occurs, leading to the expression of an αβTCR. These cells also acquire CD4 and CD8 and become double-positive (DP) thymocytes committed to

the αβ T lineage (Fig. 5). Cells that have acquired the γδT cell fate do not enter the DP stage [100]. Subsequently, the DP cells go through positive and negative selection by interacting with cortical thymic epithelial cells presenting self-peptides on their MHCI or MHCII molecules. Interaction with MHCI or MHCII with moderate affinity leads to positive selection and commitment to CD8 or CD4 single positive (SP) cells, respectively. No binding leads to death by neglect, and too strong binding leads to negative selection. CD8 and CD4 SP cells then migrate to the medulla, where they interact with medullary thymic epithelial cells (Fig. 5) [95, 101]. These epithelial cells express a large variety of self-antigens, so called tissue-restricted antigens (TRAs) [102]. Expression of TRAs is regulated by the transcription factor autoimmune regulator (AIRE) [103]. The cells that bind to the self-peptide/MHC complexes will be negatively selected, while the cells that do not bind will survive [95, 101], constituting the second tolerance checkpoint. After thymic selection, the naïve $CD8^+$ and $CD4^+$ cells are exported to the periphery. In order to become activated, the T cell needs to interact with a cell carrying a MHC molecule presenting the antigen specific for the TCR.

T-cell effector functions

Cytokine production

$CD4^+$ T cells are termed helper T (Th) cells, since they provide help to other immune cells through production of cytokines. $CD4^+$ T cells recognize antigens presented on MHCII molecules, expressed on APCs. Th activation requires TCR signalling and co-stimulation through interaction between CD28 on Th cells and CD80 and CD86 on APCs. After the APC interacts with the $CD4^+$ T cell, the T cell differentiates into one of the Th subsets, which is directed by the surrounding cytokine environment. The cytokines are produced by APCs as well as other cells. Traditionally, Th cells were thought to differentiate into two subsets; T helper 1 (Th1) cells and T helper 2 (Th2) cells, categorized by their different cytokine profiles and functions [104]. Presence of IL-12 and IFNγ causes activation of the transcription factor Tbet and differentiation into Th1 cells which produce IFNγ [105] that activates $CD8^+$ T cells. Th1 cells also produce granulocyte-macrophage colony-stimulating factor (GM-CSF) that activates macrophages (Fig. 5). Thus, Th1 cells are important for defence against intracellular pathogens. Presence of IL-4 induces activation of the transcription factor GATA3 leading to differentiation into Th2 cells that produce IL-4 and IL-13 [106] (Fig. 5), cytokines important for humoral immunity and protection against parasites. More recently, IL-17-producing T cells were defined as a distinct Th subset, termed Th17 cells [107]. Th17 cells provide protection against extracellular bacteria and differentiation is induced by IL-6 together with TGFβ and activation of the transcription factor RORγt [108, 109] (Fig. 5). $CD4^+$ T cells can also develop into Tfh cells, which are crucial for the formation and regulation of the GC reaction and hence play an important role in humoral immune response. Tfh differentiation is dependent

on the transcription factor Bcl-6, IL-21 and IL-6 [63]. In addition, $CD4^+$ T cells can differentiate into inducible Tregs. This cell type will be described under "T cell immune regulation".

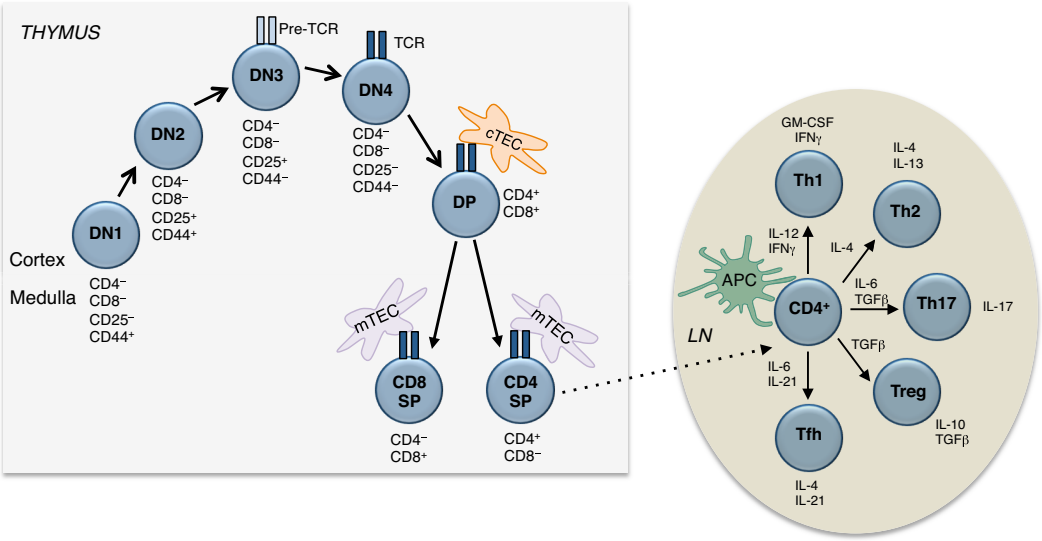

Figure 5. Schematic overview of T-cell development and Th differentiation.
T cells develop in the thymus and interaction with epithelial cells leads to positive and negative selection and differentiation into CD4+ or CD8+ T cells. CD4+ T cells interact with APCs in secondary lymphoid organs and can acquire Th1, Th2, Th17, Treg or Tfh phenotype based on the cytokine environment. DN, double- negative; pre-TCR, pre-T cell receptor; TCR, T-cell receptor; DP, double-positive; SP, single positive; cTEC, cortical thymic epithelial cell; mTEC, medullary thymic epithelial cell; LN, lymph node; APC, antigen-presenting cell; Th, helper T; Treg, regulatory T, Tfh,

Cytotoxicity

$CD8^+$ T cells are referred to as cytotoxic T cells based on their ability to induce apoptosis and necrosis of infected cells and tumour cells, making them important for the protection against intracellular microbes such as viruses. The $CD8^+$ cell is first primed through interaction with an APC expressing an MHCI molecule carrying the TCR-specific antigen. Since MHCI is expressed on all nucleated cells, this enables infected cells to display the antigen and activate primed $CD8^+$ T cells. This leads to cell death of the target cells either through the secretion of granules containing cytotoxic proteins or the expression of Fas-ligand, inducing apoptosis.

