Arthritis and immune-mediated bone loss

-role of estrogen signaling pathways

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Abstract

Objective: Rheumatoid arthritis (RA) is associated with immune-mediated bone loss and thereby increased risk for fractures. Estrogen and selective estrogen receptor modulators (SERMs) ameliorate not only the incidence and progression of experimental RA but also the immune-mediated bone loss. The aim of this thesis was to elucidate estrogen signaling pathways in arthritis and the associated immune-mediated bone loss.

Methods: Arthritis and bone mineral density (BMD) were evaluated in two experimental models of arthritis, collagen-induced arthritis (CIA) and antigen-induced arthritis (AIA). Specific estrogen receptor (ER) agonists and transgenic mouse models (total ERα knockout (KO), cartilage-specific ERα KO and ERE-luciferase reporter mice) were used, and the resulting phenotypes were examined by histological evaluation and peripheral quantitative computerized tomography.

Results: The ameliorating effect of estrogen on arthritis and associated bone loss was mediated via ERα, as determined by CIA using a specific ERα agonist and confirmed in total ERα KO mice using AIA. Furthermore, the amelioration of joint destruction was mediated via ERα in non-chondrocytes but for synovitis via ERα in chondrocytes. AIA resulted not only in bone erosions, but also in decreased periarticular BMD and can be used as a model to study periarticular bone loss. The SERM raloxifene exerted its effects by inducing the classical genomic estrogen signaling pathway in bone *in vivo*.

Conclusions: ERα mediates estrogens ameliorating effect on arthritis and immune-mediated bone loss. Estrogen ameliorates joint destruction and synovitis via ERα by two different mechanisms.

Long-term treatment with estrogen is associated with significant side effects. Thus increased understanding of the mechanisms behind the beneficial effects of estrogen and SERMs is important in the search for novel treatments of arthritis, including postmenopausal RA, and immune-mediated bone loss.

Keywords: Arthritis, Bone, Estrogen

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List of publication

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

I. Cecilia Engdahl, Caroline Jochems, Sara H Windahl, Anna E Börjesson, Claes Ohlsson, Hans Carlsten, Marie K Lagerquist
Amelioration of collagen-induced arthritis and immune-associated bone loss through signaling via estrogen receptor alpha, and not estrogen receptor beta or G protein-coupled receptor 30.
Arthritis Rheum. 2010 Feb; 62(2): 524-33 *

II. Cecilia Engdahl, Anna E Börjesson, Annica Andersson, Alexandra Stubelius, Andree Krust, Pierre Chambon, Ulrika Islander, Claes Ohlsson, Hans Carlsten, Marie K Lagerquist
The role of total and cartilage-specific ERα expression for the ameliorating effect of estrogen on arthritis
Manuscript

III. Cecilia Engdahl, Catharina Lindholm, Alexandra Stubelius, Claes Ohlsson, Hans Carlsten, Marie K Lagerquist
Periarticular bone loss in antigen-induced arthritis
Manuscript

IV. Cecilia Engdahl, Caroline Jochems, Jan-Åke Gustafsson, Paul T van der Saag, Hans Carlsten, Marie K Lagerquist
In vivo activation of gene transcription via oestrogen response elements by a raloxifene analogue.
Journal of Endocrinology. 2009 Dec; 203(3): 349-56 *

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Content

Abbreviations

AIA	Antigen-induced arthritis
ALP	Alkaline phosphatase
APC	Antigen-presenting cells
BM	Bone marrow
BMD	Bone mineral density
BMP	Bone morphogenetic proteins
CIA	Collagen type II-induced arthritis
DPN	Diarylpropiontrile
ER	Estrogen receptor
ERE	Estrogen response element
FACS	Fluorescence-activated cell sorting
GPR-30	G coupled protein receptor 30
HRT	Hormone replacement therapy
IFN	Interferon
IGF	Insulin-like growth factor
IL	Interleukin
KO	Knockout
mBSA	Methylated bovine serum albumin
MIA	Mycobacteria-induced arthritis
M-CSF	Macrophage colony stimulating factor
MHC	Major histocompatibility complex

NADPH	Nicotinamide adenine dinucleotide phosphate
OPG	Osteoprotegerin
OVX	Ovariectomy
PIA	Pristine-induced arthritis
PMA	Phorbol myristate acetate
pQCT	Peripheral quantitative computed tomography
PPT	Pyrazoletriol
RA	Rheumatoid arthritis
RANKL	Receptor activator of NF-κB ligand
ROS	Reactive oxygen species
SERM	Selective estrogen receptor modulator
SIA	Squalene-induced arthritis
TCR	T cell receptor
TGF	Transforming growth factor
TRAP	Tartrate-resistant acid phosphate
TNF	Tumor necrosis factor
Wnt	Wingless type
WT	Wild type

Bone

The skeleton is a living tissue that supports muscles, protects vital internal organs, stores calcium and is vital for production of hematopoietic cells. It consists of a framework of elastic fibers of collagen and crystals of calcium minerals that harden and strengthen the framework. Without question, bone is the ultimate biomaterial. It is light, strong, can adapt to different functional demands and be repaired. The skeletal tissue consists of two different bone structures, the cortical compact bone and the trabecular spongy bone. These two types of bone are composed of the same types of cells, osteoclasts, osteoblasts and osteocytes, but there are functional differences. The cortical bone is predominantly found in the long bones of the extremities. Trabecular bone is mainly found in the vertebrae, pelvis and in the ends of the long bones. The trabecular bone in the long bone ends is found in the epiphyses and in the metaphyses (*Fig 1*).

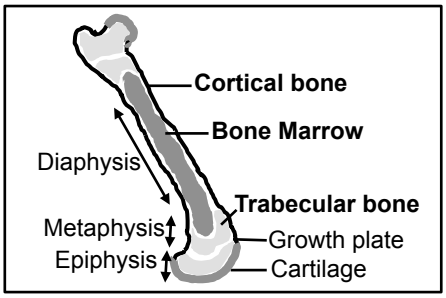

Figure 1. Schematic view of a longitudinal section of a long bone.

Osteoclasts

The osteoclast is derived from monocytes originating from hematopoietic stem cells. Its primary function is to resorb bone. The osteoclast is multinuclear and attaches to the bone surface where it forms a ruffled border. The resorption area under the osteoclast is sealed off, creating an acidic microenvironment with various lysosomal and proteolytic enzymes, which digest the skeleton. During resorption, collagen type I is degraded and fragments are released into the serum. Two factors are essential for the development of osteoclasts, macrophage colony stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL) [1]. M-CSF, secreted from osteoblastic stromal cells and monocytes, binds the c-fms receptor on preosteoclasts and stimulates proliferation [2]. Binding of RANKL to its receptor RANK on preosteoclasts and osteoclasts is required for proliferation, survival and for inhibition of apoptosis [3]. RANKL is either soluble or membrane-bound and is expressed in a variety of

cells including osteoblasts [4], synovial fibroblasts [5], chondrocytes [6] and endothelial cells [7], as well as in immune cells including T cells [8], B cells [9], macrophages [10] and neutrophils [11]. Recently it was demonstrated that membrane-bound RANKL on osteocytes is essential for normal bone homeostasis [12]. Mice deficient in one of these vital components for developing osteoclasts, RANK, RANKL or M-CSF, suffer from osteopetrosis [1, 13, 14].

