Estrogens and interleukin-17 in arthritis and associated osteoporosis

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ABSTRACT

Rheumatoid arthritis (RA), a disease characterized by persistent joint inflammation and joint destruction, is frequently associated with generalized osteoporosis. A female preponderance (3:1) is present in RA, and conditions with sex hormone alterations such as pregnancy and menopause influence the disease. Estrogen-containing hormone replacement therapy (HRT) in postmenopausal RA reduces disease activity and prevents osteoporosis; however, use of HRT is restrictive due to risk of adverse effects. Selective estrogen receptor modulators (SERM) utilize positive effects of estrogens – prevent osteoporosis and reduce menopausal symptoms – with minimized side effects. SERM are also combined with estrogens to achieve a tissue-restricted estrogenic response (tissue-selective estrogen complex [TSEC]). Effects of new SERM and TSEC have not been studied in RA. Thus, the first aim of the thesis was to elucidate effects of new SERM and TSEC on arthritis and associated osteoporosis in an experimental arthritis model. The T cell cytokine interleukin-17A (IL-17) mediates both joint inflammation and bone degradation in RA; however, if IL-17-producing T cells can be regulated by sex hormones have been scarcely studied. Thus, the second aim of the thesis was to study influence of estradiol (E2) on IL-17-producing T cells in experimental arthritis.

To address these aims, ovariectomized ("postmenopausal") female mice were subjected to collagen-induced arthritis (CIA). E2, SERM, and TSEC therapy in CIA mice dramatically reduced joint inflammation and destruction, and prevented osteoporosis, compared with placebo control. Moreover, E2 reduced IL-17-producing Th17 and γδT cell numbers in joints, in contrast to lymph nodes where E2 increased their numbers. In line with modulated cell distribution, the migration-associated phenotype of IL-17-producing T cells was altered by E2. In conclusion, this thesis increases the understanding of sex hormonal influence in arthritis. Furthermore, the experimental evidence obtained herein motivates initiation of clinical trials evaluating addition of SERM or TSEC to postmenopausal women with RA at risk for osteoporosis.

Keywords: arthritis (experimental), osteoporosis, interleukin-17, estradiol, estrogens, selective estrogen receptor modulators
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SAMMANFATTNING PÅ SVENSKA

Reumatoid artrit (RA), även kallad *ledgångsreumatism*, är en vanligt förekommande kronisk sjukdom som ca 0.5–1% av befolkningen lider av. Artrit är den medicinska termen för ledinflammation, och det är artrit i flertalet leder i händer och fötter som är kännetecknande för sjukdomen. Sjukdomen beror på ett felriktat immunförsvar, där immunförsvarets celler angriper kroppsegen vävnad i lederna, med ledförstörelse, smärta och handikapp som följd. Sjukdomen är vanligare hos kvinnor än hos män, och kraftiga hormonella förändringar under en kvinnas liv (t.ex. graviditet, menopaus) påverkar ledsjukdomen. De mest betydelsefulla könshormonerna hos kvinnor är östrogener. När östrogenproduktionen är hög, som under graviditet, blir ledinflammationen hos RA-patienter lindrigare. Avtagande östrogenproduktion under klimakteriet verkar däremot vara relaterat till utveckling av RA. En vanlig följdsjukdom till RA är benskörhet (osteoporos) med ökad risk för benbrott. Benskörhet är framförallt vanligt hos kvinnor som passerat menopaus, eftersom den låga östrogenproduktionen hos dem leder till att bentätheten försämras.

Syftet med denna avhandling har varit att öka kunskapen om varför och hur östrogener påverkar ledinflammation. Vidare var också målet att undersöka effekten på ledinflammation av nya östrogenlika läkemedel, som annars används mot benskörhet och övergångsbesvär i samband med klimakteriet. För detta ändamål har vi använt försöksdjur (möss) hos vilka vi först har framkallat ett klimakteriellt tillstånd och sedan ledinflammation. Mössen har sedan behandlats med ett östrogen (östradiol), östrogenliknande läkemedel (lasofoxifen, bazedoxifen eller bazedoxifen-östrogenkombination) eller kontrollsubstans (placebo) under experimenten. Graden av inflammation och förstörelse av lederna har bedömts på flera olika sätt, och mössens generella bentäthet har mätts. Noggranna studier av immunförsvarets celler i mössen har också utförts.

Experimenten som utförts inom detta avhandlingsarbete resulterade i fem artiklar publicerade i vetenskapliga tidskrifter. I **arbete I** och **II** fann vi att nya östrogenlika läkemedel, som idag används för att förebygga och behandla benskörhet efter klimakteriet, också var effektiva som behandling mot ledinflammation samt mot benskörheten som uppkom i samband med ledsjukdom hos möss. Detta leder fram till slutsatsen att det är motiverat att göra studier på patienter, där man prövar ett tillägg med östrogenliknande läkemedel till befintlig behandling mot RA hos kvinnor som passerat

menopaus. En sådan behandling skulle då kunna förebygga och behandla benskörhet, och förhoppningsvis också dämpa ledinflammationen.

I **arbete III** visade vi att en experimentell modell för inducera artrit hos möss, med ett mycket kort tidsförlopp, var tillräcklig för att framkalla benskörhet, vilket underlättar studier i detta fält.

I **arbete IV och V** kunde vi påvisa att östradiolbehandling hos möss med ledinflammation påverkar immunceller som producerar ett aggressivt protein, IL-17. Detta protein har visats driva både inflammation och förstörelse av lederna i RA. Hos två olika typer av IL-17-producerande T-celler verkade östradiol kunna förändra cellernas förmåga att förflytta sig till lederna, där dessa celler gör skada. Östradiolbehandlingen gjorde att färre IL-17-producerande T-celler fanns i lederna och därmed blev också ledsjukdomen påtagligt lindrigare hos dessa möss, jämfört med kontrollgruppen. Dessa resultat utgör viktig kunskap i förståelsen för hur kvinnliga könshormoner påverkar RA.

LIST OF PAPERS

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

I. Andersson A, Bernardi AI, Stubelius A, Nurkkala-Karlsson M, Ohlsson C, Carlsten H, Islander U.
Selective oestrogen receptor modulators lasofoxifene and bazedoxifene inhibit joint inflammation and osteoporosis in ovariectomised mice with collagen-induced arthritis.
Rheumatology (Oxford). 2016; 55(3): 553-63.

II. Andersson A, Bernardi AI, Nurkkala-Karlsson M, Stubelius A, Grahnemo L, Ohlsson C, Carlsten H, Islander U.
Suppression of experimental arthritis and associated bone loss by a tissue-selective estrogen complex.
Endocrinology. 2016; 157 (3): 1013-20.

III. Grahnemo L, Andersson A, Nurkkala-Karlsson M, Stubelius A, Lagerquist MK, Svensson MN, Ohlsson C, Carlsten H, Islander U.
Trabecular bone loss in collagen antibody-induced arthritis.
Arthritis Res Ther. 2015; 25; 17:189.

IV. Andersson A, Stubelius A, Karlsson MN, Engdahl C, Erlandsson M, Grahnemo L, Lagerquist MK, Islander U.
Estrogen regulates T helper 17 phenotype and localization in experimental autoimmune arthritis.
Arthritis Res Ther. 2015; 13; 17:32.

V. Andersson A, Grahnemo L, Engdahl C, Stubelius A, Lagerquist MK, Carlsten H, Islander U.
IL-17-producing γδT cells are regulated by estrogen during development of experimental arthritis.
Clin Immunol. 2015; 161 (2): 324-32.

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ABBREVIATIONS

ACPA	anti-citrullinated protein antibodies
AF-1/2	activation function-1/2
AIA	antigen-induced arthritis
APC	antigen-presenting cell
BCR	B cell receptor
BMD	bone mineral density
Breg	regulatory B cell
CAIA	collagen antibody-induced arthritis
CCL	CC chemokine ligands
CCR	CC chemokine receptor
CD	cluster of differentiation
CEE	conjugated equine estrogens
CHD	coronary heart disease
CI	collagen type I
CIA	collagen-induced arthritis
CII	collagen type II
COMP	cartilage oligomeric matrix protein
CRP	c-reactive protein
CTLA-4	cytotoxic T-lymphocyte associated protein 4
CTX-I	C-terminal telopeptides of type I collagen
CVD	cardiovascular disease
DBD	DNA-binding domain
DC	dendritic cell
DN	double negative
DP	double positive
DXA	dual energy x-ray absorptiometry
E1	estrone
E2	17β-estradiol
E3	estriol
EAE	experimental autoimmune encephalomyelitis
ER	estrogen receptor
ERE	estrogen response elements
ESR	erythrocyte sedimentation rate
FLS	fibroblast-like synoviocyte
FO	follicular
GC-MS/MS	gas chromatography-tandem mass spectrometry
GPER-1	G protein-coupled estrogen receptor-1
HLA	human leukocyte antigen
HRT	hormone replacement therapy
IFN	interferon
IL	interleukin
KO	knockout
LDB	ligand-binding domain
LPS	lipopolysaccharide
M-CSF	macrophage colony-stimulating factor

mBSA	methylated bovine serum albumin
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
MS	multiple sclerosis
MZ	marginal zone
NET	neutrophil extracellular trap
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
OBL	osteoblast
OC	oral contraceptive
OCL	osteoclast
OPG	osteoprotegrin
OVX	ovariectomy/ovariectomized
PAD	peptidylarginine deiminases
pDC	plasmacytoid dendritic cell
PINP	N-terminal propeptide of type I procollagen
pQCT	peripheral quantitative computed tomography
pre-B	precursor B cell
pro-B	progenitor B cell
PTPN22	protein tyrosine phosphatase, non-receptor type 22
RA	rheumatoid arthritis
RANKL	receptor activator of nuclear factor kappa-B ligand
RF	rheumatoid factor
RORγt	RAR-related orphan receptor gamma t
SERM	selective estrogen receptor modulator
SHBG	sex hormone-binding globulin
SP	single-positive
TCR	T cell receptor
Tfh	T follicular helper cell
TGFβ	transforming growth factor β
Th	T helper
TLR	toll-like receptor
TNF	tumor necrosis factor
TRAP	tartrate-resistant acid phosphatase
Treg	regulatory T cell
TSEC	tissue-selective estrogen complex
WHI	Women's Health Initiative
μCT	micro computed tomography

1 INTRODUCTION

The disease rheumatoid arthritis (RA) is characterized by chronically inflamed and often destructed joints. The prevalence of RA is 0.5–1% with a female predominance. RA is caused by an autoimmune response located to the joints; however, the triggering factors and the pathogenesis of RA are not fully understood. Animal models of RA are useful tools to clarify the immunological mechanisms as well as to test therapeutic efficacy of new drugs. In this thesis, experimental arthritis models in mice have been utilized to study effects of estrogens and estrogen-like drugs in autoimmune arthritis. In the attempt to make a long story short; the main aims of the thesis are graphically presented in fig. 1. However, some basics facts are needed to be able to understand the aims;

- The female sex hormone **estradiol** ameliorates arthritis in mice and influences disease activity in women with RA.
- **Osteoporosis**, generalized bone loss, can be caused by estrogen deficiency due to menopause, as well as a consequence to severe inflammation, due to *e.g.* RA.
- Estrogens, synthetic estrogen-like drugs such as selective estrogen receptor modulators (**SERM**), and combined estrogens and SERM (tissue-selective estrogen complex [**TSEC**]) reduce osteoporosis in postmenopausal women
- Interleukin-17 (**IL-17**) is a proinflammatory cytokine contributing to inflammation and destruction of joints as well as bone erosions and osteoporosis, in RA.

The questions raised in *aim 1* are; do the third generation SERM or TSEC inhibit arthritis development and associated osteoporosis in experimental arthritis? (fig. 1). The answers are found in Paper I and II. Furthermore, the central question in *aim 2* is; does estradiol regulate IL-17-producing T cells in experimental arthritis? Paper IV and V are answering this question, as a contribution to the elucidation of mechanisms in estradiol-mediated inhibition of arthritis. Of note, the aim of paper III is not represented in figure 1; is the collagen antibody-induced arthritis (CAIA) model associated with generalized bone loss? *For the very curious reader; jump to section 7 to obtain all the answers directly.*

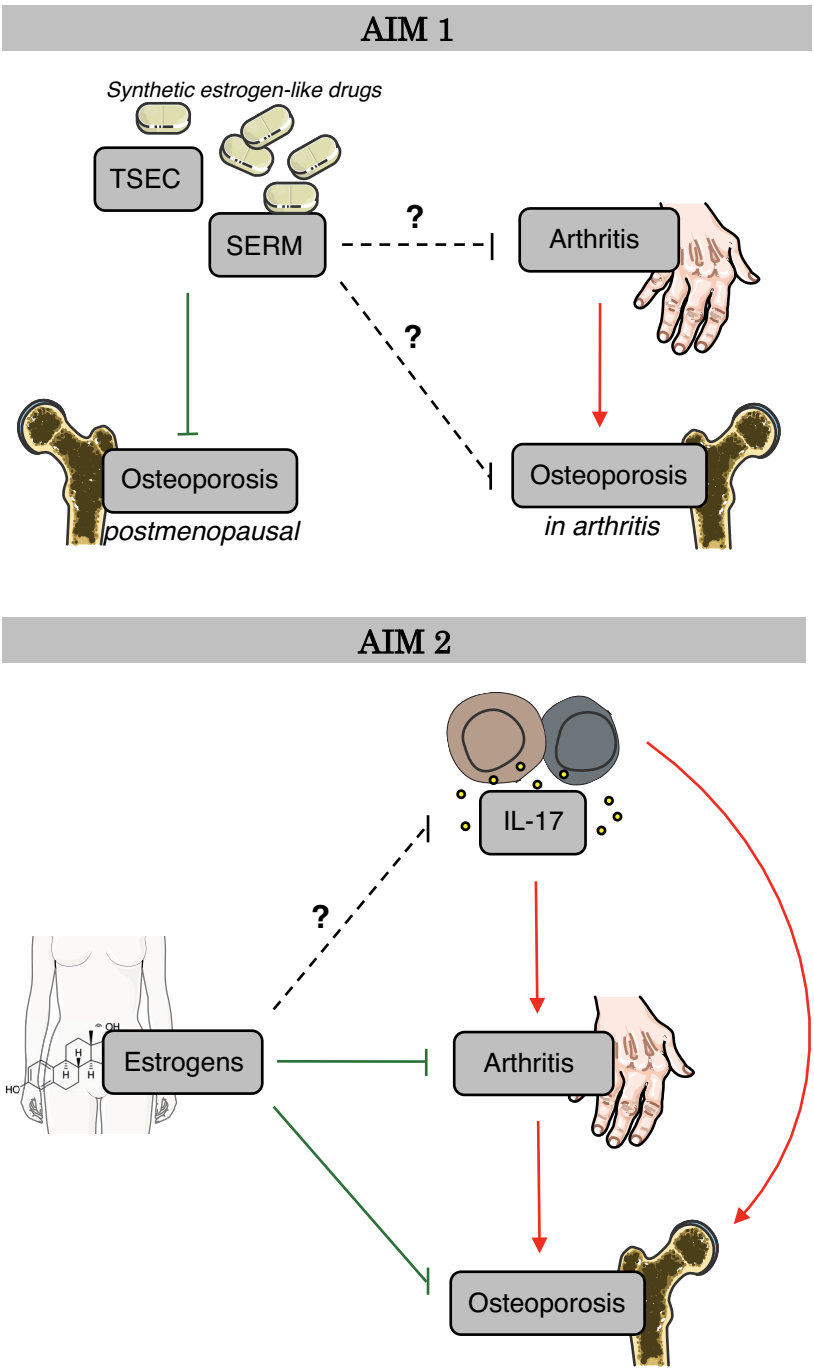

Figure 1. Illustration of the main aims of the thesis. Question marks pinpoint areas of investigation in the work of this thesis. All studies are performed in experimental arthritis in mice. Red arrows indicate known stimulatory effect, green blunted lines represent known inhibitory effect. TSEC: tissue-selective estrogen complex; SERM: selective estrogen receptor modulator; IL-17: interleukin-17.