T-cell mediated immune regulation

In 1995, $CD4^+CD25^+$ T cells were found to be essential for suppression of autoimmunity, since lymphopenic mice reconstituted with $CD4^+CD25^-$ T cells developed severe autoimmunity whereas co-transfer of $CD4^+CD25^+$ T cells provided protection from autoimmunity [110]. The transcription factor forkhead box P3 (FoxP3) was later determined as a unique marker of $CD4^+CD25^+$ Tregs that controls both development and the suppressive functions of this population [111]. Tregs can be

derived either from the thymus, i.e. natural Tregs or be generated in the periphery, i.e. induced Tregs (Fig. 5). In the thymus, the selection into natural Tregs has been suggested to be an alternative to deletion since the thymocytes developing into Tregs bear a TCR with a high affinity for self-antigen [112]. The generation of induced Tregs in the periphery is thought to occur in the presence of TGFβ (Fig. 5), and involves interactions with non-self antigens and some degree of co-stimulation from APCs [113]. Tregs can regulate immune responses by using cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) to block the interaction between CD80/CD86 on APCs and CD28 on T cells, thus inhibiting T cell activation [114]. Furthermore, Tregs can produce anti-inflammatory cytokines such as IL-10 and TGFβ [115].

T-cell dependent inflammation

Cell-mediated inflammation is characterized by the interaction between cells of the innate immune system and T cells where T cells are crucial as cytokine producers. The delayed-type hypersensitivity (DTH) model is useful for assessing cell-mediated inflammation, also referred to as T-cell dependent inflammation. The cutaneous DTH reaction comprises a sensitization step and an elicitation step (Fig. 6). In the sensitization step, the hapten, a naturally occurring or synthetic small molecule, which is not immunogenic in itself, binds to endogenous proteins [116]. This leads to cytokine production by keratinocytes and subsequent activation of APCs (Langerhans cells, dermal DCs and tissue-resident macrophages), which internalize and process the hapten-protein complex (fig. 6, step 1-2) [117, 118]. These cells then migrate to the lymph nodes and during migration they mature to APCs capable of effectively presenting hapten-peptides to T cells in the lymph nodes, which leads to clonal expansion of hapten-specific T cells [119] (Fig. 6, step 3-5). The elicitation phase is induced by re-exposure to the hapten and leads to migration of antigen-specific T cells to this site (Fig. 6, step 6-7). Here, the T cells start to produce cytokines derived from Th1, Th2 and Th17 cells (Fig. 6, step 8). Mice devoid of IFNγ, IL-4, or IL-17 all show a decreased DTH response, implying that all these Th subtype associated cytokines play an important role in mediating the inflammatory reaction [120]. In addition, the T cells also trigger cells resident at the site of the challenge to produce chemokines such as MCP-1 (monocyte chemo-attractant protein 1), altogether leading to massive infiltration of leukocytes, importantly monocytes, neutrophils and macrophages (Fig. 6, step 9).

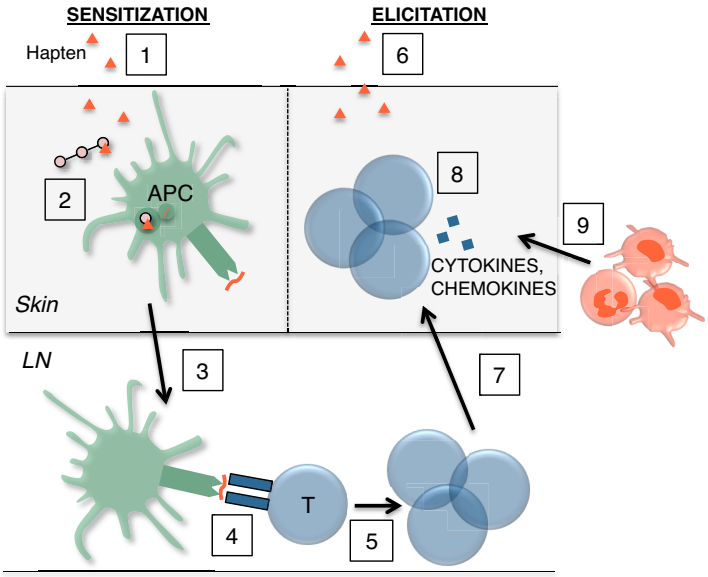

Figure 6. Schematic overview of DTH reaction.
The delayed-type hypersensitivity (DTH) reaction comprises a sensitization phase and an elicitation phase. The sensitization phase includes 1) hapten application, 2) binding of the hapten to endogenous proteins and uptake by APCs, 3) APC migration to the LN and 4) presentation of the hapten to T cells followed by 5) clonal expansion of hapten-specific T cells. The elicitation phase includes 6) re-application of the hapten, 7) migration of hapten-specific T cells to this location, 8) secretion of cytokines and chemokines by T cells and 9) leukocyte infiltration. APC, antigen-presenting cell; LN, lymph node