Osteoblasts

Osteoblasts build the skeletal tissue by secreting different bone proteins and collagen that construct the bone matrix [15]. Osteocalcin is secreted into the serum and is often used as an activity measurement of osteoblasts [16]. To give the skeleton stability, the osteoblasts mineralize the matrix and this process involves alkaline phosphatase (ALP) [17]. The osteoblasts originate from mesenchymal stem cells in the bone marrow [18]. Important growth factors for osteoblast differentiation are bone morphogenetic proteins (BMPs) [19], transforming growth factor β (TGFβ) [20] and the wingless type (Wnt) family [21].

Osteocytes

Some osteoblasts become trapped in the bone matrix and develop into osteocytes. Osteocytes are long-lived, do not divide and are the most numerous cell-type in the bone (95%). Osteocytes lie in lacunae and form a network with each other via long cytoplasmic extensions in canaliculi, where they exchange nutrition and signaling molecules. Osteocytes are involved in the regulation of bone remodeling through various mechanosensory signals [22].

Bone remodeling

Bone remodeling is a constantly ongoing process, with a total exchange of the adult skeleton every 10 years. Remodeling is a cyclic event, with resorption preceding formation, and this requires a tight regulation and cross talk, referred to as coupling, between osteoblasts and osteoclasts. The remodeling cycle starts with an activation of bone-lining cells on the surface for degradation and a subsequent attraction of preosteoclasts (*Fig 2*). The preosteoclasts fuse on the surface, forming multinuclear mature osteoclasts, which start resorbing bone at the resorption site. After an intermediate process, called the reversal phase, preosteoblasts migrate to the resorption site, differentiate into mature osteoblasts, produce matrix and finally mineralize the bone. Several factors affect bone remodeling, including cytokines like interleukin-1 (IL-1), IL-6, IL-17, tumor necrosis factor α

(TNFα) and TGFβ. The whole bone remodeling cycle takes about half a year in humans, 4-6 weeks for resorption and 4-6 months for formation. Trabecular bone remodeling appears at the surface of trabeculae in resorption pits while in the cortical bone, the osteoclasts form a tunnel through the bone.

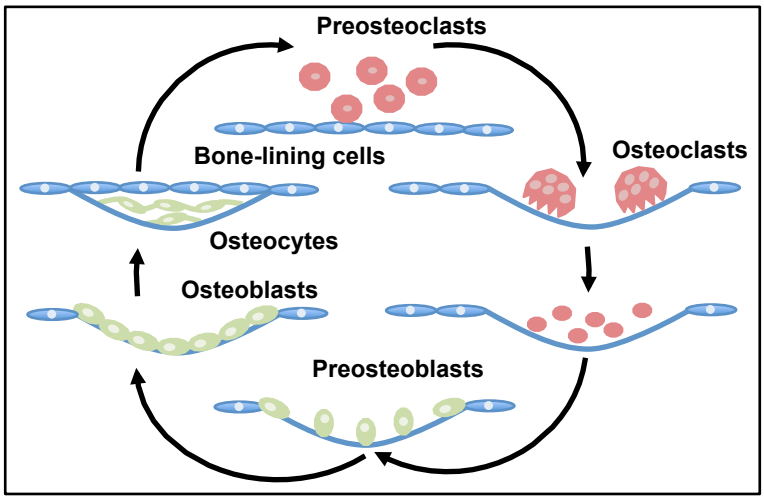

Figure 2. The bone remodeling cycle starts with recruitment of preosteoclasts. The osteoclast formation is followed by bone resorption, removal of osteoclast and then recruitment of preosteoblasts. The osteoblast formation is followed by bone formation and osteoblasts trapped in the bone matrix differentiate into osteocytes.

Osteoporosis

Osteoporosis is a condition characterized by reduced bone mass, leading to an increased risk of fractures. It is a leading cause of morbidity and affects over 75 million people worldwide. Osteoporosis is often called a silent disease because it usually progresses without any symptoms until a fracture occurs or a vertebra collapses. The disease affects 55% of the western population above the age of 50 years and around 80% of them are women. Osteoporosis occurs if there is a remodeling imbalance between bone resorption and bone formation or if there is an increase in the number of active remodeling units. As a result there is an increased porosity of cortical bone and an increase in the resorption area on the trabecular surface, which can lead to thinning and perforation of trabeculae [23].

The immune system

Our body is constantly exposed to foreign substances and infectious agents. The immune system protects us by immunological recognition, isolation of the pathogen and clearing of the infection. The elimination of the antigen has to be kept under control so that it does not cause self-damage.

The innate immune system

The initial defenses against infection are physiological and chemical barriers that prevent infectious agents from entering the body. If the agents should overcome these barriers, the innate immune system is initiated. Phagocytic cells are the first to respond and are able to ingest and kill pathogens and cell debris by producing toxic chemicals and powerful degradative enzymes (*Fig 3*).

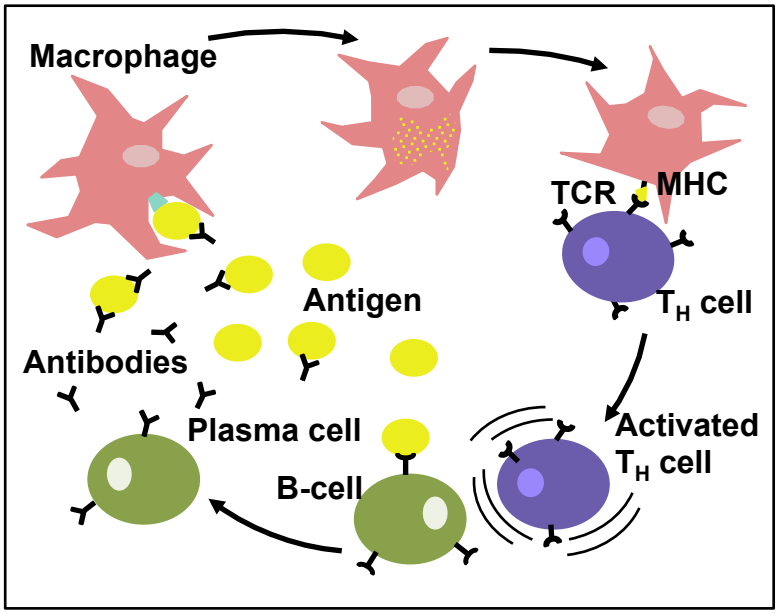

Figure 3. Macrophages phagocyte, degrade and present antigens via the major histocompatibility complex (MHC). Specific T_H cells with T cell receptors (TCRs) are activated by the antigens and trigger for example specific B cells to become activated plasma cells. The plasma cells produce antibodies towards the antigen, which facilitates the phagocytosis.