2 THE IMMUNE SYSTEM

2.1 Inflammation

What are the threats to mankind? Spontaneously, one would think of natural disasters such as heavy storms and earthquakes. However, the most powerful threats to mankind are tremendously smaller than that. Historically, microorganisms have extinguished large human populations. The importance of our immune system, as defense against foreign invaders, is undisputable. We are constantly exposed to microorganisms but are very seldom seriously ill. What are the weapons of the immune system? What are the drawbacks of harboring such a powerful defense?

When you get a wound, the barrier (the skin) is damaged and bacteria are able to enter. Invading pathogens are immediately recognized by cells of **the innate immune system** – tissue-resident **macrophages** and **dendritic cells** (DCs) – via their pattern recognition receptors (PRR). PRRs bind pathogen-associated molecular patterns (PAMP) present in the bacterial cell wall, *e.g.* lipopolysaccharide (LPS) from gram-negative bacteria, binding to toll-like receptor 4 (TLR4) on the macrophage. The macrophage is activated and produces alarm signals – *e.g.* cytokines, chemokines, and prostaglandins – to alert the immune system and to facilitate immune cell transportation to the site of pathogen invasion. Blood vessel endothelium surrounding the injury gets more permeable and expresses adhesion molecules, directing white blood cells. Moreover, the injured dermal cells leak endogenous alarm signals due to necrosis (uncontrolled cell death). **Neutrophils**, the first cells that arrive to the site of infection, eradicate pathogens by phagocytosis and by releasing the content in their granules; reactive oxygen species and various enzymes toxic to microorganisms. **Monocytes**, also recruited from the blood stream, differentiate into macrophages at the site of infection. Macrophages are professional phagocytes and engulf whole bacteria as well as damaged cells. Altogether, these processes result in heat, pain, redness and swelling – the hallmarks of **inflammation.**

While the innate immune system rapidly recognizes and starts to eliminate the invading pathogen, a second line of defense is activated later on – **the adaptive immune system** – enabling support, specificity and amplification of the immune response. The DC provides a link between the innate and adaptive immune responses via its function as an antigen-presenting cell (APC). The DC patrols tissue and engulfs pathogens by phagocytosis and migrates to **lymph node** via lymphatics. Therein, the DC presents processed parts of the pathogen (**antigen,** usually a peptide) on major histocompatibility complex class II (MHC, or human leukocyte antigen [HLA]) to the naïve **T cell**. Naïve T cells have previously been educated in thymus (presented in detail in section 4.5). A set of chemokines and corresponding receptors, such as CC chemokine ligands (CCL) 19 and 21 and CC chemokine receptor (CCR) 7, directs circulating naïve T cell from high endothelial venules into the T cell zone of lymph node paracortex. Herein, the T cell encounters the APC and becomes activated as described in fig. 2.

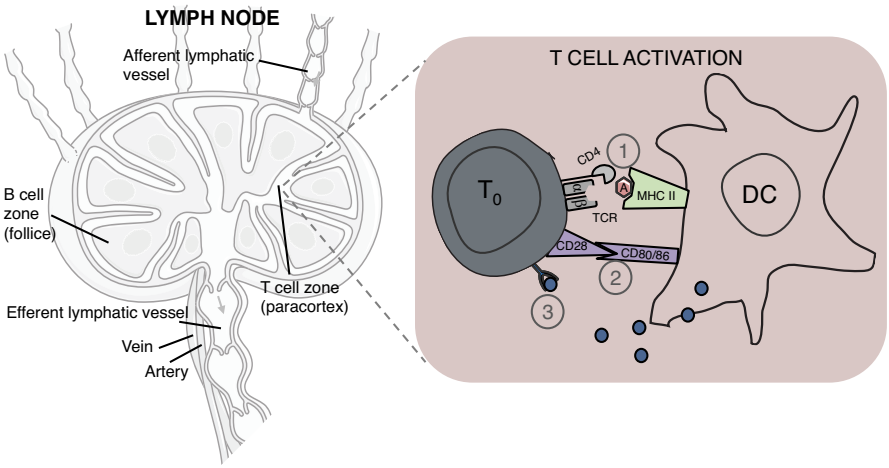

Figure 2. Initiation of the adaptive immune response. An antigen-presenting cell, *e.g.* a dendritic cell (DC) has processed a foreign protein, traveled to the lymph node where it encounters a naïve T cell (T_0). The APC presents the antigen to the T cell which will differentiate into an effector T helper cell if it receives correct signals, which are: 1) the T cell receptor (TCR) matches the antigen/MHC complex (there is one TCR for each possible antigen); 2) the APC expresses correct co-stimulatory signals, *e.g.* CD80/CD86 binding to CD28 on the T cell; 3) certain cytokines are present. T_0: naïve T cell; DC: dendritic cell; CD: cluster of differentiation; MHC: major histocompatibility complex; TCR: T cell receptor.

Differentiated **T helper cells** ($CD4^+$) undergo clonal expansion and exit the lymph node via efferent lymphatics, mediated by sphingosine-1-phosphate receptor (S1PR) signaling. These cells are now highly efficient cytokine producers, thereby supporting the innate immune response. The term 'helper' stems from the function of T cells to help B cells to become activated and produce antibodies. Several subclasses of T helper cells have been defined based on cytokine profile (fig. 3); however, the plasticity between Th lineages has gained a lot of attention lately (reviewed in [1]), truly challenging this categorization.

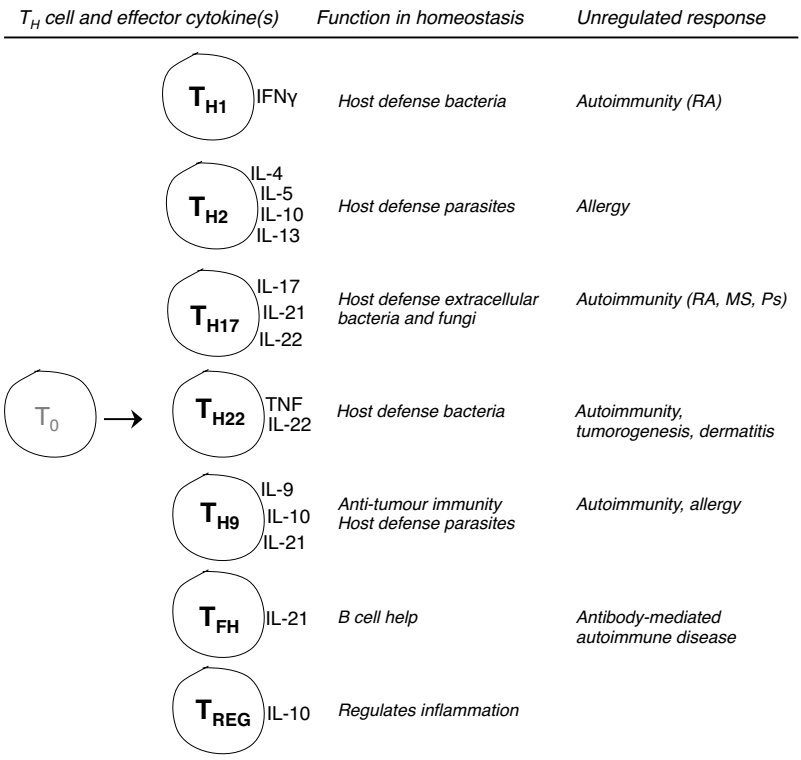

Figure 3. T helper cell types, their signature effector cytokine(s) and functions in health and disease. Th: T helper; Tfh: T follicular helper; Treg: regulatory T cell; IL: interleukin; IFN: interferon; TNF: tumor necrosis factor; RA: rheumatoid arthritis; MS: multiple sclerosis; Ps: psoriasis.

$CD8^+$ T cells – **cytotoxic T cells** – are important in fighting viral and intracellular bacterial infections. These cells kill host cells not expressing self (expressing pathogen components instead) on MHC class I (all nucleated cells express MHC I), after initial activation by an APC and after T helper cell cytokine production.

The main effector function of the **B cell** is to produce immunoglobulins (Ig) (**antibodies**). Antibodies bind to and neutralize pathogens, activate the complement system, and activate innate cells via their Fc-receptors, altogether facilitating eradication of pathogens. B cell development occurs in bone marrow (presented in detail in section 4.5). The immature naïve B cell leaving the bone marrow expresses the B cell receptor (BCR), consisting of a membrane-bound IgM antibody, allowing the B cell to respond in an antigen-specific manner. Antigen bound to BCR is internalized and presented on MHC II, whereby T helper cell recognition of this antigen will result in cell contact (via CD40-CD40L interaction) and subsequent B cell activation, in the secondary lymphoid organs. T helper cell-mediated activation of **follicular B cells** (FO B) cause differentiation of **plasma cells** and memory B cells. The plasma cells then produce high affinity antibodies (isotype-switched: IgG, IgA, IgE) towards the protein antigen that initially activated the B cell and the T helper cell. Non-protein antigens (*e.g.* bacterial polysaccharides, lipids) cannot activate T cells; instead these antigens directly activate splenic **marginal zone** (MZ) B cells or **B1** B cells in the peritoneal cavity or at mucosal surfaces. Subsequently, these cells mature into short-lived plasma cells producing low-affinity IgM antibodies. In addition, B cells also produce cytokines, and under certain circumstances B cells can activate naïve T helper cells via antigen presentation on MHC II.

Some of the T and B cells remain after the inflammation has resolved, providing the immune system with a memory function. The memory cells enable a faster and more efficient pathogen clearance upon reinfection. The most prominent differences between innate and adaptive immunity are thus the specificity and memory (provided by T and B cells), and the time span (innate response is rapid).

2.2 Autoimmunity

What is foreign and what is self? This is a central question in maintaining a competent immune response that eradicates danger but is unresponsive to tissue that is self – **self tolerance**. The importance of the immune system's ability to discriminate self from non-self cannot be stressed enough. If T and B cells incorrectly become activated by self-antigens, break of self tolerance

occur, resulting in autoimmunity and potentially severe disease. Autoreactive B cells produce **autoantibodies** directed towards self structures.

Thus, several processes must prevent autoimmunity to arise. During T cell lymphopoiesis in the thymus, autoreactive T cells are eliminated through selective processes, maintaining **central tolerance**. The immature $CD4^-CD8^-$ ("double-negative") T cells are selected based on affinity of their TCR to self-antigens:

- Weak recognition of MHC II + self-antigen results in *positive selection* – a mature naïve $CD4^+$ T cell
- No recognition of MHC II + self-antigen leads to apoptosis (so-called death by neglect)
- Strong recognition of MHC II + self-antigen gives rise to apoptosis (*negative selection*).

$CD8^+$ T cells are selected in the same manner, but are responding to antigens presented on MHC I instead. Although T cell selection takes place in the thymus only, the diversity of self-antigens is ensured by the unique expression of a self-antigen repertoire in thymus, controlled by the transcription factor autoimmune regulator (AIRE). Autoreactive B cells undergo selection based on BCR affinity to self, primarily in the bone marrow. Autoreactive B and T cells can be further controlled in the periphery, *e.g*. by incomplete signaling during activation in secondary lymphoid organs – **peripheral tolerance**. In addition, T helper cells with suppressive capacity, **regulatory T cells** (Treg), can control autoimmune responses – **regulatory tolerance** (find out more about Tregs in section 5.4).

Besides the fact that autoreactive cells can escape tolerance checkpoints and become activated by self-antigens, modification of self-antigens can also occur and thereby the signature of self is lost. One example is citrullination, a posttranslational modification of endogenous proteins, resulting in peptides that can be recognized by the immune system as non-self. Environmental factors, such as smoking, can cause these modifications [2]. How citrullination is linked to autoimmune disease is further discussed in section 5.3.

Around 5 % of the western population is affected by autoimmune disease. An autoimmune response can arise in virtually all tissues in the body, *e.g.* joints (RA), central nervous system (multiple sclerosis), pancreas (diabetes mellitus) and skin (psoriasis). Immune responses differ greatly between men and women. In general, women mount stronger immune responses than men,

reflected in the female predominance in the incidence of autoimmune diseases. In particular, women are more prone to develop *e.g.* systemic lupus erythematosus, Sjögren's syndrome and RA. Instead, men are more susceptible to malignant cancer diseases (cancers of reproductive organs excluded) [3].

2.3 Introducing IL-17

IL-17 is actually a family of cytokines – IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (IL-25), and IL-17F. IL-17A is the most studied and of most relevance in autoimmunity. From now on, IL-17A will be referred to as "IL-17".

Several autoimmune diseases were considered to be caused by Th1 cells, until the **T helper 17 (Th17)** cell lineage was discovered a decade ago [4, 5]. The development of the Th17 cell lineage depends on IL-23, and IL-23 was the clue in the discovery of Th17 cells in autoimmunity [4, 6, 7]. Beyond antigen-specific TCR activation, certain cytokines are needed for Th17 cell differentiation (different cytokines between mice and men). IL-6 is critical in both species but the role of TGFβ is controversial and IL-1β is needed in humans but not in mice [8-10]. Th17 cell differentiation is dependent on expression of the transcription factor RAR-related orphan receptor γt (RORγt) and is inhibited by interferon (IFN) γ [11]. Except from producing IL-17, Th17 cells also secrete tumor necrosis factor (TNF) α, IL-22 and IL-21, and their migration is mainly controlled by the expression of CCR6 [12].

Another major producer of IL-17 is the **γδ T cell**, particularly in certain bacterial infections [13]. γδ T cells express γ and δ chains of the TCR, instead of α and β as T helper cells. This T cell originates from the thymus, does not generally express CD4 and CD8, and is considered as a more innate-like T cell type since its activation occur primarily in an antigen-independent fashion [14]. "Natural" thymus-derived γδ T cells rapidly respond to PAMP via TLR1 and 2 and produce cytokines, *e.g*. IL-17 and IFNγ upon activation [15, 16]. However, some reports indicate that IL-17 production from γδ T cells can be induced by antigen recognition and these cells have been termed "inducible" γδ T cells [17, 18]. Similarly to Th17 cells, IL-17-producing γδ T cells express RORγt, IL-23R and CCR6 [16] (paper V). γδT cells reside mainly on epithelial surfaces and in skin, but are also found in lymph nodes and in inflamed joints [19-21].

Other cells reported to produce IL-17, although not studied in the work of this thesis, are for example mast cells, neutrophils, group 3 innate lymphoid cells and natural killer cells [22-25].

The role of IL-17 in RA is discussed in section 5.4, and later on also effects of estrogen on IL-17-producing cells in RA are presented (section 6.3).

3 OSTEOPOROSIS

3.1 Clinical introduction

Osteoporosis naturally occurs as a consequence of age and declining sex steroid levels, in both sexes. However, the rapid decrease in estrogens during menopause renders women at an immensely higher risk of developing osteoporosis and subsequent fragility fractures, compared with men. Around 40% of postmenopausal women are affected by osteoporosis; however, the disease is asymptomatic until a fracture occurs [26]. Osteoporotic fractures are most frequent in hip, forearm and spine [27]. Besides sex hormones, a number of other hormones and factors are systemically controlling bone metabolism, *e.g.* parathyroid hormone, vitamin D, calcitonin, and thyroid hormones. **Secondary osteoporosis** represents the bone loss occurring due to a primary disease, such as RA, further discussed in section 5.5. Moreover, osteoporosis can also develop as a side effect due to treatment, *e.g.* glucocorticoid therapy. **Clinical diagnosis** of osteoporosis is set by bone mineral density (BMD) measurement. BMD can be assessed with dual energy x-ray absorptiometry (DXA), defining osteoporosis as a T score of less than minus 2.5, which means more than 2.5 standard deviations below the average of a young adult [28]. The first hand choice of treatment is bisphosphonates, combined with calcium and vitamin D(3) supplementation. Biologic agents such as monoclonal antibodies against receptor activator of nuclear factor kappa-B ligand (RANKL) treatment are newer options (the role of RANKL in bone is discussed in section 3.2) [29]. Selective estrogen receptor modulators (SERM) such as raloxifene and bazedoxifene are used less frequently in osteoporosis therapy than bisphosphonates [30, 31]. Moreover, combined SERM and estrogens in the tissue-selective estrogen receptor complex (TSEC) was recently approved as postmenopausal osteoporosis prevention in the US. SERM and TSEC are presented in detail in section 4.4.