Estrogen, SERMs and T cells

In both animals and humans, the thymus involutes dramatically during pregnancy. [121]. Histological studies of the mouse thymus have shown that during pregnancy, the size of the cortex is reduced while the medulla is increased, implying a loss of cortical thymocytes [122]. The decrease in thymocytes was shown to be due to a block in T-cell development with a preferential loss of DP cells, an effect that has been at least partly ascribed to the increase in pregnancy-associated hormones such as estrogen [123]. Indeed, when mice were treated with estrogen, thymic T-cell development was suppressed, seen as a decrease in T-cell numbers and a reduced proportion of DP cells, but an increase in the percentage of $CD4^+$ and $CD8^+$ SP cells and of DN cells [124, 125]. When DN cells were divided into subpopulations, a clear increase in the earliest stage, the DN1 stage, was noted, while the remaining stages DN2, DN3 and DN4 were reduced by estrogen [125]. Conversely, removal of endogenous estrogen by ovx results in increased weight and cellularity of the thymus [126]. The increase in thymus cellularity is associated with a shift towards increased DP cells and a decrease in DN and SP cells [126]. Several mechanisms have been suggested by which estrogen induces thymic atrophy, e.g. an increased apoptosis was observed in thymocytes after a single-dose of estrogen [127].

However, other studies have failed to detect an increase in apoptosis, and instead found a lower amount of thymic homing progenitors in the bone marrow as well as a reduced proliferation of thymocytes [124].

Estrogen has multiple effects on peripheral T cells. In low doses, estrogen increases the proliferation of antigen-specific $CD4^+$ T cells in lymph nodes and the IFNγ production from these cells [128]. However, in higher doses, similar to pregnancy levels, estrogen has shown to inhibit the TNF production from T cells [129], and to increase IL-4 secretion and expression of GATA3 in $CD4^+$ T cells [130], rather indicating that estrogen increases anti-inflammatory responses. In line with this, estrogen also stimulates the induction of Tregs and increases FoxP3 expression [131, 132]. Indeed, during pregnancy there is an increased secretion of Th2-related cytokines [133] and an expansion of Tregs [134], thus providing maternal tolerance to the fetus.

In addition to the effects on thymic T-cell development and T-cell effector functions in a non-inflammatory setting, estrogen also has well-documented effects on T cell-dependent inflammation. In mice, estrogen treatment potently inhibits cutaneous T-cell dependent delayed type hypersensitivity (DTH) reaction [135, 136] (Fig. 7).

The mechanism for the suppressive effects of estrogen on DTH is not clear, but estrogen does not directly target T cells in DTH, as female SCID (severe combined immunodeficient) mice reconstituted with thymocytes from estrogen-treated mice did not show a decrease in the DTH response [137]. Instead, the estrogen-mediated inhibition of DTH is believed to involve a decrease in the antigen presentation to T cells, as APCs from estrogen-treated mice induced a lower proliferation of hapten-specific T cells in response to the hapten *in vitro*, compared to controls [138]. In addition, there was a decreased production of IL-2 and IFNγ, but an increased production of IL-10, in lymph nodes of estrogen treated mice subjected to DTH [138, 139]. This suggests that an altered cytokine profile together with a decreased APC function are two important mechanisms by which estrogen mediates the suppression of T-cell dependent inflammation.

The effects of SERMs on T-cell development differ from the effects of estrogen. Treatment with raloxifene leads to a minor thymus atrophy, but the DN1-4, DP and SP populations remain unchanged ([136], Paper II). Lasofoxifene, but not bazedoxifene, also induces a reduction in thymus weight, but none of the third-generation SERMs mediate any changes in the thymic T-cell populations (Paper II).

The effects of SERMs on T-cell effector functions remain to be studied, however, it is clear that raloxifene, lasofoxifene, and bazedoxifene all completely lack suppressive properties on T-cell dependent inflammation, as treatment with these compounds leads to a normal DTH response ([136], Paper II).

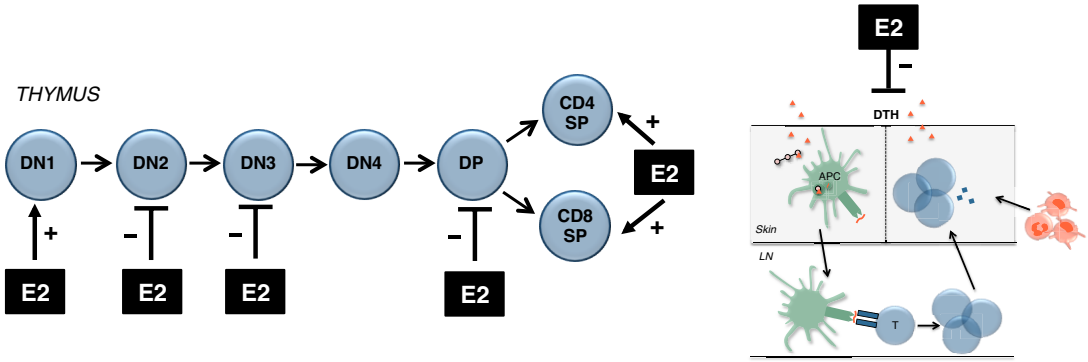

Figure 7. Effects of estrogen and SERMs on T-cell development and DTH.
Estrogen increases the proportion of DN1 cells, decreases the proportion of DN2, DN3 and DP cells and increases the proportion of CD4 and CD8 SP cells. Estrogen also suppresses the DTH reaction. SERMs do not alter any thymic T cell populations and do not inhibit suppress the DTH reaction. E2, estradiol; DN, double-negative, DP, double-positive; SP, single-positive; DTH, delayed-type hypersensitivity.

BONE AND OSTEOIMMUNOLOGY

Bone

The skeleton has a number of functions; it provides structural support for the body, protection of inner organs, and functions as storage for calcium and phosphate, thereby controlling mineral homeostasis. Bone also serves as the location for hematopoiesis, as the bone marrow is located on the inside of long bones (Fig. 8). Bone consists of a collagen protein scaffold, hardened by the mineral hydroxyapatite, and of three different cell types; osteoblasts, osteoclasts, and osteocytes. There are two types of bone, both composed of the same constituents, but with functional differences; trabecular (or spongy) bone, which is more porous and metabolically active, while cortical (or dense) bone is the hard outer layer of bones with less metabolic activity (Fig. 8).