Neutrophils

The neutrophils are the most frequent white blood cells in the circulation and are often the first cells at the site of an infection. They attack any invaders, dead or injured tissue by phagocytosis until they are forced to apoptosis. Phagocytosis is a form of endocytosis where the cell absorbs material into vesicles that are subsequently isolated and destroyed. The phagocytic cells recognize opsonized structures, which can be either antibodies or byproducts of the complement system (*Fig 3*). Elimination of invaders is either via oxygen dependent degradation with nicotinamide adenine dinucleotide phosphate (NADPH) and reactive oxygen species (ROS) or via oxygen independent degradation with proteolytic enzymes [24, 25].

Monocytes

Macrophages, dendritic cells and osteoclasts are all derived from monocytes. Monocytes, macrophages and dendritic cells have three distinguished functions; phagocytosis, antigen presentation and cytokine production (*Fig 3*). They mainly phagocytose opsonized structures and play a key role in alerting the rest of the immune system. They produce large amounts of immune regulating cytokines such as IL-1, IL-6, IL-8, IL-10, IL-12, TNFα, interferon (IFN)α, IFNγ, M-CSF, and TGFβ, which attract immune cells [26].

The acquired immune system

A unique feature of the acquired immune system is that it is capable of recognizing all agents, enabling an immediate, strong response and establishing an immunological memory. The antigen-presenting cells (APCs) are dendritic cells, macrophages and B cells that digest and present antigens to T cells (*Fig 3*). The antigen presentation requires the major histocompatibility complex (MHC) and with the right set on T cell receptor (TCR) and signals of co-stimulatory molecules the activation of T cells occurs. There are two different forms of MHCs, MHC class I, expressed on the surface of all nucleated cells displaying intracellular peptides, and MHC class II expressed only on APCs displaying extracellular peptides that have been endocytosed.

T cells

T cells develop from circulating lymphoid progenitors in the thymus, where they receive signals from the stromal cells, securing them to the T cell lineage. In thymus, the TCR on the cell surface is tested in two ways: positive selection tests for recognition of MHC molecules and negative selection eliminates self-reactive T cells. T cells are separated into two main groups, CD8-positive cytotoxic T (T_C) cells and CD4-

positive T helper (T_H) cells. T_C cells recognize antigens presented by MHC class I and need stimulation from T_H cells to be activated. T_H cells recognize antigens presented by MHC class II on APCs and are central for regulation and activation of other immune cells (*Fig 3*). T_H cells are further divided into different subtypes depending on their function and interaction with other cells. T_H1 cells produce IFN-γ and IL-12, activating macrophages. T_H2 cells are characterized by secretion of IL-4, IL-5 and IL-13, stimulating B cells and their production of antibodies. T_H17 cells produce IL-17, which is important in recruiting neutrophils to the site of inflammation and finally regulatory T cells (T_{REG}) produce TGFβ that is important for inhibiting and regulating immune responses.

B cells

B cells are an important part of the acquired immune system. They produce antigen-specific antibodies, a variety of cytokines and they are also professional APCs. B cells are developed in the bone marrow (BM) from a lymphoid stem cell. Stromal cells present self-antigens and by clonal deletion, the auto-reactive B cells are edited or forced to apoptosis before they leave the BM [27]. In peripheral lymphoid organs, the mature B cells meet their specific antigens, migrate and form germinal centers with T cells where both cell types are activated. B cells and T cells start to communicate and trigger each other to produce cytokines, to proliferate and to differentiate. B cells can be divided into effector B cells and regulatory B cells depending on their cytokine expression. Effector B cells produce IL-2, IL-4, TNFα, IL-6, IFNγ and IL-12, whereas regulatory B cells produce IL-10 and TGFβ [28, 29]. Continued exposure to the same antigen results in large quantities of high affinity antibodies that are of great importance to prevent and fight infections.

Osteoimmunology

The term osteoimmunology represents a relatively new way to view the connection between immunology and skeletal metabolism. Traditionally, the endocrine system has been believed to be the main regulator of bone remodeling. Accumulating evidence has however shown that the osteoclasts and immune cells share a number of regulating molecules and thereby influence each other. In fact, patients with excessive activation of the immune system are at high risk of experiencing concomitant osteoporosis, as is the case in various autoimmune diseases including rheumatoid arthritis (RA). Other medical conditions in which osteoimmunology is relevant are osteoporosis and osteopetrosis, where an immune disturbance often occurs, and bone metastases, where immune cells often are targeted. In addition, mice deficient in immunomodulatory molecules often develop an abnormal skeletal phenotype [30].

Bone marrow

The BM is located in the medullary cavity, mainly in the central part of the long bone shaft, but also in ribs, skull, sternum, vertebral column, and pelvis, and creates the physiological opportunity for interaction between immune cells and bone cells. The maturation of hematopoietic and mesenchymal stem cells occurs in the BM. Bone lining cells form a nursing microenvironment defined as a niche, where the hematopoietic stem cells are protected and provided with signals that maintain their quiescent, slow-dividing and stationary state [31, 32]. Detachment from these niches is believed to be associated with entering the cell cycle, proliferation and differentiation, which are accompanied by migration to the circulation. The proliferation and maturation of stem cells is regulated and stimulated by cytokines.

Cytokines

Already in the middle of the 1970s immune cells were revealed to produce soluble factors that stimulate osteoclastic bone resorption [33]. Some important factors affecting osteoclastogenesis are RANKL, TNFα, IL-1, IL-6, IL-17 and TGFβ (*Fig 4*) [23, 34]. When inflammatory cytokines are elevated, the balance of bone remodeling is affected, contributing to increased risk of developing osteoporosis.