Mice do not go into menopause naturally; therefore, surgical removal of ovaries, **ovariectomy** (OVX), is a commonly used method to induce postmenopausal osteoporosis in mice. Mice were subjected to OVX throughout all experiments in this thesis.

3.2 Bone physiology

The skeleton protects internal organs and supports the body with strength and motility. Moreover, bone also constitutes the nursery for hematopoiesis and storage of calcium and phosphate. Bone is a rigid but still flexible construction, due to its unique composition of a mineralized extracellular matrix. Collagen type I (CI) fibers are the main organic components in the matrix, other proteins are osteocalcin and osteopontin. Hydroxyapatite crystals constitute the inorganic bone compartment. Two types of bone structure, with different properties due to the extent of mineralization, are present in bone. **Cortical** bone is compact and dense and constitutes the hard outer shell of a bone (fig. 4), comprising 80% of all bone tissue. The remaining 20% of bone is **trabecular** (cancellous) bone, a sponge-like network found within the bone.

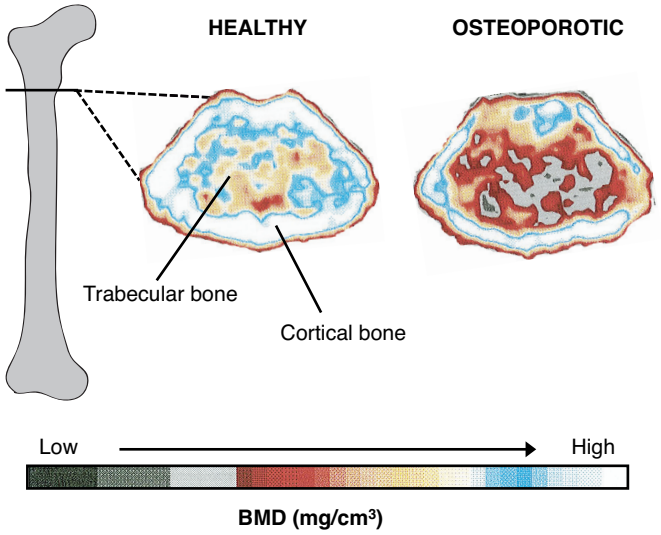

Figure 4. Illustration of bone structure and osteoporosis. Cross sections of femurs from ovariectomized DBA/1 mice, obtained by peripheral quantitative computed tomography scans. Treatment with estradiol represents "healthy" and placebo control represents "osteoporotic". Color bar indicates bone mineral density, with higher density in cortical bone compared with trabecular bone, and higher density overall in healthy bone vs. osteoporotic bone.

Bone is not an inactive tissue; instead it is constantly remodeled. Several bone cells operate the metabolism of bone. **Osteoblasts** (OBL), originate from mesenchymal stem cells, form new bone by secreting CI and other bone matrix proteins, and mineralize the matrix by expressing the enzyme alkaline

phosphatase. During bone formation, some OBL are trapped within small spaces in the bone (lacunae), and become **osteocytes**. Osteocytes, the most abundant cell type in bone, sense loading of bone, *e.g.* mechanical pressure due to movement. The bone-resorbing **osteoclasts** (OCL) originate from the hematopoietic lineage, from precursors of monocyte/macrophage phenotype. OCL differentiation is dependent on RANKL and M-CSF [32], where mononuclear precursors fuse and form multinuclear OCL. The OCL resorbs bone in two steps, first by demineralization via secretion of hydrochloric acid, and secondly by producing matrix-degrading proteolytic enzymes. OBL produce RANKL but also osteoprotegrin (OPG), which inhibits RANKL by acting as a decoy receptor. OCL can be detected and counted in bone sections by staining for the OCL-specific enzyme tartrate-resistant acid phosphatase (TRAP) (paper III). Metabolites generated during bone remodeling can be quantified in serum to get a reflection of bone formation vs. bone resorption. In papers I–III, levels of N-terminal propeptide of type I procollagen (PINP), and C-terminal telopeptides of type I collagen (CTX-I), were assessed as measurements of ongoing bone formation and bone resorption, respectively. The immune system and bone closely interact via the influence of *e.g.* cytokines on the bone remodeling process. This area is called *osteoimmunology* and is further discussed below as well as in the context of RA (section 5.5).

3.3 Pathogenesis

After menopause, the bone turnover rate is generally accelerated; however, the net effect results in increased bone resorption. Estrogens directly reduce differentiation of OCL and induce OCL apoptosis, thus explaining the increased bone resorption after menopause [33, 34]. Furthermore, estrogens induce OPG production from OBL, thereby inhibiting RANKL-driven osteoclastogenesis [35]. In addition, estrogen deficiency negatively regulates bone metabolism systemically as estrogen increases intestinal calcium absorption and serum 1,25-dihydroxivitamin D levels [36, 37].

During the last decade, the role of the immune system in the pathogenesis of postmenopausal osteoporosis has been appreciated, in particular T cells and their cytokines. Menopause is associated with elevated serum levels of IL-1, IL-6, and TNFα [38]. Bone marrow T cells producing TNFα are elevated after OVX in mice and $TNF^{-/-}$ mice are resistant to OVX-induced osteoporosis [39]. Moreover, IL-17 has also been implicated in postmenopausal bone loss. Th17 cell numbers are increased in OVX mice compared with sham-operated controls [40]. In accordance, IL-17 serum

levels are elevated in postmenopausal women, compared with premenopausal controls, and high IL-17 levels are associated with low estradiol levels [41]. When comparing anti-IL-17, anti-TNF and anti-RANKL treatments in OVX mice, targeting IL-17 was found to be most efficient regarding protection of bone [42].

4 ESTROGENS

Estrogens are the primary sex hormones in women. Progesterone and testosterone are other sex hormones of significant importance in females. In the following section, estrogens and later on also synthetic estrogen-like drugs, will be presented.

4.1 Biosynthesis and function

Estrogens have multiple biological functions and are most prominently involved in maturation and development of reproductive organs in women, *i.e.* breast and uterus. Moreover, estrogens have profound effects on bone as previously discussed, both in females and males. In addition, estrogens influence the central nervous, cardiovascular and immune systems. Owing to the proliferative actions of estrogens on uterus and breast, estrogen-containing therapy increases the risk of cancer in reproductive organs (table 1).

Estrogens have a steroid tetracyclic structure with an aromatic A ring (fig. 5A). The biosynthesis of estrogens starts with cholesterol, which several steps later result in the androgens androstenedione and testosterone that are aromatized into estrogens (by the enzyme aromatase). Thus, expression of aromatase is a key factor in determining local estrogen levels. Levels of sex hormone-binding globulin (SHBG) determine the bioavailable amount of estrogens and androgens in serum, and the majority of sex steroids in humans are bound to SHBG. However, rodents lack SHBG [43].

Estrogens is the collective term for **estrone (E1)**, **estradiol (E2),** and **estriol (E3)** where

- E1, the primary circulating estrogen after menopause, is present in low levels in the fertile woman. E1 is mainly produced at extragonadal sites such as adipose tissue.
- E2 is produced by granulosa cells in the ovarian follicles, and is the most abundant and potent estrogen. Some production of E2 also occurs in the adrenal cortex (in humans, but not in mice).

- The placenta produces E3 during pregnancy and E3 is also the major estrogen metabolite in urine.

4.2 Receptors and signaling

All steroids are lipophilic and thus readily pass over the cell membrane. Estrogens bind to nuclear receptors, the **estrogen receptor alpha (ERα)** (fig. 5B) and **beta (ERβ)**, which to some extent overlap in structure and function. As a ligand-activated transcription factor, the ER has a ligand-binding domain (LBD) and a DNA-binding domain (DBD). Ligand and receptor binding result in ER dimerization (hetero or homodimers) and the ER-estrogen complex translocates to the nucleus and binds to estrogen-response elements (ERE) in DNA. This pathway of estrogen signaling is denoted the classical transcriptional pathway (fig. 5C:1). In addition, coactivator or corepressor proteins bind to the ER-E2 complex to further regulate transcriptional activity. In the non-classical transcriptional pathway, ER-estrogen complex binds alternative transcription factors (such as AP-1 or SP-1) bound to non-ERE sites in DNA (fig. 5C:2). Subsequently, both classical and non-classical pathways result in induction or repression of gene transcription. However, ER can also be membrane-bound (mER). In addition, another type of ER has been found; a membrane-bound G protein-coupled estrogen receptor-1 (GPER-1). Both mER and GPER-1 mediate estrogenic signaling via rapid non-genomic responses influencing intracellular signaling cascades (fig. 5C:3) [44]. Studies with mice lacking either ERα or ERβ, or double ERαβ knockout (KO) mice, have clarified the importance of each receptor in various tissues and diseases. ERα mediates the main effects of estrogens on bone [45] and in reproductive organs, thus $ER\alpha^{-/-}$ mice have a disturbed reproductive function [46]. $ER\alpha^{-/-}$ mice were used in paper IV and V. In addition, conditional KO mice where the ER is selectively deleted in a specific cell type, using the Cre-Lox recombination technology, has also been useful in research concerning estrogens.

4.3 Estrogen replacement after menopause

Estrogens or therapy modulating ER response are used for treatment of climacteric symptoms and postmenopausal osteoporosis. Furthermore, these compounds are exploited as oral contraceptives, fertility agents, and breast cancer therapeutics. However, in this section, focus will be on the role of estrogen and estrogen-related drugs in treatment of symptoms and diseases associated to menopause.

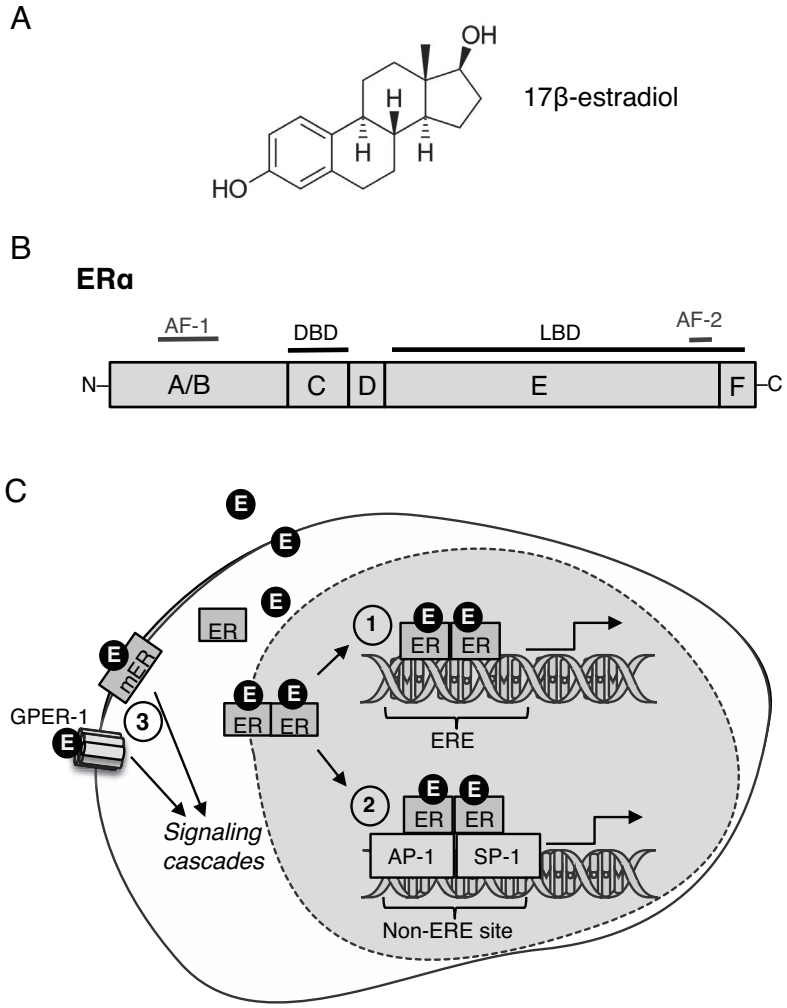

Figure 5. Estradiol, the ERα protein and ER intracellular signaling pathways. (A) The molecular structure of 17β-estradiol, a steroid structure with the aromatic A ring typical for estrogens. **(B**) the ERα protein with 5 domains: A/B with transcriptional AF-1; C with the DBD; D is the hinge region; E/F contains the LBD and AF-2. **(C)** Describes estrogen signaling via 1) the classical transcription pathway, 2) the tethered or non-classical transcription pathway and, 3) membrane-associated ER and GPER-1, resulting in rapid non-genomic responses.
ER: estrogen receptor; mER: membrane-bound ER; GPER-1: G protein-coupled estrogen receptor-1; AF: activating function; DBD: DNA-binding domain; LBD: ligand-binding domain; E: estrogen; TF: transcription factor; AP-1: activator protein-1; SP-1: specificity protein-1.
Illustration in C was adapted with permission from PhD Angelina Bernardi.

Production of E2 and progesterone from ovaries start to cease after the age of 40, ultimately resulting in a physiologic state called **menopause** around the age of 50, referring to pause of menstrual cycling. Beyond accelerated bone loss with subsequent osteoporosis as discussed previously, the rapid decrease in ovarian function also result in atrophy of uterine endometrium and vaginal epithelium, elevated risk for hypertension and atherosclerosis, vasomotor symptoms (hot flushes, sweating) and loss of fertility. **Hormone replacement therapy (HRT)** is prescribed to reduce menopausal symptoms, and has additional beneficial effects on bone health. HRT usually consists of an estrogenic part, *e.g.* synthetic estradiol or ”natural estrogens” (conjugated equine estrogens [CEE]) extracted from horse urine, and a progesterone (P) part, *e.g.* progestin. Progesterone is necessary to prevent estrogen-induced endometrial hyperplasia of the uterine lining, thus, in hysterectomized women estrogens alone can be administered as HRT.

HRT was readily used until reports from the large *Women's Health Initiative* (WHI) study in the US and the *Million Women Study* in the UK came in the early 2000s. The primary aim of the WHI study was to assess effect of continuous HRT (CEE+P) on coronary heart disease (CHD) in postmenopausal women, and secondly, to evaluate breast cancer risk. However, the study was terminated early due to increased events of breast cancer and lack of protective effect on CHD [47]. Results from the UK study confirmed the increase in breast cancer risk [48]. In contrast, in the estrogen-alone arm of the WHI study, CHD risk was not affected and breast cancer risk tended to be lower after CEE treatment [49]. The conclusions drawn from the WHI study received substantial criticism, amongst them questioning the inclusion of rather old women (up to 79 years of age). Re-evaluation of study results showed that CHD risk was instead nearly reduced in ”young” postmenopausal women in receiving CEE+P [50]. The inclusion of subjects in the Million Women Study was criticized for being biased; subjects were enrolled to the study when attending breast cancer screening mammography units.

Current recommendations are that short term HRT can be used to alleviate moderate to severe vasomotor symptoms in women with recent transition into menopause. HRT also improves bone health but is not recommended to use as treatment of osteoporosis.