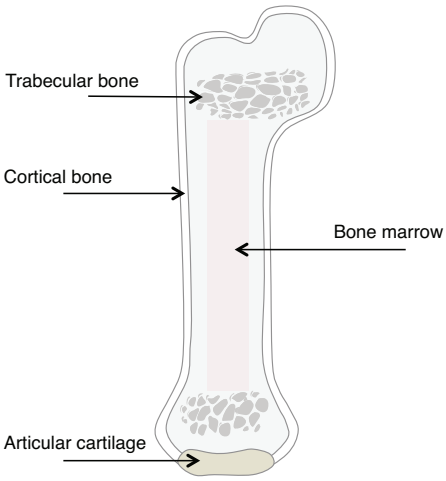

Figure 8. Longitudinal section through femur.
Illustration of the structure of long bones.

Bone is a highly dynamic structure, subjected to constant remodelling. Osteoblasts are the cells responsible for bone formation. They develop from mesenchymal progenitor cells and several signalling pathways are of importance for osteoblastogenesis such as the wnt (wingless type) and BMP (bone morphogenetic protein) signalling pathways [140, 141]. Osteoblasts produce extracellular proteins including osteocalcin and type I collagen. This extracellular matrix, or osteoid, is then mineralized through accumulation of hydroxyapatite, a process controlled by osteoblast expression of alkaline phosphate [142]. Bone formation can be assessed by measuring the concentration of osteocalcin and procollagen pro-peptides such as procollagen I intact N-terminal (PINP) in serum [143]. When osteoblasts are entombed in the bone matrix, they develop into osteocytes, which sense mechanical loading on the bone and adjust bone remodelling via up- or down-regulation of sclerostin, a potent inhibitor of bone

formation [144]. Osteoclasts are bone-resorbing cells, which originate from hematopoietic stem cells. Osteoclastogenesis is dependent on two factors; macrophage colony stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL) [145]. M-CSF and RANKL binds to receptors on pre-osteoclasts and stimulates differentiation, proliferation, and survival [146]. The binding of RANKL to its receptor RANK is inhibited by the decoy receptor osteoprotegerin (OPG) [147]. RANKL is expressed by many different cell types in response to osteoclastogenic factors, such as inflammatory cytokines. Resorption of bone involves the degradation of collagen I, and serum concentration of the collagen I fragment C-terminal telopeptide (CTX-I) can be used as a measurement of bone resorption.

Cartilage

At the ends of long bones, the presence of articular cartilage provides a smooth surface towards the joint, which reduces friction during movement. Articular cartilage consists of a single cell type, the chondrocyte, together with an extracellular matrix, which is composed of structural macromolecules such as collagens, proteoglycans, and non-collagenous proteins. Collagen type II is the main collagen in articular cartilage making up collagen fibrils that gives the cartilage its tensile strength. Proteoglycans bind to fluid to provide stiffness of the tissue. Among the non-collagenous proteins present in articular cartilage, cartilage-oligomeric matrix protein (COMP) is a well-studied component, which functions to stabilize the collagen network. COMP can be measured in serum as a marker of cartilage degradation [148].

Osteoporosis

Osteoporosis arises as a consequence of decreased net bone formation, resulting in a reduction of BMD and bone strength, which causes an increased susceptibility to fractures. According to the WHO classification, the diagnosis criterion for osteoporosis is a BMD lower than 2.5 standard deviations below the mean value of young adults [149]. The risk of developing osteoporosis increases with age; after the age of 40, bone resorption starts to exceed bone formation. Consistent with the important role of estrogen as a positive regulator of bone metabolism, the prevalence of osteoporosis is highest in postmenopausal women. A number of other factors also contribute to an increased risk of developing osteoporosis, e.g. lifestyle factors such as low nutrition and tobacco use, metabolic or inflammatory conditions (hyperparathyroidism, RA), and the use of medications such as glucocorticoids [150]. Osteoporosis therapy includes bisphosphonates, strontium ranelate, estrogen-containing HRT and SERMs. Bisphosphonates act to induce apoptosis in osteoclasts, while strontium ranelate increases bone formation through osteoblasts and reduces the bone resorptive function of osteoclasts [151, 152]. To assess osteoporosis and evaluate anti-osteoporosis therapy, guidelines have been postulated that describe useful serum markers of bone resorption and bone formation [153].

Osteoimmunology

Osteoimmunology is an interdisciplinary field encompassing the interactions between the skeletal system and the immune system. An obvious reason for the interaction between these two systems is the location of the bone marrow, the site of immune cell formation, in the interior of long bones. Indeed, a number of regulatory pathways have been identified that are shared between the skeletal system and the immune system. One factor of particular interest in the field of osteoimmunology is RANKL, a crucial cytokine for osteoclastogenesis. RANKL is expressed by the bone-forming osteoblasts [154], but also by immune cells such as activated T cells, and has shown to play an important role in the interaction between T cells and DCs by enhancing the function of DCs [155]. Mice deficient in RANKL develop severe osteopetrosis due to a block in osteoclastogenesis, but also show an immunological phenotype with abnormal development of secondary lymphoid organs, thus highlighting the important role of this cytokine in both systems [156]. RANKL expression by osteoblasts can be induced by vitamin D3 and parathyroid hormone [157], but also by cytokines that increase during inflammatory conditions, such as TNFα [158]. Also, IL-1, IL-6 and IL-17 are osteolytic cytokines [159-161]. The RANKL decoy receptor OPG is not only produced by osteoblasts, but also by B cells, and the production is stimulated by CD40-CD40L ligation [162]. Indeed, mice deficient in B cells, CD40 or CD40L present with osteoporosis, suggesting that B cells and B-T-cell interactions are important in bone homeostasis [163]. In addition, transcription factors such as the NFκB and NFAT families, as well as the signal transducer and activator of transcription 1 (STAT1), have implications in both bone and the immune system [164].