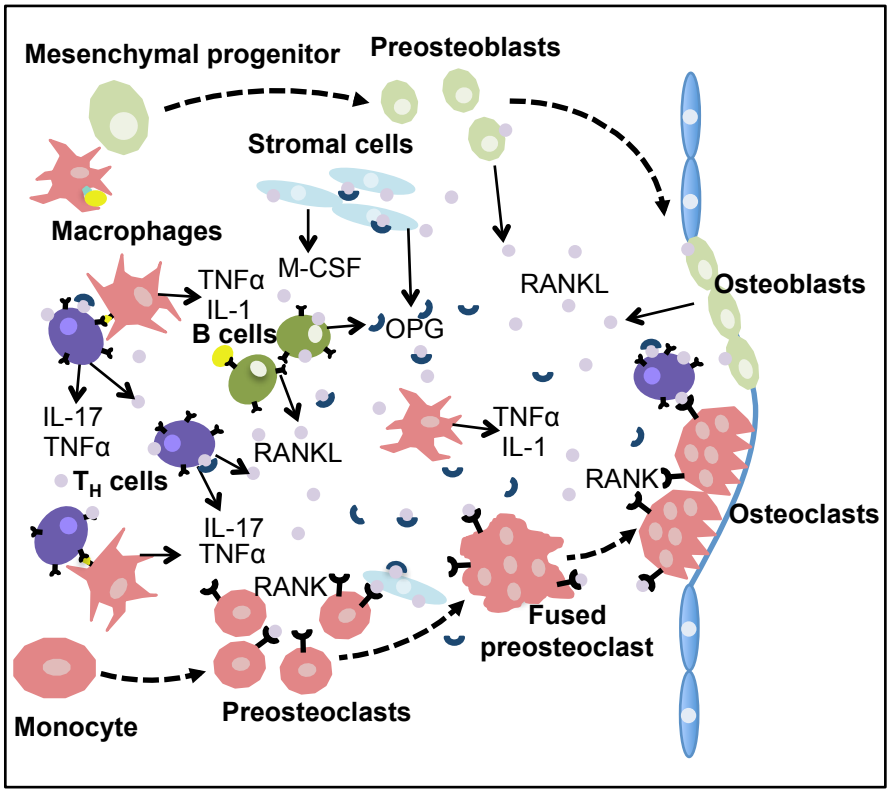

Figure 4. During inflammation there is a strong enhancement of osteoclast-mediated bone loss facilitated via an induction of RANKL, IL-1, IL-17 and TNFα from the activated immune cells that are recruited.

RANKL/OPG

RANKL was discovered, unconnectedly, by different groups working with both immunology as well as bone biology [14, 35]. In the immune system, RANKL serves as an activating molecule expressed by T_H cells stimulating dendritic cells, and in bone biology as an essential stimulatory factor of osteoclast maturation and activation. Today it is clear that RANKL is produced in a large variety of cells [4-12]. In addition, a decoy receptor that binds and neutralizes RANKL, osteoprotegerin (OPG), is produced by several cells including B cells [36]. The OPG/RANKL/RANK system is the target for treatment of various bone diseases and the OPG/RANKL ratio determines the degree of osteoclast activation. Activated T_H cells express RANKL but also many inhibitors of osteoclastogenesis, OPG, IFNγ, IL-4 and TGFβ, providing mainly an inhibitory effect on osteoclasts [37-39]. However, T_H17 cells express high levels of RANKL and IL-17 that is a strong

inducer of RANKL in other cells [40] (*Fig 4*). Also B cells affect the OPG/RANKL ratio by secretion of OPG and recently the expression of RANKL in B cells was shown to be important for induced bone loss [41, 42] (*Fig 4*).

IL-1

There are two IL-1 molecules, IL-1α and IL-1β. Both are proinflammatory and stimulate bone resorption [43]. IL-1 promotes osteoclast fusion, activation and prolongs survival [43, 44]. Furthermore, IL-1 promotes osteoblast apoptosis [45]. A variety of cells produce IL-1, including cells in the monocyte linage. The IL-1 receptor antagonist can hamper inflammation and bone resorption [46].

TNFα

TNFα is a proinflammatory cytokine produced mainly by macrophages and T cells for recruitment and activation of neutrophils. This cytokine is produced in large amounts locally in inflammatory diseases, such as rheumatoid arthritis (RA), and induces resorption indirectly by affecting RANKL and OPG production from osteoblasts and stromal cells [47]. TNFα also induce bone formation via promotion of Wnt signaling on osteoblast [48].

IL-17

IL-17 is mainly produced locally by T_H17 cells as a proinflammatory cytokine [49] and is significantly elevated in the synovial fluid of RA patients [50]. IL-17 exerts many effects on bone cell cultures, including stimulation of both soluble and membrane-bound RANKL [51].

TGFβ

TGFβ is a growth factor involved in proliferation and differentiation of most cell types including osteoclasts and osteoblasts. TGFβ is stored in an inactive form in the bone matrix, making bone the most abundant source of TGFβ in the body. TGFβ reduce osteoclast function by inhibition of formation, activation and increased osteoclast apoptosis [52, 53]. TGFβ also stimulates osteoblast proliferation and differentiation [54].

Immune-mediated bone loss

Several autoimmune diseases are associated with increased risk of osteoporotic fractures adding significantly to the morbidity and mortality of these conditions. Bone remodeling is controlled not only by the regulation of osteoblasts and osteoclasts but also by the immune cells and cytokines that are elevated in autoimmune diseases as earlier described [55]. The levels of proinflammatory cytokines are involved in fracture repair and high levels of proinflammatory cytokines are associated with an elevated risk of fractures [56]. Several investigations have been performed to identify patterns of circulating and locally produced cytokines, however still no constitutive pattern has been correlated to bone loss [23]. The OPG/RANKL ratio is affected by several cells and factors, and is associated with bone loss [57].

A class of drugs commonly and effectively used for treating underlying autoimmune inflammation is corticosteroids [58, 59]. Corticosteroids have several adverse skeletal side-effects including suppression of osteoblastogenesis as well as induction of osteoblast and osteocyte apoptosis [60]. Corticosteroids can also increase bone resorption by a stimulatory effect on RANKL expression and an inhibitory effect on OPG expression together leading to bone loss and increased risk of fracture [58]. However, treatment with low dose corticosteroids used in the initial phase of RA shows no effect on bone loss but effectively reduces the progression of joint damage, controls inflammation and improves physical function in RA patients [59].

Arthritis

Arthritis is defined by inflammation in one (monoarthritis), a few (oligoarthritis) or more (polyarthritis) joints and can be acute or chronic. The major issues for individuals who suffer from arthritis are constant joint pain, due to inflammation that occurs in and around the joint, tissue destruction and fatigue due to a more activated immune system.

Monoarthritis

Monoarthritis is characterized by one inflamed joint at a time and is seen in crystal arthropathy or arthritis caused by infections, such as septic and reactive arthritis.

Gout is the most common form of crystal arthropathy and is caused by an excess of uric acid in the joint. It is a relapsing disease, which may develop into polyarthritis [61]. Gout primarily affects young to middle age men. Female sex steroids can influence the levels of serum uric acid and after menopause, when female sex steroid levels drops, the incidence equals up between men and women [62].

Septic arthritis is a severe, rapidly progressing erosive disease with high morbidity and mortality [63]. The most common bacteria that cause septic arthritis is *staphylococcus aureus*, which is spread via the blood stream to different compartments including the joint.

Reactive arthritis refers to an arthritis caused by an immune reaction to an infection, but is not directly attributable to the infection itself [64, 65]. Several microbes with harmful effects in the tissues may cause reactive monoarthritis, including intestinal infection with e.g. *Salmonella* or *Campylobacter* and sexually transmitted infections with e.g. *Chlamydia* or *Neisseria*.