4.4 Selective estrogen receptor modulators (SERM)

SERM are synthetic estrogen-like molecules developed for several therapeutic purposes. SERM can either be utilized when ER-agonistic effects are desired, or to achieve ER-antagonistic effects, or both simultaneously. SERM pharmacology is influenced by many factors: *e.g.* relative binding affinity for ERα and ERβ; the tissue-specific ERα and ERβ expression; influence on ER conformation and binding of coregulators, and the ERE sequence within the target gene (reviewed in [51]). In comparison to the molecular structure of E2, SERM have long bulky side chains which influence conformation of ER upon binding; specifically preventing the formation of a transcriptionally active AF-2 region in the LBD of ERα [52]. However, it was recently established that the anti-osteoporotic effects of SERM in OVX mice are dependent on both the AF-1 and AF-2 regions of ERα [53, 54].

The first commercially used SERM was **tamoxifen**, as an ER-antagonist for adjuvant treatment of ER-positive breast cancer [55]. However, as tamoxifen increased bone mineral density in breast cancer patients, it was established that tamoxifen exerted mixed ER antagonist-agonist properties [56]. Unfortunately, tamoxifen had agonistic properties also in uterus. Altogether, this prompted researchers to find a SERM with antagonistic effects in uterus and breast, but agonistic effects in bone, in order to be useful as treatment of postmenopausal osteoporosis. **Raloxifene** (initially named keoxifene) was the first approved SERM for treatment of postmenopausal osteoporosis [30]. Later on raloxifene proved to be effective as breast cancer prevention [57]. More recently, the third generation SERM, lasofoxifene and bazedoxifene has been approved as osteoporosis therapy in postmenopausal women. **Lasofoxifene**, was the first SERM to prevent non-vertebral fractures [58]. Moreover, lasofoxifene decreased risk of ER-positive breast cancer but increased vaginal bleeding (but not endometrial cancer) [58, 59]. Although lasofoxifene was approved in the EU year 2009, it has not been marketed, thus approval has been withdrawn. **Bazedoxifene**, currently used in EU and Japan, prevents vertebral fractures and also non-vertebral fractures in high-risk patients (femoral neck T-score ≤-3.0 and/or earlier vertebral fracture]) [31]. Bazedoxifene is not an ER-agonist in uterus or breast tissue [60]. Another new SERM is **ospemifene,** approved for the treatment of severe vulvar and vaginal atrophy and subsequent dyspareunia. However, ospemifene is not yet evaluated in terms of anti-osteoporotic properties [61].

As an option to classic HRT with estrogen and progesterone, the combination of conjugated estrogens (CEE) and bazedoxifene has turned out very

successful and was recently approved as prevention of postmenopausal osteoporosis (in US only) and treatment of vasomotor symptoms (hot flushes) (in the EU and US) [62]. This combination is named the **tissue-selective estrogen complex (TSEC).** In addition, TSEC improved lumbar spine and hip BMD but has not been evaluated in terms of influence on fracture risk yet [63]. Bazedoxifene acts as an ER antagonist on reproductive organs thus blocking estrogenic effects therein, and an ER agonist on bone, but presumably exert low blood-brain barrier penetrance, as opposed to estrogens. Thus, estrogens exert positive effects on central vasomotor regulation.

A summary of the clinical characteristics of SERM and TSEC is found in table 1.

Table 1. Clinical characteristics of SERM and estrogen replacement therapy in postmenopausal women

		SERM *Osteoporosis treatment*			**Estrogen replacement** *Osteoporosis and menopausal symptom treatment*[g]	
		RAL	**LAS**	**BZA**	**HRT**[a]	**TSEC**[b]
Fracture prevention	Vertebral	Yes	Yes	Yes	Yes	ND[c]
	Non-vertebral	No[d]	Yes	No[d]	Yes	ND[c]
Vasomotor regulation	Hot flushes symptom relief	No[e]	No[e]	No[e]	Yes	Yes
Breast	Cancer prevention	Yes	Yes	No	No	No
	Cancer risk	No	No	No	Yes	No
Uterus	Endometrial cancer risk	No	No	No	No	No
	Endometrial thickness increase	No	Yes	No	No	No
Ovary	Cancer risk	ND	No	No	Yes	ND
Vagina	Vulvar and vaginal atrophy treatment	No	Yes	No	Yes	Yes
Heart/ circulation	Overall CVD risk	No	No[f]	No	Yes	No
	Stroke incidence increase	Yes	No[f]	No	Yes	No
	VTE incidence increase	Yes	Yes	Yes	Yes	No
References		[30, 57, 64, 65]	[58, 59, 64, 66]	[31, 64, 67-69]	[47, 70-76]	[63, 77-79]

a Continuous regimen with 0.625 mg conjugated equine estrogens (CEE) + 2.5 mg medroxyprogesterone acetate (MPA) in the Women's Health Initiative study
b 0.625 mg or 0.45 mg conjugated equine estrogens (CEE) + 20 or 40 mg bazedoxifene
c Fracture risk not yet assessed; however, TSEC increased BMD in lumbar spine, hip and femoral neck
d Reduced non-vertebral fracture risk in women at high-risk; having previous vertebral fracture
e Increases hot flushes
f Lasofoxifene reduced these events
g Besides use of HRT as menopausal symptom relief, HRT is approved as prevention of postmenopausal osteoporosis but is not recommended for this indication in Sweden
ND: not determined; SERM: selective estrogen-receptor modulator; RAL: raloxifene; LAS: lasofoxifene; BZA: bazedoxifene; HRT: hormone replacement therapy; TSEC: tissue-selective estrogen complex; CVD: cardiovascular disease (*e.g.* myocardial infarction, angina, stroke); VTE: venous thromboembolism.

4.5 Estrogen and SERM in adaptive immune development

Estrogens exert profound effects on the development and function of the immune system. ERs are expressed by virtually all immune cells (reviewed in [80]). Generally, E2 at high doses are believed to be anti-inflammatory whereas low doses can be pro-inflammatory, instantly revealing the complexity of the role of estrogens in the immune system (excellently reviewed in [81]). In the following section, effects of estrogens and SERM on the homeostatic immune system with emphasis on adaptive immune development will be discussed. Immunologic effects of estrogens and SERM in RA are presented later (section 6.3).

Estrogens cause involution of thymus, due to both **inhibited T lymphopoiesis** and influence on thymic stromal cells, summarized in fig. 6 [82, 83]. In accordance, OVX results in increased thymic weight and cellularity [84]. Briefly, E2 arrests early T cells in the first $CD4^-CD8^-$ double-negative stage (DN1), thereby reducing transition into DN2/3 stages. Subsequently, the levels of $CD4^+CD8^+$ DP cells are decreased, but single-positive (SP) $CD4^+$ and $CD8^+$ cells increase [85]. Interestingly, previous studies from our lab demonstrated that none of the SERM influences T cell developmental stages; nevertheless, raloxifene and lasofoxifene, but not bazedoxifene, reduce thymus weight in OVX mice [85, 86]. Estrogenic effects on effector T cells are not as established as those described in T lymphopoiesis. E2 but not SERM, suppresses T-cell dependent inflammation in the classic delayed-type hypersensitivity (DTH) model [85, 86]. Effects of E2 are often described as biphasic; low E2 levels result in pro-inflammatory effects while high levels are anti-inflammatory [81]. For instance, high E2 doses as during the third trimester of pregnancy, is believed to induce **Th2** response and reduce **Th1** response, via stimulatory effects on IL-4 production and inhibitory effects on IL-12, respectively [87-89]. Instead, low dose E2 treatment in ovalbumin-immunized OVX mice resulted in enhanced antigen-specific Th1 response [90]. Studies of the effects of estrogens on **Th17** cells in healthy mice are few and inconsistent, *e.g.* both E2-mediated induction and inhibition of RORγt expression and subsequent Th17 differentiation have been demonstrated [91, 92]. Nevertheless, E2 inhibits IL-6 and IL-1β in humans, necessary for Th17 differentiation [93, 94]. Estrogenic influence on Th17 cells and IL-17 has been more closely studied in the field of osteoporosis (section 3.3) and autoimmune diseases (section 6.3). Of note, $CD4^+$ T cells express higher levels of ERα than ERβ, whereas the ER expression is the opposite in B cells [95].

Estrogens have profound effects on **B cells** and the influence of SERM on B cells has been clarified in previous studies from our laboratory [96]. As illustrated in fig. 6, E2 arrests early B cell development in the bone marrow, resulting in elevated number of progenitor B cells (pro-B) and thus reduced populations of precursor B cells (pre-B). SERM do not influence pro-B cells, but still reduce the pre-B developmental stages. The membrane-bound antibody-expressing immature B cell is also numerically reduced after E2 or SERM treatment.

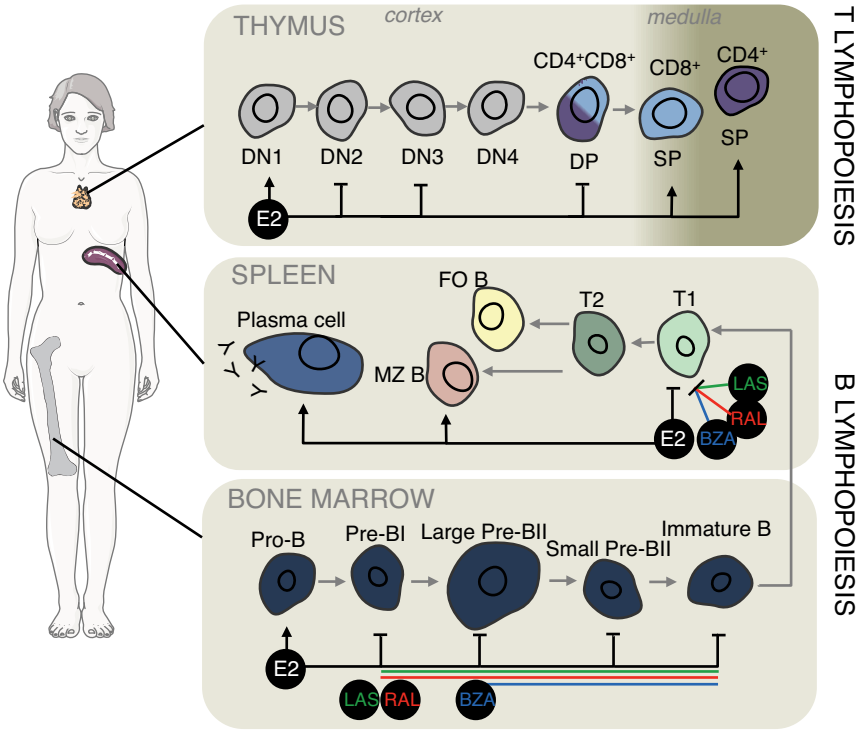

Figure 6. Influence of estradiol and selective estrogen receptor modulators on T and B lymphopoiesis. SERM completely lack effect on T lymphopoiesis, while having some effects on B lymphopoiesis. Black arrows indicate stimulatory effect, blunted lines represent inhibitory effect. The majority of data has been obtained in ovariectomized mice. DN: double-negative ($CD4^-CD8^-$); DP: double-positive; SP: single-positive; MZ: marginal zone; FO: follicular; T1: transitional 1; T2: transitional 2; pro-B: progenitor B cell; pre-B: precursor B cell; E2: estradiol; LAS: lasofoxifene; RAL: raloxifene; BZA: bazedoxifene.

After selection processes based on BCR affinity, the B cells leave the bone marrow and enter spleen for further selection and activation. Both E2 and SERM inhibit the first developmental stage in spleen, the formation of transitional (T) 1 B cells, but do not influence T2 B cells. T2 B cells differentiate into FO B cells or MZ B cells, as mentioned in section 2.1. Only E2, and not SERM, stimulate the formation of MZ B cells. Moreover, the stimulatory effects of estrogens on general Ig production from plasma cells are well known; however, none of the SERM increase number of Ig-secreting cells in spleen or bone marrow [96-98].

From an evolutionary perspective, estrogenic modulation of the immune response is probably important during pregnancy. Suppression of T lymphopoiesis and cell-mediated immunity reduces the risk of fetal rejection, while increase in humoral immunity (leading to antibody production) improves protection from infections as a compensatory mechanism.

5 RHEUMATOID ARTHRITIS

5.1 Clinical introduction

”Rheumatoid” originates from the Greek word *rheum*, which means flow, and ”arthritis” is the medical term for inflamed joint. The prevalence of RA is 0.5–1% worldwide. RA is characterized by tender, swollen and dysfunctional joints. Predominantly small joints of hands and feet are afflicted, often in a symmetrical fashion. Morning stiffness and fatigue are other common features of RA. Systemic inflammation and destruction of cartilage and bone in the joints result in life-long pain, disability and impaired quality of life. One study showed that after five years since RA diagnosis, around 30% of the patients could not manage a full time job [99]. In addition, RA is associated with elevated risk of developing osteoporosis [100, 101] and increased mortality, mainly due to higher incidence of cardiovascular disease in this group of patients [102]. The overall female to male ratio is 3:1 for RA. The typical newly diagnosed RA patient is a woman in the age of 50–60 years. Children can also be afflicted by autoimmune arthritis, so-called juvenile idiopathic arthritis.

The **diagnosis of RA** is based on criteria determined by American College of Rheumatology (ACR) and European League Against Rheumatism (EULAR) [103]. Briefly, to be diagnosed with RA, at least two large joints or one small joint should be swollen. Moreover, positive serology test for autoantibodies (Rheumatoid Factor [RF] and/or anti-citrullinated protein antibodies [ACPA]), as well as elevated acute-phase reactants (CRP and/or ESR) are also critical for diagnosis. Symptom duration longer than 6 weeks further strengthens the RA diagnosis. To quantify disease activity a score (DAS28) is calculated based on the examination of 28 joints together with ESR or CRP levels, and the patient’s global health.

5.2 Joint physiology

The joint is crucial for functional mobility. The surface of bone ends in joints is covered with articular cartilage, composed of chondrocytes embedded in matrix. Type II collagen (CII) and proteoglycans are the main components of cartilage matrix. Serum cartilage oligomeric matrix protein (COMP), is an established biomarker of cartilage degradation, assayed in papers I–III [104].

The synovium – the inner lining layer of the joint capsule – provides structural support, produces lubricating synovial fluid, and transports nutrients to the cartilage. The synovium is only two to three cell layers thick and consists of macrophage-like synovial cells and fibroblast-like synoviocytes [105].

5.3 Etiology

The etiology of RA is unknown, but it is believed that the disease arises due to interaction of several factors of genetic, environmental and infectious origin. Genes are important for development of RA, as 15% monozygotic twin concordance has been reported [106]. Furthermore, several risk genes related to immune regulation are associated with RA, such as the T cell activation pathway protein tyrosine phosphatase non-receptor type 22 (PTPN22) [107]. In particular, gene variants encoding for HLA are strongly linked to RA susceptibility, especially a specific amino acid motif in the HLA-DR4β region; the so-called **shared epitope** [108].

Citrullination, a post-translational modification where the amino acid arginine is exchanged to citrulline, is mediated by peptidylarginine deiminase (PAD) enzymes present at mucosal surfaces. Citrullination of various proteins, *e.g.* fibrinogen, vimentin, and α-enolase, results in peptides that can be recognized by the immune system as non-self. Thus, citrullinated peptides can result in activation of autoreactive T and B cells and ACPA production. **Smoking** increases PAD expression in lungs, thereby inducing citrullination [2]. Moreover, the shared epitope is linked to the presentation of citrullinated peptides [109]. In accordance, having the shared epitope and being a smoker clearly increase the risk of developing ACPA-positive RA, but not ACPA-negative RA [110]. Not only smoking contributes to the citrullination process; *Porphyromonas gingivalis*, a bacterium associated with **periodontal disease**, also expresses PAD enzymes with relevance for RA [111, 112].

In addition, other **infections** might initiate autoimmune disease through a process called **molecular** or **epitope mimicry** – when a foreign antigen shares structural similarities with a self-antigen (reviewed in [113]). Moreover, the general composition of gut and oral microbiota is altered in RA patients, compared with healthy controls, further implying a role for bacteria in the etiology of RA [114].