Although much attention has been paid to the influence of immune cells and cytokines on the skeletal system, conversely, bone cells have also shown to be able to regulate the immune system. Osteoblasts have been identified in the hematopoietic stem cell niche and have shown to correlate with the number of hematopoietic stem cells [165]. Furthermore, osteoclasts, which similarly to DCs derive from the monocyte/macrophage lineage, have been reported to be able to up-regulate co-stimulatory molecules for T-cell activation and function as APCs [166]. In addition, B-cell apoptosis is increased in the absence of sclerostin expression from osteocytes [167].

Estrogen, SERMs and bone

Estrogen receptors are found on all types of bone cells [168] and estrogen functions as an important regulator of bone growth in adolescence and of bone remodelling in adults. Estrogen deficiency is the main cause of bone loss in postmenopausal women, and also contributes to the development of osteoporosis in elderly men [169]. Bone resorption is decreased by estrogen in multiple ways; estrogen directly inhibits osteoclast activity and induces osteoclast apoptosis [170] and also down-regulates pro-inflammatory cytokines such as IL-1, IL-6 and TNFα, which induce osteoclastogenesis [171].

In addition, estrogen stimulates Treg differentiation and TGFβ production, which inhibit bone resorption by osteoclasts [172, 173] Consequently, menopause or ovariectomy in mice leads to stimulation of bone resorption. T cells have been reported to play an important role in ovariectomy-induced bone loss, as ovx mice have an increased number of TNFα-producing T cells [129]. Of note, estrogen deficiency also leads to increased bone formation through up-regulation of osteoblastogenesis [174]; however, this increase does not exceed the increased bone resorption, leading to a net loss of bone. In postmenopausal women, HRT mediates an increase in lumbar BMD and a reduction in bone turnover markers [175]. Similarly, estrogen treatment of castrated animals leads to an increase in BMD and decreased serum levels of bone turnover markers [176].

In postmenopausal women, raloxifene, lasofoxifene and bazedoxifene increase lumbar spine BMD [24, 31, 36] and also decrease bone turnover markers [177-179]. Raloxifene decreases the risk of developing vertebral fractures [23, 24], lasofoxifene decreases the risk of both vertebral and non-vertebral fractures in postmenopausal women [31] and bazedoxifene decreases the risk of vertebral fractures in postmenopausal women [36] and the risk of non-vertebral fractures in high-risk fracture patients [37].

In mice subjected to ovariectomy, all three SERMs cause an increase in total BMD ([28, 34, 35, 40], Paper I); however, markers of bone resorption and bone formation have not been assessed.

AUTOIMMUNITY

The immune system is designed to react in a controlled manner to invading pathogens, while not responding to self-tissues. A number of regulatory mechanisms function to maintain unresponsiveness, or tolerance, to self. These mechanisms include central and peripheral tolerance, where self-reactive B- and T cells are eliminated, as well as regulatory cells (e.g Tregs) that act to suppress self-reactive effector cells. However, when tolerance is abrogated, autoimmunity arises. There are a number of autoimmune diseases, where rheumatoid arthritis (RA) is one of the most prevalent.

Rheumatoid Arthritis (RA)

RA is a chronic systemic inflammatory disease characterized by synovitis, autoantibody production and destruction of bone and cartilage. The worldwide prevalence is around 0,5-1% and the female to male ratio is 3:1. Both genetic and environmental factors contribute to development of disease. Diagnosing RA involves assessing synovitis and the presence of autoantibodies and acute-phase reactants in serum according to the guidelines defined in the 2010 ACR-EULAR classification criteria for Rheumatoid Arthritis [180]. The autoantibodies included in the criteria are Rheumatoid Factor (RF) and Anti-Citrullinated Protein Antibodies (ACPA). RF is an antibody against the Fc part of IgG while ACPA is a class of antibodies against proteins where the amino acid arginine has been post-translationally modified to citrulline [181]. RA patients can be divided into ACPA-positive and ACPA-negative, which differ in risk factors, prognosis, and treatment response. For example, in ACPA-positive patients, the presence of the HLA-DRB1 locus and especially alleles with the amino acid motif QKRAA, known as the shared epitope, is associated with an increased susceptibility [182]. In addition, ACPA-positive patients are more at risk of developing erosive disease [183].

The immunopathogenesis of RA includes both cells of the innate immune system (neutrophils, macrophages and DCs) as well as cells of the adaptive immune system (B- and T cells).

B cells in RA

An obvious role for B cells in the pathogenesis of RA has been demonstrated by the efficacy of the B-cell depleting therapy rituximab a CD20-specific monoclonal antibody that depletes all B-cell subsets except plasma cells, which are $CD20^-$ [184]. The significance of B cells for disease has been further supported by the resistance of B-cell deficient mice to experimental arthritis [185]. B cells contribute to disease in multiple ways; most importantly they are responsible for the production of autoantibodies and thereby subsequent immune complex formation. In addition to ACPA, antibodies against collagen type II (CII) have been detected in RA patients [186].