Rheumatoid arthritis

RA is a chronic progressive autoimmune disease that affects approximately 0.5-1% of the population with a female dominance and a peak incidence that coincides with menopause [66, 67]. The characteristics of the disease are symmetrical polyarthritis, leading to destruction of the joint and often systemic features like fever and elevated erythrocyte sedimentation rates. Macrophages, neutrophils, T cells and B cells infiltrate the synovium, which lines the joints, and cause an inflammation. The infiltration can be very extensive and a constant recruitment of leukocytes makes it an ongoing process.

Pathogenesis of RA

The pathogenesis of RA is largely unknown. Genetic and environmental factors influence disease development and progression. The clinical diagnosis of RA is based on certain disease criteria that need to be fulfilled and include joint swelling of both large and small joints as well as serology markers present in serum [68]. Genetic studies have found that the susceptibility for RA is associated with the structures of the MHC, shared epitope, which is involved in activation of T cells [69, 70]. Furthermore, there are also several environmental factors that affect RA; like cigarette smoking with a clear association with onset of the disease [71]. The impact of infections initiating RA is observed in several patients studied in the early phase of arthritis [72]. However, there is no single virus, bacteria, yeast, fungi nor mycoplasma that is singly associated with arthritis. If an individual with risk factors, a smoker with shared epitope gets infected, the immune system recognizes the infectious intruder and responds by activating macrophages, neutrophils, lymphocytes, secretion of antibodies and production of proinflammatory cytokines. Unfortunately, parts of the joint tissue may resemble parts of the intruder, meaning that even when the infection is cleared the immune system may attack self-tissue leading to established RA.

Bone loss in arthritis

Bone loss represents a major problem in arthritis. The skeletal manifestations include focal bone erosions, periarticular osteoporosis at the site of the active inflammation and generalized bone loss with reduced bone mass [73, 74]. The proinflammatory environment at the site of inflammation may lead to differentiation of monocyte lineage cells into mature osteoclasts and subsequent focal bone erosions. The mechanism behind the periarticular bone loss is not clearly established but local aggregates of macrophages and lymphocytes in BM could lead to increased bone resorption by osteoclasts [75]. Generalized bone loss is mediated via an induced systemic pro-inflammatory cytokine profile and a decreased joint motion, in response to the inflammation [76]. The frequency of generalized osteoporosis in postmenopausal patients with RA has been reported to be over 50% [77]. Osteoclasts are the key mediators of all forms of bone loss in RA [78] and elevated TNF and IL-1 levels are found both in the joints and in the circulation in RA patients [79-82]. Furthermore, elevated levels of RANKL and IL-17 are found in the synovial fluid, but not in serum, of RA patient, which partly could explain the local bone loss [50, 83, 84].

Animal models of arthritis

Animal models of arthritis have proved to be extremely useful in elucidating pathogenic mechanisms of relevance to RA and for evaluating the effects of new treatments. However, no animal model of arthritis gives the entire picture of the complex pathogenesis of human RA. Furthermore, there are large variations in the clinical picture with high heterogeneousness among RA patients [85]. It is therefore important to choose the model that is most appropriate to answer the question posed.

Systemic models:

Collagen type II-induced arthritis (CIA)

The CIA model is the most widely studied arthritis model, largely on the basis of pathological similarities to human RA with a systemic polyarthritis [86]. MHC molecules present peptides of collagen type II in both CIA and RA patients, which activates the acquired immune system and starts a specific response. In contrast to RA, the CIA is not chronic and represents only the acute phase. The CIA development is gene restricted requiring the H2q haplotype of the MHC found in e.g. the DBA/1 strain.

K/BxN transgenic mice

These mice develop a robust spontaneous polyarthritis with synovitis and erosions at 3 weeks of age [87]. The incidence depends upon the loss of tolerance in the interaction between the MHC class II and the T cell receptor. The K/BxN model is dependent on lymphocytes and the mice produce antibodies against glucose-6-phosphate isomerase that could be passively transferred and cause a transient arthritis (see below). The mice have a reduced breeding capability and are somewhat immune compromised due to a limited diversity of the T cell receptor.

Passive transfer models

Antibodies from CIA mice and from K/BxN mice are passively transferable and induce arthritis in all recipient mice [87, 88]. The arthritis developed is dependent only on the response towards antibodies. These mice develop an acute systemic polyarthritis with both synovitis and erosions in all strains of mice. Antibody cocktails are commercially available in both cases.

TNFα transgenic mice

These transgenic mice express the human TNFα gene, which leads to development of a chronic progressive polyarthritis with 100%

incidence [89]. The onset and severity is dependent on the copy number of human TNFα gene that has been inserted. The arthritis share several features with RA such as synovial hyperplasia, immune cell infiltration as well as cartilage and bone destruction. This arthritis is independent of lymphocytes as demonstrated by the fact that TNFα transgenic mice crossed with RAG-1 knock-out (KO) mice, lacking mature lymphocytes, still develop erosive arthritis [90].

Adjuvant-induced arthritis

Mycobacteria-induced arthritis (MIA) was the first arthritis model used in several different species and it is induced by repetitive immunizations with complete Freunds adjuvant (CFA) [91]. The disease is not joint-specific but associated with widespread inflammatory infiltrates and granuloma formation. The joint inflammation develops as a consequence of an immune response towards microbial antigens in the joint and is a severe but self-limiting disease [92]. There are other adjuvant arthritis models, like squalene (SIA) [93] and pristine-induced arthritis (PIA) [94] which share many features with RA such as similar severity and chronicity of the disease. Macroscopic arthritis appears in peripheral joints in SIA and PIA but reported to be non-immunogenic and suitable for mechanistic studies.

Septic arthritis

Septic arthritis is induced by an intravenous injection with *staphylococcus* or *streptococcus* and within 24 hours both clinical and histological signs of arthritis occur [95]. The characteristics of the model closely mirror changes seen in human septic arthritis, especially with regards to high frequency and severity of local bone loss. The septic arthritis is accompanied by a rapid recruitment of neutrophils, macrophages and activated T cells into the joint.

Local model:

Antigen-induced arthritis (AIA)

The AIA is a monoarthritis induced by an intra-articular injection of an antigen, like methylated bovine serum albumin (mBSA), after sensitization [96]. This arthritis model is robust and rapid, with no strain dependence and without systemic involvement. The arthritis shows several clinical and histopathological similarities to RA and is characterized by leukocyte infiltration and synovitis together with joint destruction. The development of the condition is dependent on T_H cells as well as synovial macrophages and neutrophils [97-99].