5.4 Pathogenesis

The pathogenesis of RA has not been fully elucidated; however, some of the so far clarified immunological pathways are summarized in fig. 7 in the end of this section. RA is a heterogeneous disease, with large inter-individual differences in disease progression, activity and treatment response. Thus, understanding the disease mechanism is difficult. Established RA is the end-stage of a multistep immunological process, and Burmester *et al* have stratified the development of RA into four phases [115]:

1. **Induction.** The innate immune system is triggered by infection, injury or environmental factors such as smoking. This activation might take place in the synovia, or start elsewhere.
2. **Inflammation.** Presentation of self-antigens, *e.g.* citrullinated proteins on variants of HLA (*e.g.* shared epitope), ultimately results in differentiation of effector Th cells. Th cells activate B cells, which produce autoantibodies. Th cells further amplify synovitis by production of pro-inflammatory cytokines.
3. **Chronification.** Destructed tissue permits formation of new self-antigens derived from *e.g.* cartilage. The adaptive response is reinforced, resulting in a highly pro-inflammatory cytokine milieu, further attracting and activating neutrophils and macrophages.
4. **Destruction.** Upon cytokine stimulation, joint resident cells produce matrix-degrading enzymes and bone-resorbing osteoclasts are differentiated, whereby both bone and cartilage are damaged.

The inflammation of the synovium lining the joint – synovitis – is due to both leukocytes migrated from the circulation and proliferation of fibroblast-like synoviocytes (FLS). **T cells** constitute 30–50% of the cells in RA synovium, and most are T helper cells. As previously mentioned, before the discovery of **Th17** cells, RA was believed to be driven primarily by Th1 cells. However, when arthritis was induced in mice lacking the receptor for the Th1 hallmark cytokine, IFNγ, and these mice developed very severe arthritis it became evident that the Th1 paradigm in autoimmunity was failing [116, 117]. Later on, it was reported that mice deficient of **IL-17A** developed very mild arthritis in the collagen-induced arthritis (CIA) model compared with controls (experimental arthritis models are described in section 5.7) [118]. Accordingly, therapeutic neutralization of IL-17 in CIA reduces arthritis [119] while IL-17 overexpression aggravates CIA [120]. Altogether, these studies showed that IL-17 mediates synovitis, cartilage and bone erosions in murine arthritis. In synovial explant cultures from RA patients, IL-17 production was found already nearly 20 years ago and high levels of IL-17 in

synovial fluid have been confirmed [121, 122]. Effector mechanisms of IL-17 in RA are numerous, *e.g.* IL-17 stimulates production of IL-8, IL-6 and tissue-degrading enzymes matrix metalloproteinases (MMP) from FLS [123] and attracts neutrophils [124]. The extensive effects of IL-17 in RA are highlighted in fig. 7.

Studies of IL-17-producing γδ T cells in CIA demonstrated that in joints and draining lymph nodes, numbers of IL-17$^+$γδ T cells are equal to, or even outnumber Th17 cells, and IL-17$^+$γδ T cells increase with disease activity [20, 125] (paper IV and V). IL-17 production from γδ T cells is induced by IL-1β and IL-23, independent of TCR [125]. However, IL-17$^+$γδ T cells do not contribute to joint destruction as Th17 cells do, and have not been found in synovial tissue in human RA [125, 126].

The chemokine CCL20 and its receptor, **CCR6**, are important in T cell migration from lymph nodes to joints. CCR6 is expressed by the majority of Th17 and IL-17$^+$γδ T cells (paper IV and V). CCL20 production in RA joints drives migration of CCR6$^+$ Th17 cells and CCR6 blockage ameliorates CIA [12, 127]. Moreover, adoptive transfer of CCR6$^+$ γδ T cells enhances CIA [125]. In addition, a polymorphism in the *Ccr6* gene has been associated with RA susceptibility, and correlates with CCR6 expression and presence of IL-17 in RA serum [128]. In addition, ACPA-positive RA patients have higher proportions of peripheral memory CCR6$^+$ T helper cells than ACPA-negative patients [129].

Foxp3-expressing **Tregs** are critical regulators of autoimmunity, clearly proven by the fact that spontaneous mutations in the *Foxp3* gene result in aggressive fatal autoimmunity, both in mice and men [130, 131]. Tregs can suppress effector T cell responses, such as Th17 cell activity. Thus, in RA, it has been proposed that there is an imbalance in the Treg-Th17 cell immunity, favoring Th17-driven autoimmunity [132, 133]. Tregs mediate suppression via several mechanisms, *e.g.* secretion of IL-10, TGFβ and CTLA-4 [134]. IL-10 and TGFβ are anti-inflammatory mediators and CTLA-4, a ligand to CD80/86 on APC, abrogates CD28-mediated costimulation during T cell activation. Interestingly, CD4$^+$CD25$^+$ Tregs isolated from RA patients were unable to suppress T helper cell-produced TNFα [135]. Lately it has become increasingly clear that Th17 and Tregs are not distinct and constant lineages, as a high degree of plasticity between these cell lineages has been demonstrated in arthritis [136]. Thus, Tregs could lose Foxp3-expression and undergo differentiation into Th17 cells, with subsequent ability to produce IL-17. Moreover, these “exFoxp3 Th17” cells accumulate in inflamed joints in CIA mice and are even more pathogenic than their conventional Th17 cell

counterparts with respect to induction of osteoclastogenesis. Moreover, presence of exFoxp3 Th17 cells was confirmed in synovial samples from RA patients [136].

The role of **B cells** in RA is supported by the presence of several types of **autoantibodies**. Autoantibodies are detected in serum long before RA diagnosis [137]. Autoantibodies with diverse specificities have been found in RA, *e.g.* antibodies to citrullinated fibrinogen, vimentin, α-enolase, as well as anti-collagen type II (CII) and glucose-6-phosphate isomerase antibodies. RF is an antibody of IgM isotype that binds to the Fc portion of IgG, thus creating immune complexes that activate complement pathways and evoke subsequent innate immune response. The **antigen-presenting function of B cells** is of importance in autoimmunity [138, 139]. This is simply proven in RA where the depletion of $CD20^+$ B cells with anti-CD20 monoclonal antibody is therapeutic [140]. Since plasma cells do not express CD20, the beneficial effect of anti-CD20 treatment is not explained by reduction in autoantibody production, instead other functions of the B cell are crucial for disease activity. Generally, B cells are a minor population in the synovia; however, ectopic lymphoid follicles can occur in synovia, where B cells encounter Tfh cells and undergo affinity maturation and acquire antibody-producing function in a similar fashion as in lymph nodes [141]. Moreover, B cells produce pro-inflammatory cytokines in synovia; *e.g.* TNFα and IL-6, but a subset of B cells – **regulatory B cells** (Breg) – can produce IL-10, and thereby suppress CIA [142].

Dendritic cells are significant contributors to autoimmunity via their primary functions as professional APCs. Indeed, antigen-presentation is critical in RA – supported by the strong association between HLA genes and disease. DCs presenting self-peptides initiate autoreactive T-cell differentiation and proliferation. DCs are also present in synovia where they present antigen and produce cytokines, *e.g.* IL-12 and IL-23 [143, 144]. However, DCs can also exert anti-inflammatory effects and ameliorate arthritis; so-called **tolerogenic DCs** induced by *e.g.* glucocorticoids, vitamin D(3). CII-specific tolerogenic DCs reduce CIA by inducing IL-10 production and reducing Th17-cell proportion [145]. An early phase clinical trial with tolerogenic DC administration to RA patients indicates clinical efficacy [146]. Furthermore, plasmacytoid DCs (pDCs) produce substantial amounts of interferons, *e.g.* IFNα that is protective in experimental arthritis [147]. Thus, the role of the DC in RA is dualistic.

Macrophages also express MHC II and present antigen. Nevertheless, the major role of macrophages in RA is as cytokine producers. Macrophages

produce massive amounts of pro-inflammatory **IL-6, TNFα** and **IL-1β**, crucial cytokines in the pathogenesis of RA. Treatment with monoclonal antibodies neutralizing TNFα is up to this day the most prominent and successful RA treatment (section 5.6). **Neutrophils** are rapidly recruited to joints during the induction phase of arthritis, both indirectly and directly due to *e.g.* IL-17 [123, 124]. Usually located in synovial fluid, neutrophils contribute to RA pathology by secreting toxic substances such as reactive oxygen species (ROS) and MMP, as well as releasing neutrophil extracellular traps (NETs) containing citrullinated proteins [148]. **Mast cells** are found in RA synovia and produce TNFα and probably also IL-17 [22]. The importance of tissue-resident synovial cells such as **FLS** in RA, should not be neglected as they are both producers and responders of pro-inflammatory cytokines and chemokines (reviewed in [105]). *The overall immunological events in the pathogenesis of RA, with particular emphasis on IL-17, are depicted in fig. 7.*

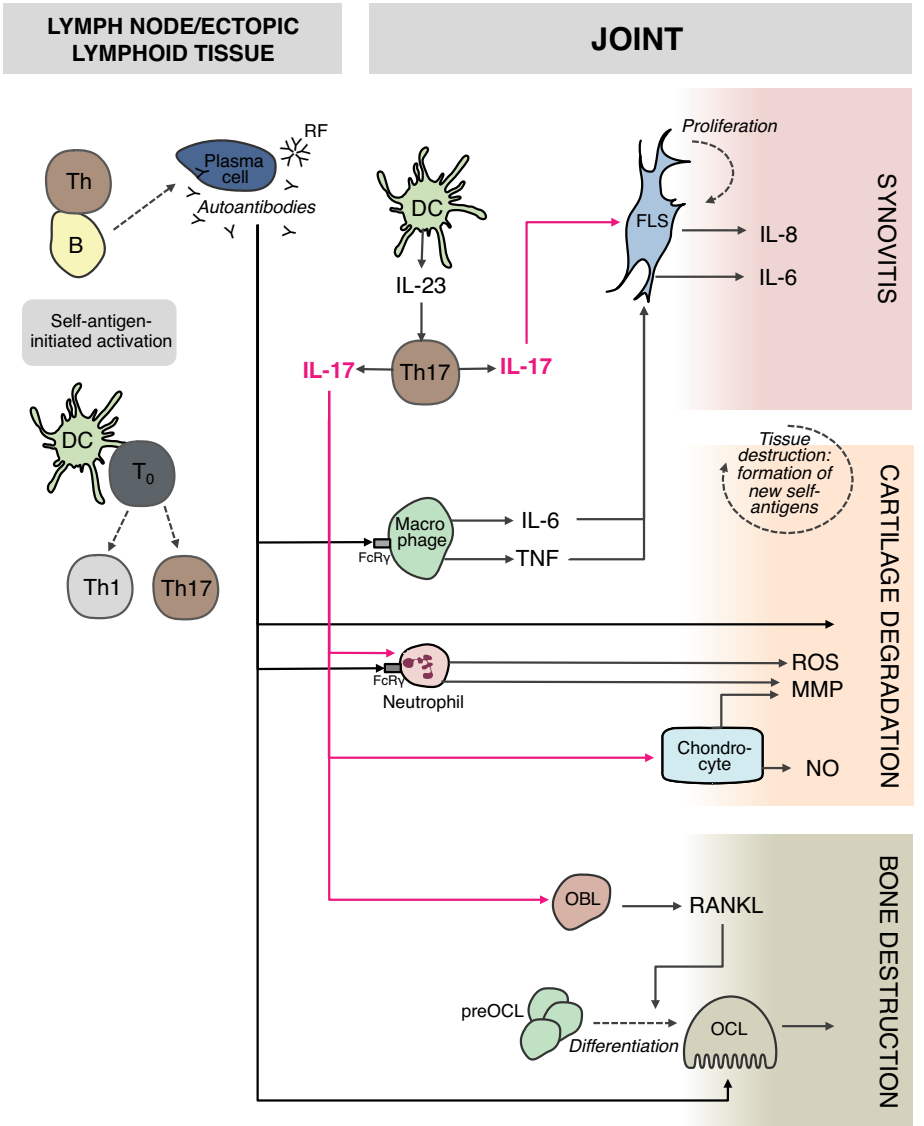

Figure 7. The central role of IL-17 and other immunological events in RA development. Autoreactive T and B cells respond to self-antigens, resulting in differentiation of effector Th cells and maturation of plasma cells. Autoantibodies include IgG with affinity for *e.g.* collagen-type II and citrullinated self-proteins, as well as the rheumatoid factor (IgM binding the Fc portion of IgG). IL-17 is central in the T cell effector response by stimulating: FLS production of IL-6 and 8; recruitment of neutrophils and their ROS and MMP production; OBL production of RANKL. Pink lines demonstrate effects of IL-17, dashed lines represent differentiation/proliferation, and black/grey solid lines show other effects/pathways. FcR: Fc receptor; MMP: matrix metalloproteinase; Th: helper T cell; TNF: tumor necrosis factor; DC: dendritic cell; T_0: naïve T cell; IL: interleukin; FLS: fibroblast-like synoviocyte; ROS; reactive oxygen species; NO: nitric oxide; OBL: osteoblast; OCL: osteoclast; RANKL: receptor activator of nuclear factor κB ligand; RF: rheumatoid factor.

5.5 Bone changes in RA

The proximity of the immune reaction in the joints to the bone enables immune cells and bone cells to interact. The highly inflammatory milieu in the RA joint cavity, with high levels of IL-6, TNF, IL-1 and IL-17, induces RANKL production from FLS and OBL, thus driving OCL differentiation and bone resorption [149]. ACPA can directly promote OCL differentiation [150], providing an explanation for the association between ACPA-positivity and erosive disease. In addition to local **bone erosions** in the subchondral bone and at the joint edges, also **periarticular osteopenia** arises due to persistent arthritis. Furthermore, systemic inflammation with increased circulating levels of pro-inflammatory cytokines contributes to generalized **osteoporosis** in RA patients. A common side effect of long-term glucocorticoid treatment in general is osteoporosis; however, the strong immunosuppressive effects of glucocorticoids in RA might also indirectly retard inflammation-associated bone loss [151, 152]. Around 50% of postmenopausal RA women are osteoporotic [100, 101]. Moreover, generalized bone loss is present already early in the disease and does correlate to disease activity [153]. Also male RA patients have reduced bone mineral density [154]. Subsequently, the fracture risk is elevated in RA, with up to a 3-fold increased risk of hip fractures [155, 156]. Osteoporosis in RA is conventionally treated with bisphosphonates, and anti-RANKL treatment has also been efficient; nevertheless, none of these treatments have beneficial effects on disease activity [157, 158].

5.6 Treatment

The cornerstone and the firsthand choice in RA treatment is **methotrexate**, a chemotherapeutic drug when used in high doses. However, methotrexate is prescribed in very low doses to RA patients, thus exerting a general anti-inflammatory effect. Other classically used drugs are chloroquine phosphate and sulfasalazine. If the patient does not respond to methotrexate therapy sufficiently, or if the patient already at onset displays severe disease, methotrexate is combined with **anti-TNF treatment**. Anti-TNF treatment was the first approved **biologic agent**, which paved the way for a new revolutionizing era in RA therapy. Since then, a plethora of biologics has been developed, with the most successful beyond anti-TNF being anti-CD20 treatment, CTLA-4-Ig, anti-IL-1R, and anti-IL-6R. Clinical trials evaluating anti-IL-17A therapy in RA have so far reported only moderate clinical efficacy; moreover, blocking the IL-17R did not influence RA disease activity at all [159-162]. Nevertheless, anti-IL-17R and anti-IL-17A therapies are efficient in psoriasis and ankylosing spondylitis, respectively [163, 164].

In addition, **glucocorticoids** can be added temporarily (considering side effects) to manage aggressive disease and **nonsteroidal anti-inflammatory drugs** (NSAID) are commonly used as analgesics.