B cells are also found in the synovia of RA patients where they contribute to the inflammation by local production of cytokines such as IL-6 and TNFα. In some patients, the synovial lymphocytes form diffuse infiltrates, while in other patients the B- and T cells form aggregates similar to the microstructures formed in secondary lymphoid organs, where GC reactions take place [187], leading to an on-going production of class-switched antibodies [188]. In RA patients, production of B-cell survival and activation factors such as BAFF occurs in synovial membranes containing ectopic GCs [189, 190]. Furthermore, the T-cell activation in the rheumatoid arthritis synovia is B-cell dependent, as in the absence of B cells, the synovial T cells remain inactivated [191]. In addition, B cells can act as APCs and antigen presentation by B cells has shown to be necessary for severe and persistent disease in experimental arthritis [192]. Interestingly, a pre-existing B-cell reactivity against CII has been detected in naïve mice. This response was mediated by MZ B cells, which rapidly expanded after immunization with CII, suggesting an important role for this subset of B cells in the initiation of arthritis [193]. In addition, arthritic mice lacking IL-10-producing regulatory B cells suffered from more severe inflammation, which was associated with an increase in Th1-and Th17 cells and a decrease in Tregs [194].

T cells in RA

RA was traditionally described as a T cell-driven disease. The synovia of RA patients contains a large proportion of T cells as well as cytokines and co-stimulatory molecules required for T-cell activation [195], suggesting that the synovitis is characterized by sustained T-cell activation and generation of both memory T cells and effector T cells. Traditionally, Th1 cells have been regarded as the main mediators of the synovial inflammation in RA, through their production of pro-inflammatory cytokines such as IFNγ. However, studies have revealed an increased susceptibility to experimental arthritis and accelerated disease in mice lacking Th1-associated genes such as the IFNγ receptor [196-198], suggesting that Th1 cells are in fact not the main contributors to disease. Instead, attention was drawn to Th17 cells, as producers of the cytokines IL-17, IL-23 and TNFα. Interestingly, mice defect in Th17-associated genes are resistant to experimental arthritis and inhibiting IL-17 leads to a suppression of disease [199, 200], while overexpressing IL-17 leads to an aggravated inflammation [201]. This strongly implies a crucial role for Th17 cells in the pathogenesis of RA. IL-17 can be implicated in the development of synovial inflammation by stimulating the production of IL-8 from synovial cells, which attract neutrophils [202]. Tregs have been shown to be able to suppress disease in animal models of RA [203]. In humans, Tregs are increased in the joints of RA patients; however, the presence of inflammatory cytokines such as IL-6 and TNF reduces the immune-suppressive function of the Tregs [204]. Also γδT cells have been implicated in development of experimental arthritis as depletion of γδT cells improved arthritis. A subset of γδT cells produces IL-17 and this subset most likely contributes to pathogenesis in arthritis [205-207].

Bone destruction and osteoporosis in RA

Several skeletal involvements are found in RA, including bone erosions and periarticular bone loss. These features are mediated by a number of factors present in the RA synovia. Expression of the osteoclastogenic factors M-CSF and RANKL [154, 208, 209], strongly contributes to an increased number of mature osteoclasts in the synovial tissue and bone resorption. In addition, local production of ACPA has been associated with an increased differentiation of osteoclast precursors into mature osteoclasts [210], which is in line with fact that RA patients positive for ACPA are more at risk of developing erosive disease [183]. Furthermore, a number of the pro-inflammatory cytokines present in the RA joint have been associated with increased development and function of osteoclasts, including TNFα [211, 212], IL-1β [213], IL-6 [214], and IL-17 [215]. Apart from local bone manifestations, RA can also lead to generalized osteoporosis, because of the systemic release of osteoclastogenic factors such as pro-inflammatory cytokines. In addition, generalized osteoporosis is also promoted by long-term treatment with glucocorticoids. Approximately 50% of postmenopausal women with RA suffer from generalized osteoporosis [216, 217]. Generalized osteoporosis is also more common in men with RA compared with the healthy population [218].

Treatment of RA

Current therapies used to treat RA include non-steroidal anti-inflammatory drugs (NSAIDs), disease-modifying anti-rheumatic drugs (DMARDs) and biologic treatments. NSAIDs and glucocorticoids are used to reduce pain, inflammation, and stiffness. DMARDs is a heterogeneous group of drugs, which include anti-metabolites such as methotrexate and anti-inflammatory and anti-microbial agents such as sulfasalazine. Biologic treatments act to selectively target pro-inflammatory cytokines, such as TNFα [219] and to inhibit B- and T-cells [184, 220].

Animal models of RA

There are several animal models of RA, each characterized by different disease mechanisms and applications.

Collagen-induced Arthritis (CIA)

CIA was first described in rats [221], and later in susceptible mouse strains carrying the MHCII haplotype H-2q [222, 223]. To date, DBA/1 mice are most commonly used for the CIA model. CII is one of the main components of articular cartilage and subcutaneous injection with CII in Freund's adjuvant leads to a cross-reactive autoimmune response to CII in joint cartilage appearing as a macroscopic polyarthritis. The model includes a primary immunization at day 0 and a booster immunization at day 21-28. Symptoms appear 18-25 days after the first immunization. The polyarthritis is characterized by synovitis and erosion of bone and cartilage. The disease generally peaks around day 40, followed by remission. T cells have a crucial role in the

pathogenesis of CIA [224]; however, CII-antibody production from B cells is also required for disease development [185]. This model is widely used and generally considered to show most similarities to human RA.

Collagen-antibody induced arthritis (CAIA)

The humoral immune system is an important contributor to experimental arthritis, as serum from mice immunized with CII, thus containing anti-CII antibodies, can induce arthritis in naïve mice [225]. Indeed, the CAIA model constitutes intravenous injection of isolated monoclonal CII-specific antibodies, potentiated by injection of lipopolysaccharide (LPS) [226-229]. This model results in mild arthritis that only represent the effector phase of the disease, as the priming of the immune system occurs before the production of antibodies. There are currently a number of combinations of monoclonal CII-antibodies commercially available to induce CAIA. Apart from representing the features of the effector phase of human RA, CAIA is also useful for the study of trabecular arthritis-induced bone loss (Grahnemo *et al.*, submitted).