Estrogen

The female sex hormone estrogen has a variety of physiological effects on reproduction, cardiovascularity, nervous system, skeleton and immune system. There are three different estrogens in the physiological system. Estrone is produced by ovaries and liver and is predominantly expressed after menopause. Estriol is important during pregnancy and is produced by the placenta. 17β-estradiol is the most potent estrogen and the focus in this thesis. Granulosa cells in the ovaries produce most of the estradiol and this production ceases after menopause. Estradiol is also produced by the adrenal cortex, adipose tissue and testicles via aromatization of testosterone, but to a lower degree. The majority of estradiol is bound to sex hormone binding proteins in the serum and only a small proportion (2-3%) is biologically active. 17β-estradiol levels in females vary between 27 and 460 pg/ml during the fertile period depending on the menstrual phase, and remains below 27 pg/ml after menopause. In mice, serum levels of 17β-estradiol vary between 25-200 pg/ml in fertile mice and below 30 pg/ml after ovariectomy [100].

Estrogen receptors

Estrogen mainly exerts its physiologic effects via the two classical nuclear estrogen receptors (ERs), ERα and ERβ, which are ligand-activated transcription factors (*Fig 5*). The distribution of the ERs vary in different tissues, with high ERα content in uterus, mammary gland, liver and the cardiovascular system, and high ERβ content in testis, ovaries and the thyroid gland. The ERs have a high homology in the DNA binding domain (97%), while a lower homology is seen in the ligand-binding domain (55%) [101]. This suggests that the ERs recognize similar DNA sequences but respond differently to different ligands. The affinity for 17β-estradiol is however similar for ERα and ERβ. A third estrogen receptor, the transmembrane G coupled protein receptor 30 (GPR-30) or more recently referred to by its functional designate, G protein-coupled ER (GPER)-1, was recently described and has been shown to have pregenomic estrogen action [102, 103] (*Fig 5*).

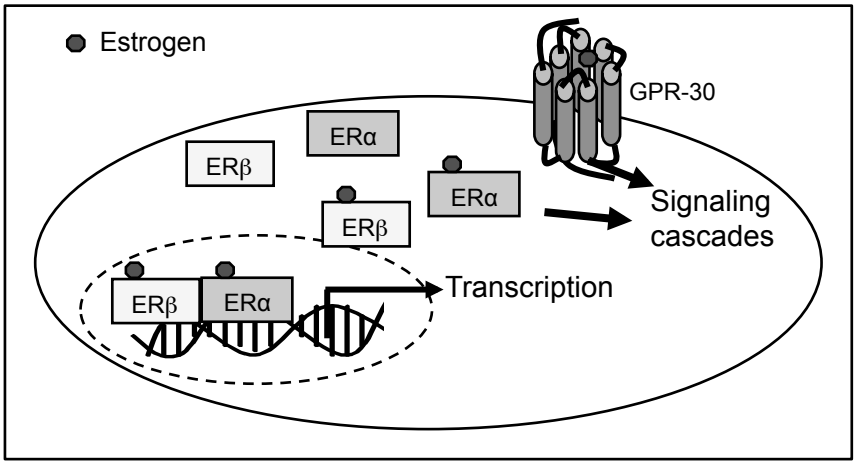

Figure 5. Estrogen signals via three defined receptors, ERα, ERβ or GPR-30. ERα and ERβ are classical nuclear receptors, mediating transcription or rapid intracellular signaling cascades. Estrogen signaling via GPR-30 mediates rapid signaling cascade changes.

Signaling pathways

Estrogen interacts with ERs and affects gene transcription in target cells via two main pathways, the classical and the non-classical pathway (*Fig 5*). Inactive ERs, with no ligand bound or no phosphorylation, are associated with binding proteins in the cytoplasma. Upon binding to the ligand, the estrogen-ER complexes form dimers and after binding to a subset of co-regulators translocate into the cell nucleus [104, 105]. In the classical pathway, the dimerized estrogen-ER complex interacts with estrogen response elements (EREs), located in the promoter region of target genes and serves as a transcription factor [106, 107]. In the non-classical pathway, the dimerized estrogen-ER complex interacts with alternative transcription factors including AP-1, SP-1 or NFκB [108-110] and thereby affects gene transcription. In addition to ligand-induced transcriptional activities of ER, ligand-independent pathways to activate ERs exist.

Several studies have shown that estrogen can exert rapid activating or repressing effects in cells without interaction with the DNA. The nature of these pregenomic receptors has been a matter of debate, but the most promising candidate receptors are the GPR-30 and membrane-associated ERα [102, 111]. Binding of these receptors leads to intracellular activation of pathways including calcium mobilization and PI3K activation, leading to pregenomic signals.

ERs can additionally be activated via phosphorylation of factors including dopamine, insulin-like growth factor-1 (IGF-1), epidermal growth factor or cyclic AMP, in the absence of estrogen [112-115].

Menopause and hormone replacement

At menopause, the hormone production from the ovaries ceases, resulting in a decline in estrogens, which can disrupt the sense of well-being and induce conditions like osteoporosis. Ovariectomy of postmenopausal women significantly decreases the serum levels of estrogen even further, demonstrating some remaining ovarian production of estrogen after menopause [116].

The loss of estrogen was earlier successfully treated with hormone replacement therapy (HRT), but in 1975 an association between estrogen and endometrial cancer was found which led to a decreased use of HRT [117]. There were several beneficial effects of estrogen reported and in combination with progesterone the treatment was still recommended for postmenopausal osteoporosis [118]. In 2002 the Women's Health Initiative study was prematurely interrupted due to severe side effects of HRT [119, 120]. Treatment with the combination of estrogen and progesterone showed an increased risk of coronary heart disease, stroke and deep vein thrombosis, in addition to previously known risks. Since then, treatment with HRT after menopause has declined and the search for drugs with only beneficial estrogenic effects continues. Aging female mice do not lose the production of sex hormones. Therefore, to mimic the postmenopausal status, ovariectomy (OVX) is performed to study the effects of estrogen deficiency.

SERMs

Selective estrogen receptor modulators (SERMs) act as agonists or antagonists depending on the cellular context, thereby displaying estrogen-like effects in some tissue while inhibiting estrogen effects in others. The selectivity of a SERM is dependent on the relative amount of ERα and ERβ, the receptor affinity of the SERM and the availability of co-regulators. Beside their ability to influence cells via ERs, SERMs can affect a number of biochemical processes in acute actions independent of ERs [121]. There are three SERMs used in clinical practice; tamoxifen for treatment of ER-positive breast cancer [122], fulvestrant, for adjuvant treatment of ER-positive breast cancer [123] and raloxifene, for prophylaxis of invasive breast cancer [124] as well as treatment of postmenopausal osteoporosis [125]. For postmenopausal osteoporosis, new SERMs have recently been

approved by the European medicine agency including basedoxifene [126] and lasofoxifene [127].

Raloxifene

Raloxifene is a SERM approved to prevent and treat osteoporosis but it has side effects including increased risk of coronary heart failure and thrombosis [125]. Raloxifene has a pronounced affinity for ERα and minimal affinity for ERβ [128, 129]. The interaction between raloxifene and ER is different compared to the estrogen-ER interaction [130] (*Fig 6*). Depending on the structure of the ligand-ER complex, different constellations of coregulators have the opportunity to interact with the complex, which causes the tissue specific agonistic or antagonistic effects of raloxifene [131].