5.7 Experimental models of RA

Experimental arthritis models in rats and mice are useful tools to initially evaluate the therapeutic effects of a new drug, but are particularly useful to perform immunologic mechanistic studies. Considering the heterogeneity of human RA, experimental models in inbred rodents, conducted in a strictly controlled environment, are indeed *models* of disease and not the rodent counterpart of rheumatoid arthritis *per se*. Nevertheless, many central aspects of rheumatoid arthritis are reproduced in some experimental arthritis models: tissue-specificity (polyarthritis of small diarthrodial joints); clinically observable joint swelling; HLA/MHC genetic association; autoantibody production; T and B cell involvement; skeletal involvement such as bone erosions and osteoporosis. Many experimental models of RA are available; however, only those models used in the work of this thesis will be presented herein.

Collagen-induced arthritis (CIA), developed already 40 years ago in rat [165] and later in mice [166], is certainly the most studied and used experimental arthritis model. This model was utilized in papers I, II, IV and V and the methodology is described in detail there. Briefly, by immunizing mice of certain genetic background (HLA haplotype H-2^q, preferably the DBA/1 mouse strain) with a heterologous or autologous cartilage component (collagen type II [CII]) together with an adjuvant (mycobacteria in mineral oil), severe autoimmune polyarthritis is induced. The mycobacterial component elicits an innate immune response and CII peptides are presented to T cells, in turn activating B cells. Thus, both B and T cells are necessary for CIA development [167, 168]. Anti-CII autoantibody production directs the immune response to joints. In addition, some studies support the presence of ACPA in CIA, and that ACPA are pathogenic in this model, although results are not consistent [169-172]. The immunization procedure is repeated after 3–4 weeks in order to aggravate the

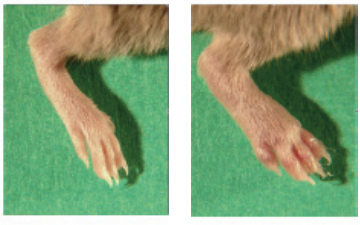

Figure 8. Photograph of a hind paw of a DBA/1 mouse, before and after induction of CIA. *Photo: Caroline Jochems*

immune response. Mice develop visible arthritis, which is clinically examined and scored, and microscopic synovitis and erosions on bone and cartilage. CIA resembles RA by comprising both induction and effector phases; however, CIA lacks the chronicity present in RA. CIA induces periarticular, as well as generalized bone loss, in both trabecular and cortical compartments [173]. Severe arthritis in a DBA/1 mouse subjected to CIA is shown in fig. 8.

Collagen-antibody induced arthritis (CAIA) is a rapidly induced poly-arthritis model with short duration. CAIA represents the effector phase, but bypasses the induction phase, of arthritis [174]. CAIA is simply induced by i.v. injection of an IgG cocktail specific for several epitopes in the CII protein, together with an immune-boosting LPS injection. Arthritis appears within days after anti-CII injection, and is usually milder than CIA, but still macroscopically scorable. CAIA is mainly innate-driven; anti-CII antibodies bind articular cartilage and form immune complexes, which bind to Fcγ-receptor-expressing macrophages or activate complement [175]. CAIA is not MHC II-restricted which enables arthritis induction in several mouse strains. C57BL/6 mouse strain, commonly used as background strain in genetically modified mice, is poorly susceptible to standard CIA protocols but can instead be subjected to CAIA. CAIA was used in paper III and V. Paper III was the first study that characterized influence of the CAIA model on general bone density. We demonstrated that despite the short duration of the CAIA model (9 days), CAIA was associated with pronounced generalized trabecular bone loss, attributed to anti-CII antibody injection (both control and CAIA groups received LPS), in OVX mice. In accordance with decreased BMD, CAIA was associated with increased osteoclasts and IL-17-producing cells.

The monoarthritic **antigen-induced arthritis (AIA)** model was utilized in paper IV and V. AIA is substantially different from CIA and CAIA with respect to lack of systemic inflammation. In AIA, a rather irrelevant antigen is used for immunization, in this case methylated bovine serum albumin (mBSA). One or two systemic immunizations with mBSA emulsified in mycobacteria-containing adjuvant are followed by an intraarticular injection of mBSA only, in one knee joint. Increased levels of T cells, neutrophils and macrophages cause a relatively moderate arthritis, preferably evaluated histologically [176]. Most mouse strains are susceptible to AIA [177]. All clinical features of manifest arthritis are present in the AIA-afflicted knee; synovial hyperplasia, cartilage and bone erosions. In addition, periarticular bone loss, but not generalized bone loss, occurs in AIA [176].

6 ESTROGENS IN RHEUMATOID ARTHRITIS

6.1 Clinical evidence of estrogenic influence on RA

Given the female predominance in RA, the influence of sex hormones on autoimmunity is often debated. Physiologic events related to alterations in sex hormones affect risk and severity of RA. **Pregnancy** has strong impact on RA severity. A recent study reported that 50% of RA patients improve during pregnancy, compared with disease activity before conception, and around 40% have disease flares post partum [178]. Older studies reported that up to 75% of RA patients experience improved disease symptoms during pregnancy [179]. However, rise in estrogens is not the only hormonal alteration that occurs during pregnancy, *e.g.* progesterone also increases, which might influence RA disease.

The rapid drop in ovarian estradiol production resulting in **menopause** might influence RA susceptibility. The peak incidence of RA is around 45–55 years of age, coinciding with menopause [180]. Furthermore, RA incidence increases after menopause and early menopause is an independent predictor of RA [181, 182]. Nevertheless, trials evaluating the influence of **HRT** on RA progression and incidence are rather inconclusive. Postmenopausal women with RA receiving HRT (estradiol + noretisterone) in a 2 year randomized controlled trial displayed reduced disease activity (DAS28 and ESR) and retarded radiological joint damage [183]. Moreover, RA patients receiving HRT had lower serum levels of proinflammatory mediators (soluble receptor for advanced glycation end product [sRAGE], soluble IL-6 receptor [sIL-6R]), compared with non-HRT RA group [184, 185]. In contradiction, the WHI study reported no influence of HRT on self-reported joint symptoms [186]. In the WHI study there was a trend towards reduced RA incidence, but non-significant. As discussed previously, the WHI study included relatively old postmenopausal women, missing out on those just transitioned into menopause. In a Swedish epidemiological study, HRT use was associated with decreased risk of developing ACPA-positive RA, compared with patients that never used HRT [187]. Furthermore, HRT was also associated with reduced ACPA levels [188]. As one might expect, HRT in RA patients resulted in increased BMD (up to 7%, dependent on site

measured), whilst BMD decreased in the control group (non-HRT RA patients) [183]. A selective ERβ-agonist was therapeutically evaluated in a placebo-controlled RA study, and no clinical response was detected at study endpoint which was only 12 weeks [189]. Current use of estradiol-containing **oral contraceptives** (OC) could decrease risk of developing RA [190]; however past OC use did not influence future RA risk [191]. Nevertheless, both past and current use of OC resulted in improved patient-reported outcomes in early RA [192]. It should be emphasized that HRT and OC do not only contain estrogens, but also progesterones, with impact on autoimmune disease as well (reviewed in [193]).

Clinical trials evaluating the influence of **SERM** on RA are rare. Incidence of RA in breast cancer patients was increased in patients using SERM, which in this case were defined as raloxifene, tamoxifen or toremifene (no subanalysis of each separate SERM) [194]. However, breast cancer and its treatment (such as chemotherapy) cannot be disregarded as confounding factors in such a study. To our knowledge, no study evaluating SERM therapy on disease activity in postmenopausal RA patients has been performed. According to clinicaltrials.gov (U.S. National Institutes of Health), one planned RA study will investigate if bazedoxifene can prevent glucocorticoid-induced osteoporosis; however, RA disease activity is not an endpoint. Moreover, there is one on-going study evaluating TSEC in MS with regards to both menopausal symptoms and inflammatory parameters.

6.2 Effects of estrogen-based therapy in arthritis models

The immunologic effects of estrogens in RA are far from elucidated. As discussed earlier, estrogens exert both stimulatory and regulatory actions on the immune system in homeostasis (section 4.5). Animal models of RA have been utilized for a long time to study mechanisms in estrogen-related effects on RA and to understand the sex bias in this disease.

As the most abundant female sex hormone, **estradiol** (E2) has been widely studied in animal models of RA. Already 50 years ago, the first reports from experimental studies on the beneficial effects of female sex steroids in arthritis were published [195]. During the 80's, Holmdahl *et al* performed a series of experiments examining the effects of E2 in OVX CIA mice, first establishing that removal of endogenous estradiol by **OVX** increased susceptibility to CIA [196]. Mice immunized with CII to induce CIA prior to **pregnancy**, developed milder arthritis than non-pregnant controls [197]. In

addition, post partum flares occurred in CIA mice as well, which were prevented with E2 treatment but not with prolactin or progesterone [198].

Both low physiological dose E2 (0.2 μg/twice a week), as well pharmacological dose (3.2 μg/twice a week), are sufficient to reduce arthritis incidence and severity in CIA [199, 200]. These doses correspond to serum E2 levels found in the mouse in estrous cycle and pregnancy, respectively [201]. Both prophylactic E2 treatment, started before immunization and continued throughout experiment (paper IV and V, [199]), as well as therapeutic treatment, started at disease onset (paper I and II, [202]), are efficient treatment regimens in CIA. For instance, therapeutic E2 treatment reduced arthritis incidence from 81% (control group) to 33% and mean severity from 5.5 to 1.2, respectively (paper I). In addition, beneficial effects of E2 in arthritis have also been confirmed in other arthritis models, such as CAIA [203, 204] (paper V) and AIA [205]. Expectedly, E2 treatment in CIA and CAIA preserved both trabecular and cortical bone (paper I and II) [204]. Pharmacologic estrogen receptor blockage with ICI 182,780, which antagonizes both ERα and ERβ, resulted in aggravated CIA in non-castrated female mice [206]. Treatment of OVX CIA mice with selective ER-agonists, and AIA experiments in E2-treated $ER\alpha^{-/-}$ mice, collectively demonstrated that ERα signaling is responsible for the beneficial effects of E2 in arthritis [205, 207].

Jochems *et al* clearly demonstrated the potency of a SERM, a **raloxifene** analogue, in CIA. Therapeutic raloxifene treatment suppressed arthritis incidence and severity dramatically [208], even in long-term experiments up to 70 days post immunization [209]. Prophylactic raloxifene treatment suppressed CIA incidence and severity when treatment was given from immunization and throughout experiment (days -2 to 45 post immunization), but no effect was observed when mice were treated from day -2 to 10 only [204, 208]. Raloxifene protected CIA mice from generalized trabecular and cortical bone loss, regardless if treatment was initiated before immunization or at onset [208, 209]. In contrast to E2, raloxifene did not influence progression or severity of arthritis in the CAIA model [204].

As new SERM with improved pharmacologic profiles have been developed, we conducted studies using **lasofoxifene** and **bazedoxifene** in experimental arthritis (paper I). OVX mice were induced with CIA and therapeutically treated with lasofoxifene or bazedoxifene, from disease onset until experiment termination (during days 18–42 post immunization). Lasofoxifene strongly suppressed arthritis severity macroscopically, and histological evaluation showed improved synovitis as well as reduced

cartilage and bone erosions. In the doses used herein, bazedoxifene reduced arthritis severity less efficient than lasofoxifene. Furthermore, incidence of arthritis was 47% in the lasofoxifene- and 56% in the bazedoxifene-treated group, compared with 81% in the placebo control group. Moreover, analysis of bone density of femurs from CIA mice demonstrated protection of bone despite inflammatory disease. Trabecular BMD was preserved after lasofoxifene and bazedoxifene treatment, but cortical bone was only protected by lasofoxifene.

Considering the previously reported anti-arthritic effects of E2 and bazedoxifene (paper I), it seemed highly relevant to evaluate the anti-arthritic potential of the newly launched HRT option **TSEC**. Treatment with combined E2/bazedoxifene in OVX CIA mice was as efficient as E2 therapy alone, regarding improved arthritis severity, microscopic synovitis and cartilage and bone erosions (paper II). Analysis of femoral BMD revealed anti-osteoporotic effects of E2/bazedoxifene despite highly inflammatory disease; both trabecular BMD and cortical thickness were substantially higher than in the control group. Importantly, when the combined treatment was administered, bazedoxifene protected the uterus from E2-induced uterine growth.

6.3 Immunological mechanisms in estrogenic suppression of RA

T cells

Estradiol has profound effects on thymic T cell development, as discussed earlier (section 4.5). Whether these effects influence the T-cell repertoire and the deletion of autoreactive T cells are unclear. Notwithstanding, E2 inhibits arthritis despite thymectomy of CIA mice [200]. Probably a sufficient pool of mature naïve T cells had already left thymus before thymectomy, since surgery was performed in adulthood. E2 reduced T cell proliferation in spleen cells after CII stimulation [199], but not T cell proliferation in lymph node cells stimulated polyclonally with concanavalin A (paper I) or with CII [210]. However, E2-treated CIA mice had reduced antigen-specific IFNγ production in spleen cells and altered anti-CII antibody response [210]. Nevertheless, no change in levels of **IFNγ-producing T cells** ($IFN\gamma^{+}\gamma\delta T$ or Th1 cells) was detected in our studies with E2-treated CIA mice (paper IV and V). IL-17-producing cells, as key players in RA, were obvious targets in our search for mechanisms in E2-mediated suppression of experimental RA. Until paper IV and V were published, data on the effects of E2 on **IL-17-producing T cells** in arthritis was scarce. Some studies showed effects of estrogens on IL-17 in

arthritis; in AIA, E2 reduced IL-17 production from polyclonally activated spleen cells, an effect that was dependent on ERα [205]. Furthermore, the estrogen metabolite 2-methoxyestradiol reduced synovial IL-17 mRNA in CAIA [211]. However, effects of E2 on Th17 cells have been more thoroughly studied in a model of MS, experimental autoimmune encephalomyelitis (EAE). In EAE, E2 inhibited splenic Th17-cell frequency and E2-mediated disease protection was lost in T helper cell-specific ERα-deficient mice (CD4-Cre crossed with $ER\alpha^{fl/fl}$) [212]. Moreover, a GPER-1 agonist (section 4.2) induced IL-10 production in Th17 cells in EAE [213].

In our studies, the number of functional IL-17-producing cells in joints of CIA mice, determined by IL-17 ELISPOT, was reduced by E2. Accordingly, the frequencies of joint Th17 cells and IL-$17^{+}\gamma\delta$T cells, determined by flow cytometry, were also reduced in established CIA (paper IV and V). Further studies of IL-17-producing cells in lymph nodes draining the joints of E2-treated CIA mice led to unexpected findings; E2 increased frequencies of both Th17 cells and IL-$17^{+}\gamma\delta$T cells in lymph nodes during the induction phase of the disease. In contrast, SERM did neither influence Th17 (paper I) nor IL-$17^{+}\gamma\delta$T cell frequency (unpublished data) in lymph nodes of CIA mice. The E2-mediated increase in lymph node Th17 cells and IL-$17^{+}\gamma\delta$T cells was confirmed in AIA and CAIA models (paper IV and V). In addition, the E2-mediated increase in Th17 and IL-$17^{+}\gamma\delta$T cells in lymph nodes was absent in AIA mice deficient of ERα. Moreover, only three days of E2 treatment was sufficient to increase Th17 and IL-$17^{+}\gamma\delta$T cells in lymph nodes, indicating a rapid effect. Therefore, analysis of receptors and ligands directing T cell migration was conducted. Divergent results were obtained regarding effects on the CCR6-CCL20 system – E2 increased CCR6 expression on lymph node Th17 cells, but not on IL-$17^{+}\gamma\delta$T cells, and increased also total lymph node tissue CCL20 mRNA expression. Moreover, expression of S1PR1 – a receptor crucial for lymph node egress [214] – on lymph node IL-$17^{+}\gamma\delta$T and Th17 cells, was induced by E2. In addition, CD69 on IL-$17^{+}\gamma\delta$T cells was upregulated by E2, and interestingly, CD69 can abrogate S1P-induced migration by inhibition of S1PR1 [215]. Based on these findings, it was hypothesized that E2 arrests Th17 and IL-$17^{+}\gamma\delta$T cells in lymph nodes, thus prevents their migration to joints. However, the net effect on migration due to altered expression of certain chemokine receptors and corresponding ligands, is hard to predict. Thus, the importance of respective pathway in the suppression of arthritis should be validated further. Furthermore, the influence of TSEC on IL-17-producing cells is completely unexplored. Effects of E2 on IL-17-producing T cells during development of CIA are summarized in fig. 9.