Antigen-Induced Arthritis (AIA)

AIA is a local mono-arthritis model induced by systemic immunization of an antigen in adjuvant, followed by intra-articular injection of the antigen. Classical antigens used in AIA are ovalbumin, methylated bovine serum albumin, and fibrin[230]. As AIA leads to decreased trabecular BMD locally in the joint together with immune cell infiltration, AIA can be used as a model to investigate the mechanisms and features periarticular inflammation–mediated bone loss [231].

Estrogen, SERMs and RA

Estrogen has a complex role in autoimmunity with aggravating effects in some conditions, but protective effects in others. In patients with systemic lupus erythematosus (SLE), pregnancy and hormone replacement therapy aggravate disease [232, 233] and similarly, estrogen treatment increases disease and increases autoantibody levels in mice with SLE-like systemic autoimmunity [234]. However, in RA, many studies have established that estrogen instead ameliorates disease.

Both clinical and experimental data support a protective role of estrogen in arthritis. The highest incidence of RA in women coincides with menopause when estrogen levels decline [235], while during pregnancy when estrogen levels are high, disease activity is often decreased [236, 237]. In addition, HRT and contraceptives have shown positive effects on disease [238, 239]. However, in other studies, HRT did not show ameliorating effects on postmenopausal RA [240], therefore the effects of HRT on RA remain inconclusive. In CIA, pregnancy has beneficial effects [241] and treatment with estrogen potently inhibits disease in CIA, CAIA and AIA [242-244]. Traditionally, since RA was considered to be a disease driven by Th1 cytokines, the shift from Th1- to Th2 response induced by estrogen was thought to play an important role in the

ameliorating effects of estrogen on disease [245]. However, since several studies have shown that Th1 cells and cytokines are not solely responsible for the pathogenesis of RA [196-198], this notion is questionable. In arthritic mice, estrogen has shown to decrease the proportion of neutrophils in the joint [246, 247] and to increase Th17 cells in the LN, possibly by retention of Th17 cells, leading to a decreased proportion of these cells in the joint [247]. Furthermore, in LN of estrogen-treated mice subjected to CIA, the B-cell population and $CD8^-$ DC population were expanded and both these cell types expressed higher levels of molecules associated with antigen-presentation compared with the same populations in healthy mice (Paper III). However, exactly how estrogen-mediated effects on DCs and B cells can be implicated in the amelioration of arthritis is unclear. When treatment with estrogen is initiated before immunization with CII, estrogen mediates a decrease in serum IL-6, but not when mice are treated therapeutically [248]. In addition to reducing the inflammation in RA, estrogen also has positive effects on inflammation-induced bone loss and cartilage destruction. HRT has shown to increase BMD in postmenopausal women with RA and treatment with estrogen increases both cortical and trabecular BMD in arthritic mice ([248, 249], Paper III) and reduces the number of pre-osteoclasts in the bone marrow (Paper III). Moreover, estrogen inhibits cartilage destruction as demonstrated by decreases levels of COMP in estrogen-treated mice with CIA ([248], Paper III)

Raloxifene, lasofoxifene and bazedoxifene all potently reduce the frequency and severity of arthritis when administered therapeutically to mice subjected to CIA [248] (Paper III). Raloxifene also delays the onset of arthritis and ameliorate disease when administered prophylactically [248] and when used as a long-term treatment of established disease [250]. Similarly to estrogen, raloxifene decreases serum IL-6 when administered prophylactically [248]. Interestingly, lasofoxifene causes a significant reduction, and bazedoxifene shows a strong tendency towards a reduction, of serum IL-6 in therapeutically treated CIA mice (Paper III). However, neither lasofoxifene nor bazedoxifene alter the proportions of all $CD4^+$ T cells, Th17 cells, B cells, or DCs, or alter B-cell expression of molecules associated with antigen presentation in LN of arthritic mice. Nevertheless, raloxifene, lasofoxifene and bazedoxifene all potently inhibit bone loss and cartilage degradation in CIA, as demonstrated by increased cortical and trabecular BMD and decreased serum COMP ([248], Paper III). Bazedoxifene, but not raloxifene or lasofoxifene also reduces the number of pre-osteoclasts in the bone marrow (Paper III).