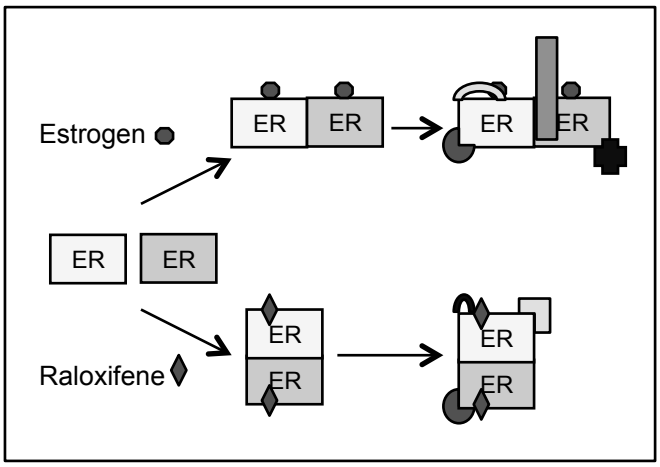

Figure 6. Depending on whether estrogen or raloxifene binds and activates estrogen receptors, the configuration shifts and thereby the opportunity for different coregulators to interact with the complex.

The role of estrogen in bone

Postmenopausal osteoporosis or ovariectomy-induced bone loss are caused by estrogen deficiency. Bone loss occurs in two phases: initially a rapid bone loss occurs, with an increase in bone resorption and trabecular thinning, resulting in decreased connection between the trabeculae [132, 133]. The rapid bone loss is followed by a phase of lower rate of bone loss, with a decrease in bone formation and trabecular thinning. The effect of estrogen deficiency on bone can either be caused by a direct effect on skeletal cells expressing ERs including osteoclasts [134], osteoblasts [135], osteocytes [136] and chondrocytes [137] or an indirect effect via a change in the cytokine

milieu. In osteoclasts, estrogen directly decreases secretion of lysosomal enzymes [138] and reduces RANK expression [139]. In osteoblasts, estrogen causes an increased expression of BMP-6 [140], TGFβ [141] and IGF-1 [142], which induces activation and differentiation of the osteoblast. In osteocytes, estrogen promotes survival [143]. Estrogen affects several factors involved in osteoclastogenesis including induced OPG expression [144], down-regulated RANKL expression and decreased production of cytokines important for osteoclast maturation including IL-1 [145], IL-6 [146] and TNFα [147]. The importance of IL-6 and TNFα for estrogenic effects on bone has been shown by the fact that mice deficient in IL-6 or TNFα do not develop ovariectomy-induced bone loss [148, 149].

The role of estrogen in the immune system

Estrogen affects the immune system in multiple ways. ERs are expressed in most cells of both the innate and the acquired immune system [150]. Estrogen has dual effects in several immunological conditions, with either a limiting or an enhancing effect [151]. Several cytokines are affected by estrogen deficiency including serum levels of IL-1, IL-6 and TNFα, which are increased after menopause [145-147, 152]. Estrogen deficiency is also associated with decreased production of OPG and TGFβ, which serves to counteract many of the effects of the factors affecting osteoclastogenesis [137, 153, 154]. Direct effects of estrogen on neutrophils include inhibition of function and adhesion to the endothelium and some studies indicate reduction of the number of circulating neutrophils [155, 156]. Monocytes and macrophages are primarily modulated by estrogen in the aspect of cytokine production and estrogen induces apoptosis in monocytes [157, 158]. Estrogen induces thymic involution, reduces T lymphopoiesis [159, 160] and stimulates proliferation and differentiation of regulatory T cells via ERs [161, 162]. B lymphopoiesis is downregulated by estrogen, but estrogen stimulates antibody production from mature B cells and stimulates mature B cell survival [163-165]. The SERM raloxifene has the same effect as estradiol on B lymphopoiesis, but no stimulatory effect on antibody production [166]. In contrast raloxifene does not affect T cells, neither T cell-dependent reactions nor induction of thymic atrophy [167].

The role of estrogen in arthritis

The peak incidence in RA coincides with the time of menopause, clearly indicating a role of estrogen in the pathogenesis [67]. In women with RA, the disease activity diminishes during pregnancy when the estrogen levels together with other sex hormones are high [168]. The role of estrogen in RA and other autoimmune diseases is complex [151] and findings have been contradictory with human studies reporting beneficial effects of estrogen on RA while others demonstrate no effect [169-171]. Animal studies in ovariectomized rodents demonstrate higher frequency and increased severity of arthritis compared to intact animals [172]. Furthermore, estrogen treatment suppresses the disease progression [173, 174]. Arthritic mice show amelioration of the disease during pregnancy and aggravation after delivery [175, 176]. The ameliorating effect of estrogen on arthritis is suppressed by the antiestrogen ICI 183,780, demonstrating that this effect is dependent on the classical ERs [177]. Raloxifene ameliorates CIA, both in the acute phase and during long-term treatment [178, 179].

Aims

The general aim of this thesis is to elucidate estrogen signaling pathways in arthritis and the associated immune-mediated bone loss. The more specific aims of the four papers included in this thesis are listed below.

Paper I
To elucidate the estrogen receptor (ER) specificity for the ameliorating effects of estrogen on arthritis and associated bone loss in collagen-induced arthritis (CIA).

Paper II
To investigate the role of ERα and cartilage-specific ERα for estrogen's ameliorating effect on antigen-induced arthritis (AIA).

Paper III
To characterize and establish AIA as a model for periarticular bone loss in arthritis and determine the importance of NADPH oxidase 2 derived ROS for local bone loss.

Paper IV
To clarify if raloxifene, a SERM, can activate the classical estrogen signaling pathways *in vivo,* in three known estrogen-responsive organs; uterus, bone and thymus.

Methodological considerations

The purpose of this section is to give an overview of the materials and methods used to collect the data for this thesis. More detailed protocols are described in the respective papers.

Animals

The transferability of research from animal to human can always be questioned. Animal models are reasonably fast and the studies can usually be detailed. Compared to cell cultures, they provide the proper environment and allow studies of interactions between tissues. We have used mouse models in this thesis to study the effects of endocrine interaction between different tissues and the complexity in arthritic diseases. One of many advantages of using mouse models is the possibility of performing transgenic modifications including deletion of specific target genes (knock out (KO) models), insertion of for example reporter genes or enhancement of the expression of specific genes. In these studies, female DBA/1 mice, total estrogen receptor α (ERα) KO mice, cartilage-specific ERα KO mice, estrogen response element (ERE)-luciferase reporter mice, unmodified C57BL/6 mice, B.10QNcf KO mice (with impaired ROS production), and corresponding B.10Q control mice, have been used. Permissions from the local animal research ethics committee, according to national animal welfare legislation, were obtained for all experiments.