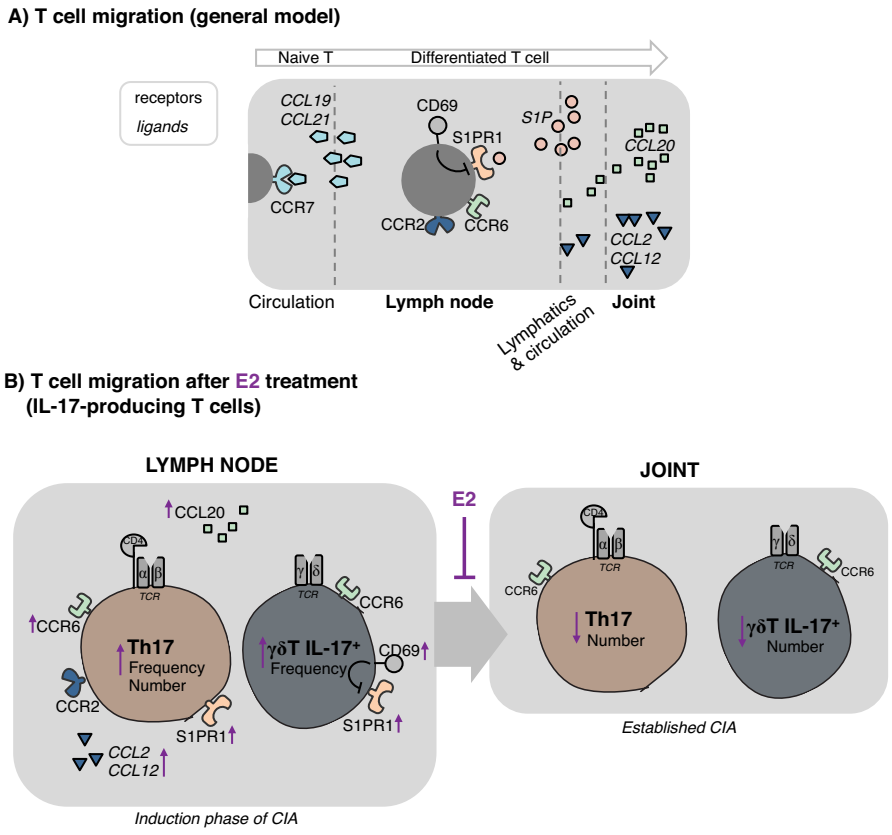

Figure 9. Description of general T cell migration and summary of E2-mediated effects on IL-17-producing T cells during development of CIA. A) A simplified model of T cell migration. More chemokines and receptors than those displayed here are obviously involved. Naïve T cells enter lymph nodes dependent on CCR7-CCL19/21. After APC-mediated T cell activation and differentiation, egress from lymph nodes are mediated via the S1P-S1PR pathway. CD69 inhibit S1PR1 function. Specific chemokines produced in the innate inflammatory response recruit T cells to the inflammation site (the joint). **B)** E2-mediated effects on migratory phenotype of IL-17-producing T cells. Purple arrows indicate effect of E2 treatment vs. placebo control, in OVX CIA mice. E2 increased levels of Th17 ($CD4^+IL\text{-}17^+$) and $IL\text{-}17^+\gamma\delta T$ cells in lymph nodes in early CIA (left) but reduced their levels in joints in established CIA (right). E2 induced CCR6 and S1PR1 on Th17, and S1PR1 and CD69 on $IL\text{-}17^+\gamma\delta T$ cells. E2 also increased lymph node CCL2, 12 and 20. Results derived from paper IV and V. Th17: T helper 17; CCL: CC chemokine ligand; CCR: CC chemokine receptor; CD: cluster of differentiation; TCR: T cell receptor; S1P: sphingosine-1-phosphate; S1PR1: sphingosine-1-phosphate receptor 1; CIA: collagen-induced arthritis; E2: 17β-estradiol.

The influence of E2 on **Tregs** are important during pregnancy, regulating the immune response at the fetal-maternal interface [216]. However, the estrogenic influence on Tregs in autoimmune disease has mostly been studied in the context of EAE, *e.g.* E2 upregulated FoxP3 expression, which increased Treg activity in EAE [217]. In paper I, neither E2 nor SERM influenced the frequency of lymph node Tregs in established CIA. E2 did not affect the proportion of Tregs during the induction phase of CIA either (paper IV). Functional analysis, *e.g.* suppression assays with Tregs and effector T cells, would be highly interesting in the search for mechanisms involved in E2-mediated arthritis inhibition.

B cells

The stimulatory effects of estrogens on general antibody production are well known [96-98]. However, in CIA mice, total anti-CII IgG levels were reduced, while anti-CII IgM increased after E2 therapy [199]. In our hands, only combined E2/bazedoxifene treatment reduced serum anti-CII total IgG significantly (paper II), although a trend towards reduction was found after E2 or SERM treatments alone (paper I).

In paper I, we sought to investigate the influence of E2 and SERM on the expression of receptors related to antigen presentation on lymph node B cells. Surprisingly, E2, but not SERM, increased CD80 and MHC II expression on B cells, which suggest an increased antigen-presenting capacity. In addition, E2 also increased B cell frequency (paper I). However, which type of B cell this was accounted for is unclear, and whether B cell cytokine production is influenced by E2/SERM was not investigated either. B regulatory cells inhibited CIA via IL-10, and IL-10-producing Bregs were increased after E2 treatment in EAE [142, 218]. However, estrogenic influence on Bregs has not been studied in the context of arthritis.

Innate cells and their products

IL-17 is a chemoattractant for **neutrophils**; subsequently, reduced IL-17-producing T cells in joints of E2-treated mice (paper IV and V) were accompanied by reduced number of joint neutrophils (CIA; paper IV and [219], AIA; [205]). Neutrophils are prominent producers of reactive oxygen species (ROS) that mediate tissue destruction, and E2 inhibits ROS production from spleen cells from CIA mice [219].

The proportion of synovial F4/80$^+$ **monocyte/macrophage** population was reduced after E2 treatment in AIA, but not in CIA [205, 219]. Estrogens regulate production of multiple cytokines from monocytes and macrophages (reviewed in [81]), *e.g.* TNF [220], IL-6 [221], IL-1β [222]. One suggested

mechanism in E2-mediated reduction in macrophage pro-inflammatory cytokine production is control of NF-κβ intracellular localization [223]. NF-κβ is a common transcription factor engaged in TLR downstream signaling. Prophylactic raloxifene or E2 therapy in CIA decreased serum IL-6 [208]. However, in our CIA studies when treatment started at arthritis onset, only bazedoxifene, but not combined E2/bazedoxifene, single SERM or E2 therapy, reduced serum IL-6 (paper I and II). Nevertheless, combined E2/bazedoxifene as well as bazedoxifene only, reduced IL-6 production from *in vitro*-stimulated bone marrow cells (paper II). Raloxifene inhibited splenic TNF mRNA levels in CIA, although no effect was seen on TNF protein levels in bone marrow or serum after E2, bazedoxifene or E2/bazedoxifene therapy (paper II) [208].

Studies with **dendritic cell**-specific ERα KO mice (CD11c-Cre x $ER\alpha^{fl/fl}$) revealed a definitive role for E2 signaling via ERα in production of IFN-α from pDC after TLR7 and TLR9 stimulation [224]. Since IFN-α is protective in AIA [147], there is a possible link for the importance of DCs in E2-mediated arthritis inhibition. We sought to study influence of E2 and SERM in two lymph node DC populations, classic $CD11c^{hi}CD8^{+}$ and $CD11c^{hi}CD8^{-}$, in CIA mice. $CD8^{+}$ DCs produce IL-12p70, thereby contributing to Th1 differentiation, whilst $CD8^{-}$ DCs are inducers of Th2 cells [225]. E2, but not SERM, induced $CD8^{-}$ DC frequency as well as their expression of CD80 (paper I), in line with the general view of E2 as an inducer of a Th2 shift.

Non-immune cells in the joint

Fibroblast-like synoviocytes (FLS) express ERα [226]. By using FLS in an *in vitro* cartilage model it was demonstrated that E2 stimulated expression of tissue-degrading MMPs [226]. However, raloxifene inhibited synoviocyte proliferation and migration *in vitro* in experiments with synoviocytes from RA patients [227].

E2, all SERM, as well as combined E2/SERM, resulted in dramatically decreased **cartilage** destruction in CIA – determined histologically and by quantification of the cartilage protein COMP in serum (paper I and II, [208]). In RA patients, COMP predicts development of joint damage [228]. Chondrocytes express ERα, and by performing AIA in cartilage-specific $ER\alpha^{-/-}$ mice (Col2α1-Cre x $ER\alpha^{fl/fl}$), Engdahl *et al* found that the E2-induced abrogation of histological joint destruction and cartilage degradation was independent of ERα in cartilage [205].

Considering the profound physiological role of estrogens in bone remodeling (section 3.3), **bone cells** such as OBL and OCL are obvious targets of

estrogenic regulation also in arthritis. In papers I and II, we defined a population of putative pre-OCL (M-CSFR$^+$ macrophage-like cells) in bone marrow of CIA mice, demonstrating that E2, bazedoxifene, and combined E2/bazedoxifene, but not lasofoxifene, reduced the frequency of pre-OCL. Speculatively, reduction in pre-OCL might depend on reduced RANKL, since RANKL is necessary for OCL differentiation; however, RANKL levels were not assessed in our studies. In accordance, raloxifene reduced RANKL mRNA in CIA [208]. Surprisingly, the bone metabolism markers PINP and CTX-I, reflecting bone formation and resorption respectively, in serum of CIA mice were unaffected by E2, bazedoxifene, lasofoxifene and combined E2/bazedoxifene (paper I and II). Time of serum sampling is a possible explanation for lack of effects on bone metabolism markers despite a distinct bone phenotype. Sera were drawn at termination only. Nevertheless, raloxifene decreased serum CTX-I and increased serum osteocalcin (another bone formation biomarker) in CIA [208].

Estrogens and SERM exert multiple but divergent effects on both immune cells and non-immune cells involved in the pathogenesis of RA. Specific effects of E2, SERM and TSEC in CIA are summarized in fig. 10.

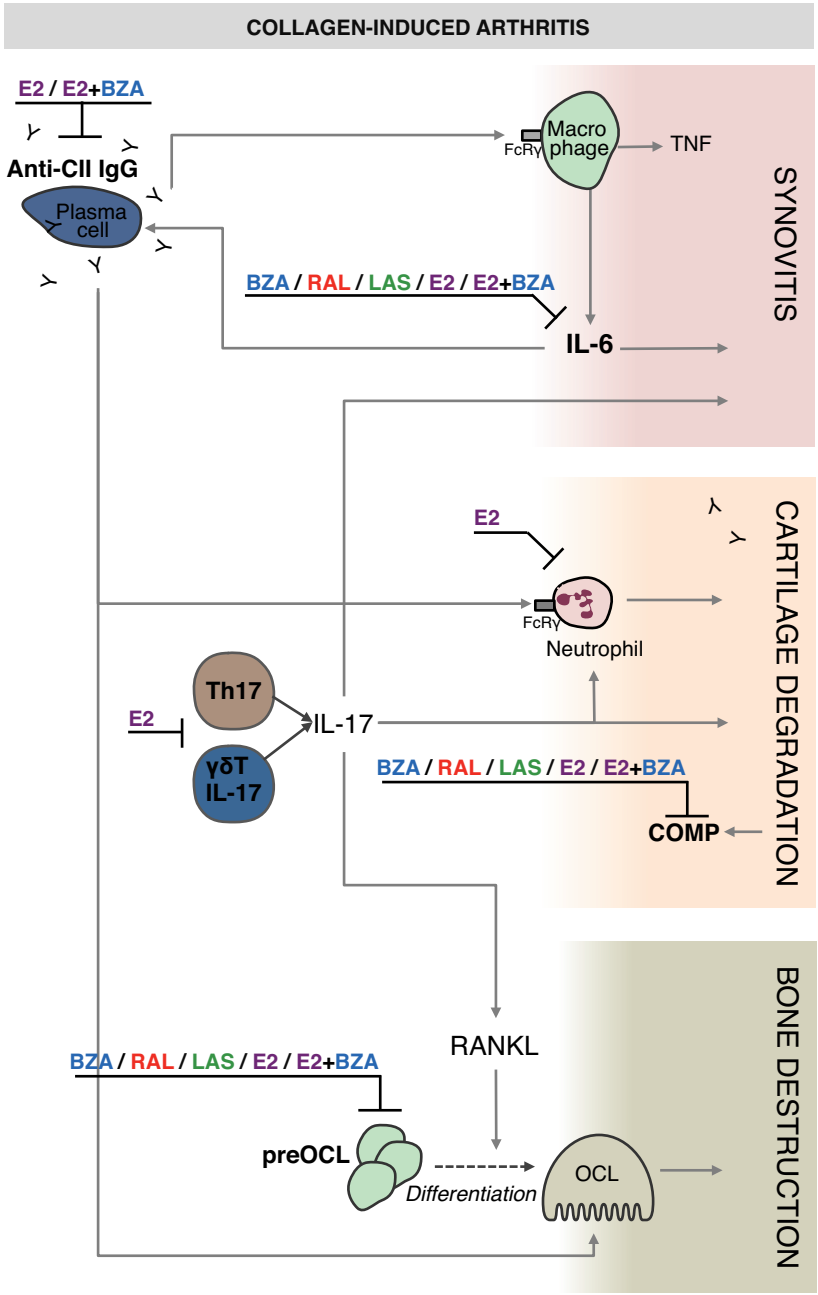

Figure 10. Specific effects of E2, SERM and combined E2+SERM (TSEC) in collagen-induced arthritis in mice. BZA: bazedoxifene; LAS: lasofoxifene; RAL: raloxifene; E2: 17β-estradiol; FcR: Fc receptor; Th: helper T cell; TNF: tumor necrosis factor; DC: dendritic cell; T_0: naïve T cell; IL: interleukin; OCL: osteoclast; RANKL: receptor activator of nuclear factor κB ligand; CII: collagen type II; COMP: cartilage oligomeric matrix protein. All data are from paper I, II, IV, and V, except raloxifene data which was obtained from Jochems *et al* [208].

7 SUMMARY OF FINDINGS IN THE THESIS

Paper I *Selective oestrogen receptor modulators lasofoxifene and bazedoxifene inhibit joint inflammation and osteoporosis in ovariectomised mice with collagen-induced arthritis* constitutes the first report on the beneficial effects of third generation SERM lasofoxifene and bazedoxifene in arthritis. SERM treatment, started at arthritis onset, efficiently suppressed arthritis in mice, seen as reduced joint swelling, synovitis, cartilage and joint erosions. Moreover, SERM prevented trabecular and cortical (lasofoxifene only) bone loss. In accordance, bazedoxifene reduced pre-OCL in bone marrow. Immunological effects of SERM included reduction of IL-6 and modulation of antigen-presenting capacity of DC and B cell populations.

Paper II *Suppression of experimental arthritis and associated bone loss by a tissue-selective estrogen complex* encompasses a similar study to paper I; however, in this study combined treatment with estradiol (E2)+bazedoxifene was utilized in collagen-induced arthritis. The combination of estrogens and SERM are termed "tissue-selective estrogen complex" (TSEC), which constitutes a new therapy for menopausal symptoms and prevention of postmenopausal bone loss. The TSEC was as efficient as E2 in ameliorating arthritis severity and incidence in mice. Furthermore, TSEC protected mice from trabecular and cortical bone loss, in accordance, bone marrow pre-OCL number and IL-6 levels were reduced by TSEC. TSEC treatment also reduced serum anti-CII antibodies. Importantly, the bazedoxifene part of TSEC successfully ensured blockage of E2-mediated uterine growth.