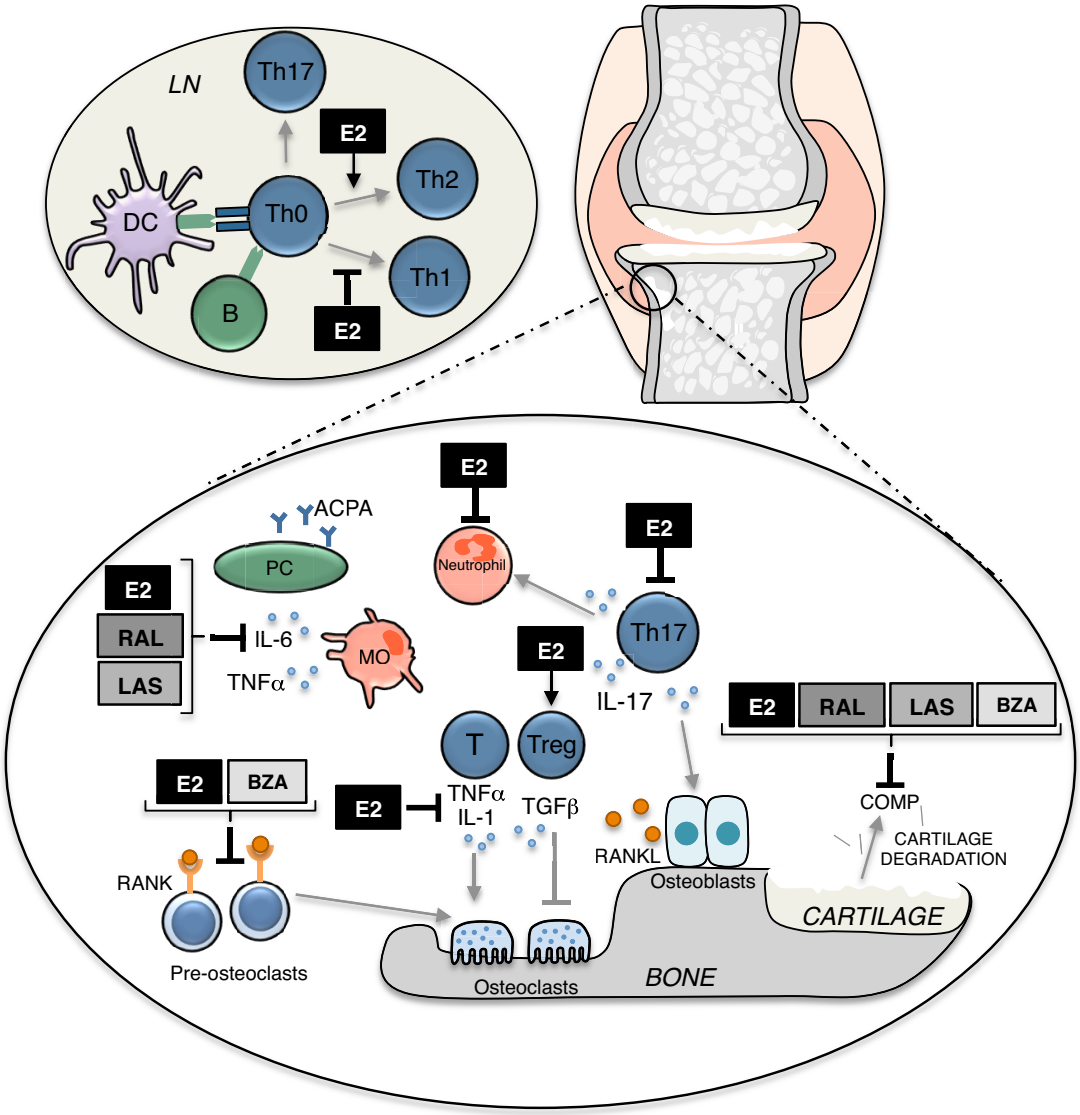

Figure 9. Illustration of the effects of estrogen and SERMs on the pathogenesis of RA.
Estrogen has a number of effects on synovitis and bone destruction in RA, in this figure a selected number of these effects are illustrated, together with the known effects of SERMs. Estrogen causes a shift from Th1 to Th2 cells and reduces the number of Th17 cells and neutrophils in the synovia. Estrogen and bazedoxifene reduces the number of pre-osteoclasts in the bone marrow. Estrogen decreases TNFα and IL-1 production. In addition, estrogen increases Treg differentiation and TGFβ secretion, leading to decreased osteoclast differentiation and activity. Estrogen, raloxifene and lasofoxifene decrease serum IL-6. Estrogen, raloxifene, lasofoxifene and bazedoxifene all reduce serum COMP. DC, dendritic cell; Th, helper T; E2, estradiol; ral, raloxifene; las, lasofoxifene; bza, bazedoxifene; PC, plasma cell; MO, macrophage; ACPA, Anti-Citrullinated Protein Antibodies; Treg, regulatory T cell; RANK, receptor activator of NF-kB; RANKL, receptor activator of NF-kB ligand; COMP, cartilage-oligomeric matrix protein.

CONCLUDING REMARKS

The increased prevalence of autoimmunity in women compared to men has prompted extensive analysis of the immune-modulating role of estrogen. Indeed, the stimulating effects of estrogen on both antibody production and the innate-like MZ B-cell subset can be related to the aggravating effect of estrogen on the antibody-driven disease SLE. Furthermore, estrogen-mediated effects on T-cell cytokines and T-cell mediated immune regulation have been implicated in the ameliorating properties of estrogen on RA. However, the clinical use of HRT has decreased due to side effects and has been, at least partly, replaced by SERMs, most recently the third-generation SERMs. Although the effects of third-generation SERMs on bone and on the female reproductive system have been thoroughly documented, the immunological properties of these compounds have remained undetermined. In the studies included in this thesis, we reveal a puzzling complexity of the immunological effects of the third-generation SERMs. Similarly to estrogen, these compounds inhibit the development of B cells, however, the suppressive effects on B-cell development was found at a later developmental stage for the SERMs compared with estrogen. This suggests an alternative mechanism of suppression, which, in contrast to what has been shown for estrogen, might not involve stromal cells. In addition, these compounds showed a striking absence of suppressive effects on T-cell development. Furthermore, the ameliorating effects of the third-generation SERMs on experimental arthritis was surprisingly not accompanied by the ability to suppress T-cell dependent inflammation or an altered lymphocyte composition in lymph nodes of arthritic mice. Therefore, further investigations are needed to determine the target for the suppressive effects of the third-generation SERMs on arthritis. We found it interesting and promising that these compounds revealed such powerful ameliorating effects on experimental postmenopausal arthritis and suggest future consideration of these compounds as treatment of postmenopausal RA, where they can function both as anti-arthritic and anti-osteoporotic therapy.

Furthermore, the finding that third-generation SERMs in contrast to estrogen do not increase antibody production or expand MZ B cells raises the question whether these compounds lack the aggravating effects on SLE seen with estrogen. As many patients with SLE suffer from osteoporosis due to systemic inflammation and treatment with glucocorticoids, bone-protective treatments are needed in this patient group. Future studies of the effects of SERMs on experimental models of SLE will reveal if SERMs can mediate inhibition of bone loss together with an absence of disease aggravation.

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