Paper III *Trabecular bone loss in collagen antibody-induced arthritis* is a methodological study, where the presence of generalized bone loss is described in the rapid collagen antibody-induced arthritis (CAIA) model. Injection of anti-CII antibodies resulted in rapidly developing mild arthritis and substantial generalized trabecular bone loss, demonstrated as reduced BV/TV ratio, trabecular thickness, trabecular number, and increased trabecular spacing. Accordingly, femoral OCL number was increased in CAIA, as well as IL-17-producing cells. Results from this study constitute a valuable addition to the knowledge and usability of the CAIA model.

Paper IV *Estrogen regulates T helper 17 phenotype and localization in experimental autoimmune arthritis* presents new data on the ability of estrogens to regulate Th17 cells. The discovery of Th17, as the main IL-17-producing cell, has been groundbreaking in the research concerning many inflammatory diseases. Up until this study was published, very little was known regarding the influence of estrogens on Th17 cells. We found that E2 accumulated Th17 cells in the lymph nodes draining the joints in an ERα-dependent manner, possibly due to altered expression of migratory receptors such as S1PR1 and CCR6. E2-mediated increase of lymph node Th17 cells, also confirmed in the antigen-induced arthritis model, was present during the asymptomatic phase of arthritis. Subsequently, fewer Th17 cells were found in the joints of E2-treated mice and thus also reduced neutrophil infiltration into joints, in line with the anti-arthritic effects of E2.

Paper V *IL-17-producing γδT cells are regulated by estrogen during development of experimental arthritis* was a parallel study to paper IV and investigated the effects of estrogens on IL-17-producing γδT cells in collagen-induced arthritis. Although classified as a T cell, the γδT cell is more innate-like, rapidly responding to stimuli, and established as a significant IL-17 producer in murine arthritis. Interestingly, E2 influenced IL-17^+ γδT cells in a similar fashion as Th17 cells; IL-17^+ γδT cells were elevated in lymph nodes draining the joints but reduced in joints of CIA mice. E2-mediated lymph node accumulation of IL-17^+ γδT cells was confirmed both in AIA and CAIA, and was ERα-dependent.

Fig. 11 presents the main simplified conclusions drawn from the thesis work, answering the questions posed within the aims in fig. 1.

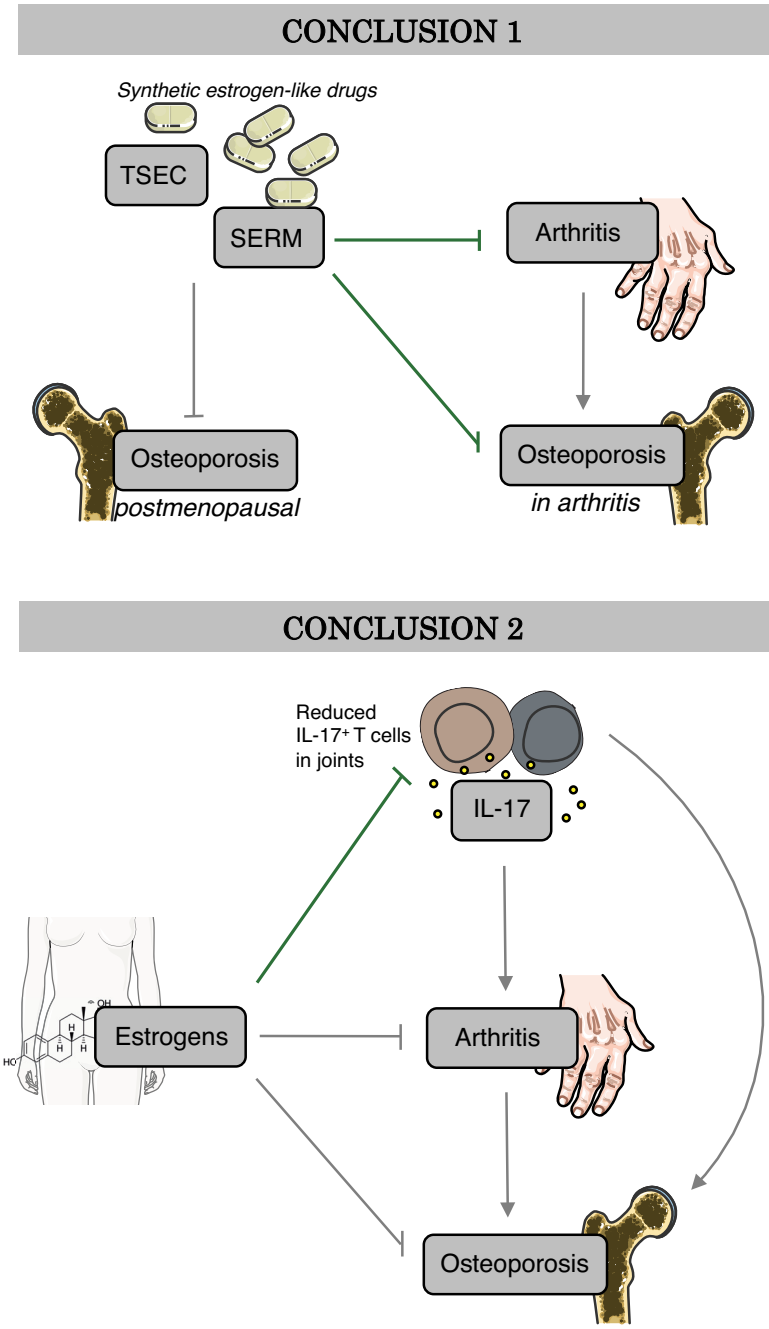

Figure 11. Illustration of the main simplified conclusions of the thesis. Green blunted lines represent new discoveries obtained in the thesis, from studies of experimental arthritis in mice. Grey arrows indicate previously known stimulatory effect, grey blunted lines represent previously known inhibitory effects. TSEC: tissue-selective estrogen complex; SERM: selective estrogen receptor modulator; IL-17: interleukin-17.

8 CONCLUDING DISCUSSION

In this section the author's thoughts about, and future implications of, thesis results are elaborated. There is an obvious contradiction in the high female incidence of RA and the herein highlighted inhibitory role of estrogens in arthritis. Let's flip the coin by posing the question: what does protect men from developing RA? **Androgens** cannot be disregarded in this context, and indeed, testosterone is protective in RA [229, 230]. Low levels of androgens have been reported in male RA patients, compared with osteoarthritis controls [231]. Moreover, low testosterone levels in men are associated with increased risk of developing RA [232]. Of note, serum E2 levels are actually lower in postmenopausal women than in elderly men [233]. In contrast to the stability of male sex hormone levels during life, extensive changes in sex hormone levels are naturally occurring during the woman's life. Thus, states of low estrogen levels might amplify immune activation and permit the rise of autoimmunity. Nevertheless, it must be emphasized that the sexual dimorphism of autoimmune diseases may also depend on other factors than sex hormones, such as **sex chromosomes**. This is demonstrated in males with Klinefelter's syndrome, caused by an extra X chromosome, in which the immune response resembles that of a woman (*e.g.* higher Ig levels) [234]. In contrast, women with only one X chromosome, or substantial deletions in X chromosomes (Turner's syndrome), display lower Ig levels than normal XX women [235]. In addition, both these syndromes are associated with an increased risk of developing autoimmune diseases, further highlighting the link between the X chromosome and autoimmunity [236-238].

Numerous studies of effects of estrogens in experimental arthritis models, in this thesis work and from other studies, show consistent and convincing results – E2 is a strong inhibitor of arthritis *in mice*. Nevertheless, studies of estrogen replacement to postmenopausal *women with RA*, or RA women taking oral contraceptives, have yielded conflicting results, with some studies showing decreased RA disease activity and some reporting no effect at all [183, 239, 240]. Speculatively, the discrepancy between **mice and men** in this context might, to some extent, depend on **dose of estrogen**. In fact, in one of the aforementioned studies with HRT in RA, improved disease activity (reduced articular index and ESR) was found only in those patients with serum E2 levels of >100 pmol/L (around 60% of the patients) [239]. Thus, this suggests that conventional HRT results in borderline E2 levels, with respect to anti-arthritic efficiency. In our experimental studies, the

administered E2 doses (0.83–1 μg/day) were initially believed to result in physiological E2 levels comparable to levels in the intact mouse during menstrual cycling, according to Offner *et al* [241]. However, they used radioimmunoassay for quantifying sex hormones, which is not as accurate as gas chromatography-tandem mass spectrometry (GC-MS/MS). Thus, when Nilsson *et al* [242] evaluated serum E2 levels with high sensitivity GC-MS/MS in mice after treatment with 0.83 μg E2/day, it became clear that E2 doses used in our studies rendered supraphysiological serum levels; five-fold higher than E2 levels during proestrus phase of the menstrual cycle. In contrast, HRT in postmenopausal women resulted in low serum E2 levels that did not even reach the lowest E2 levels present during menstrual cycle in premenopausal women [183, 243]. **Doses of SERM/TSEC** have not been studied as thoroughly as E2; thus, it is difficult to interpret the clinical relevancy of their anti-arthritic effects in CIA. Nevertheless, careful consideration was undertaken when deciding which doses of lasofoxifene, bazedoxifene, and combined E2 and bazedoxifene as a TSEC, to use in CIA. First, body surface area calculations facilitated a reasonable dose translation from human to mice [244]. Second, these doses were confirmed as anti-osteoporotic in OVX mice [53, 96]. A major drawback is though the route of delivery; humans are treated with SERM per orally while mice received s.c. injections. Nevertheless, in paper I and II, SERM and TSEC respectively, were administered to CIA mice in a therapeutic regimen; initiated at arthritis onset which is truly relevant from a clinical point of view.
The vast immunological effects of estrogens in general make it difficult to pinpoint ***the*** **mechanism underlying inhibitory effects of estradiol and SERM in arthritis**. Speculatively, estrogens and SERM target several immune cells in this disease. Results in paper IV and V demonstrated totally new knowledge concerning effects of estradiol on IL-17-producing T cells, filling a gap in the autoimmune research community. Most importantly, the comprehensive approach in these papers is very valuable; studying several populations of IL-17-producing T cells, in several arthritis models, at several time points during arthritis development, and in several tissues. Nevertheless, findings from these papers must be extrapolated to functional studies, *e.g.* using KO mice. Although beyond the scope of this thesis, one must not forget that estrogens also exert multiple effects on non-immune cells that might be targets in estrogen-mediated arthritis inhibition; *e.g.* vascular endothelial cells and stromal cells in joints (section 6.3) [226, 245], and possibly also lymphatic endothelial and stromal lymph node cells.

As for SERM-mediated inhibition of arthritis, there are some clues to the underlying immunological mechanisms derived from the direct comparison to effects of E2 (fig. 10). As opposed to E2, SERM lack effects on T

lymphopoiesis, T cell dependent DTH, and levels of Th17 cells in CIA [85] (paper I). However, more thorough studies of effects of SERM on IL-17-producing T cells in arthritis are needed. SERM exert similar, but weaker, inhibitory effects as E2 on B lymphopoiesis, but lack effect on Ig production in healthy mice. Serum IL-6, possibly produced by *e.g.* macrophages and fibroblasts, was reduced by lasofoxifene and raloxifene in CIA (paper I) [208, 246]. Beneficial effects of TSEC in arthritis are even more complex to clarify, considering the mix of competitive ER agonistic effects and BZA-mediated antagonistic effects, of this treatment. In line with results from the SERM study, IL-6 was also reduced by TSEC (most pronounced in bone marrow). IL-6 increases maturation of B cells and their differentiation into antibody-producing plasma cells and stimulates antibody production [247, 248]. In accordance, despite lacking effects on general antibody production in healthy mice, TSEC treatment resulted in reduced anti-CII IgG (paper II), and lasofoxifene tended to reduce anti-CII IgG as well (paper I), in CIA. Thus, one proposed mechanism of SERM/TSEC-mediated inhibition of arthritis is via reduced IL-6 and subsequent decrease in plasma cell differentiation, and lower autoantibody production. This is further supported by the fact that the severity of arthritis in CAIA, caused by injection of anti-CII antibodies, is not influenced by SERM [204].

All results presented in this thesis were derived from experimentally induced arthritis in mice. Thus, it is of outmost importance to discuss **the relevance of results from experimental arthritis studies for human RA**. Whole genome sequencing of the common laboratory mouse strain C57BL/6, revealed that 99% of the protein-coding genes have a sequence match in the human genome [249]. Despite the conservation between mouse and human genome, many species-specific functional differences are found in the immune system. Among these differences, several are of undisputable importance in RA, *e.g.* that mice display higher levels of circulating lymphocytes; lower neutrophil levels; different FcR expression patterns; different IgG isotypes; altered TLR expression; altered γδ T cell response; as well as different costimulatory receptor expression on T cells, in comparison to humans (reviewed in [250]). Among the used arthritis models in this thesis, CIA is considered to resemble RA most. The general similarities between CIA and RA have already been outlined (section 5.7). However, the main disadvantages of the CIA model, with respect to similarity to RA, are lack of chronicity; dependence on anti-CII antibodies; abundance of neutrophils in synovia (macrophages are the most abundant cell type in the RA synovia), and requirement of bacteria for induction [251]. Although certain infections are associated to triggering of RA, the mycobacterial component in CIA immunization is probably too immunogenic to be

considered as relevant in this aspect. Differences between mice and men are often devastatingly exemplified in studies of new drug targets, where the translation of beneficial effects in an animal model has failed in the human disease [252]. Probably, this discrepancy becomes particularly evident when drug targets increase in selectivity. The general belief is that estrogens mediate many rudimentary functions, such as actions on bone, reproduction, and immune system, similarly in mice and men. Therefore, the relevance of the anti-arthritic and anti-osteoporotic effects of SERM and TSEC is probably high (paper I and II). Speculatively, these compounds act via multiple pathways, utilizing redundant pathways considering immunological differences in mice and men. In contrast, in papers IV and V, more specific effects of estradiol were found on IL-17-producing T cells, which then might be at higher risk of being species-dependent. Since the role of IL-17^{+}γδ T cells in human RA is rather unclear, the clinical relevance of results in paper V can be disputed [125]. On top of that, function and distribution of γδ T cells in general differ between mice and men [250]. Notwithstanding, Th17 cells are established as pathogenic in RA. However, as previously discussed, Th17-cell differentiation requires different cytokines in mice and men. Fewer neutrophils were found in the joints of E2-treated CIA mice, probably due to reduced IL-17-producing T cells therein; however, since neutrophils are not as abundant in joints in RA as in CIA, the clinical relevance of this finding is unclear. To conclude, it would be highly interesting to evaluate the effect of *e.g.* HRT on phenotype and function of Th17 cells in peripheral blood and synovial tissue from RA patients.

To summarize, this thesis generated new comprehensive knowledge concerning the role of endogenous estrogens, SERM and combined estrogens and SERM (TSEC) in experimental arthritis. Considering the high frequency of osteoporosis in postmenopausal women (50%), clinical trials evaluating the addition of a SERM or TSEC to conventional RA therapy in postmenopausal women are highly motivated. Such an additional therapy could possibly have beneficial effects on osteoporosis, RA disease activity and general menopausal discomforting symptoms. In addition, work within this thesis established that generalized osteoporosis arise during the short CAIA model. Furthermore, it was established that estradiol has multiple effects on the primary types of IL-17-producing cells, which could aid in the understanding of sex bias in RA. The results concerning estrogenic effects on IL-17-producing T cells could be of potential interest in other IL-17-associated autoimmune diseases where a female bias is present, such as MS and Sjögren's syndrome [253-255].

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*

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