ERα KO

The generation of total ERα KO mice is somewhat complex since both female and male homozygous ERα KO mice are infertile. Therefore heterozygous mice were mated, resulting in wild type (WT), heterozygous and homozygous ERα KO offspring. These mice have a deletion of exon 3 of the ERα gene and do not express any of the isoforms of the ERα protein [180]. Cartilage-specific ERα KO mice were generated by crossing mice in which exon 3 of the ERα gene is flanked by loxP sequences, with mice with Cre driven by the Col2α1 promoter, which is specifically expressed in chondrocytes [181]. Thus, the Cre-loxP system is used to generate conditional tissue-specific ERα KO mice. This Cre-loxP system is a site-specific recombinase technology, widely used to carry out deletions, insertions, translocations and inversions and allows DNA modifications in specific cell types. The cartilage-specific ERα has a 62% reduction of ERα protein levels in cartilage, but no reduction in other organs [182].

The total and cartilage-specific ERα KO mice were used to investigate the role of estrogen in arthritis amelioration (paper II).

Estrogen response element-luciferase reporter mice

The ERE-luciferase mouse has a luciferase reporter gene under the control of three consensus EREs coupled to a minimal TATA-box [183] (*Fig 7*). The activated complex, with ligand bound to ER, initiates transcription of the luciferase gene and the amount of transcription can be estimated using an enzymatic reaction enabling detection of classical estrogen signaling *in vivo*. The ERE-luciferase mice were used to determine the ability of raloxifene to affect estrogen-responsive tissues via the classical estrogen signaling pathway (paper IV).

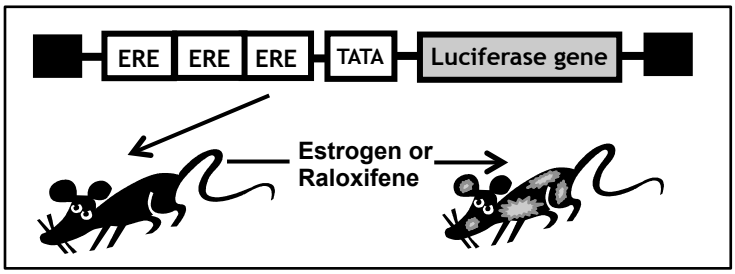

Figure 7. Estrogen response element (ERE)-reporter mice have a luciferase gene inserted in the DNA under the control of three EREs. When the classical estrogen signaling pathway is activated in these mice, the reporter gene is transcribed.

Ovariectomy and hormone treatments

Ovariectomy (OVX) is a common model for studying the effects of sex steroid deficiency in animals, and is associated with decreased bone mineral density (BMD) corresponding to the decline in BMD seen in postmenopausal women. The reduced estrogen levels in OVX mice are associated with a dramatic decrease in uterus size.

In paper I, the selective ER agonists, propyl-pyrazoletriol (PPT, 175μg/mouse) specific for ERα, diarylpropionitrile (DPN, 105μg/mouse) specific for ERβ and GI (5μg/mouse) specific for GPR-30 or 17β-estradiol-3-benzoate (1μg/mouse) were subcutaneously injected 5 days per week, starting one day before arthritis booster. The treatment lasted until study termination. In paper II, a subcutaneous slow-release pellet with 17β-estradiol (0.83μg/mouse/day) or a corresponding placebo pellet were inserted into unmodified C57BL/6, total ERα KO and cartilage-specific ERα KO mice, from the time of OVX until termination. In paper IV, ERE-luciferase mice were subcutaneously injected with a single dose of raloxifene (60μg/mouse)

or 17β-estradiol-3-benzoate (1μg/mouse) or subjected to long-term (3 weeks) treatment, 5 days per week of raloxifene (60μg/mouse) or 17β-estradiol-3-benzoate (1μg/mouse), 2 weeks after OVX. All treatment levels correspond to physiological levels seen in female mice [100].

Arthritis models

Two different arthritis models were used in this thesis (*Fig 8*): CIA in DBA/1 mice treated with different specific estrogen receptor agonists (paper I) and AIA in ERα KO animals (paper II) and C57BL/6 mice (paper III). In CIA, mice develop a systemic polyarthritis and the experiment extends over 39 days. In AIA, the experimental model is shorter and extends over 14 days. The arthritis caused by AIA is only visible microscopically in one joint. Both models share similarities to RA but none of the models develop chronicity. The CIA was observed macroscopically and blinded for the grading of frequency and severity on a scale 1-3 on each paw where 1=swelling or erythema in 1 joint, 2= swelling or erythema in 2 joints and 3=severe swelling of the entire paw or ankylosis. This is a rather blunt grading system, but for the purpose in paper I, the effects of the ameliorating compounds were clearly observed. The microscopic evaluation of arthritis was examined blinded in both CIA and AIA (paper I, II, III). The evaluation was performed either in entire paws in CIA or in one knee joint in AIA, using the 1-3 grading scale and separating synovitis and joint destruction. The joint destruction was further separated into bone erosions and cartilage degradation in paper II. This 1-3 grading scale is blunt, but adequate for detecting estrogenic amelioration of arthritis (paper I, II) and differences between arthritic and non-arthritic joints (paper III).

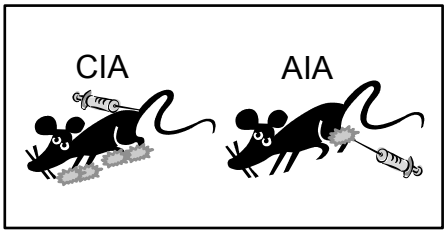

Figure 8. Two different animal models of arthritis. Collagen-induced arthritis (CIA) is a polyarthritis model caused by repetitive systemic immunizations with collagen type II. Antigen-induced arthritis (AIA) is a monoarthritis model caused by re-challenge with mBSA intraarticularly.

Bone assays

Bone parameters were thoroughly quantified in this thesis. Bone and cartilage remodeling was determined in serological specimens by measurement of C-terminal fragments of type I collagen (bone resorption), osteocalcin (bone formation) and cartilage oligomeric matrix protein (cartilage degradation) (paper I). The number of

osteoclasts was determined using immunohistochemistry and staining for the osteoclast-specific enzyme cathepsin K, in the epiphyseal parts of the distal femur and proximal tibia (paper III).

Peripheral Quantitative Computed Tomography

Peripheral quantitative computed tomography (pQCT) is a tool for measuring different bone compartments in both humans and animals. It is based on a rotating x-ray around the specimen, giving a three-dimensional measurement. The classical pQCT measurement of the cortical BMD is in the mid-diaphyseal section of the long bones (paper I, III, IV) (*Fig 9*). In the metaphyseal section of the distal femur and proximal tibia, the trabecular BMD is determined in the inner 45% of the total area (paper I, III, IV). In this thesis the Stratec pQCT XCT Resarch M, specifically modified for use on small bone specimens (version 5.4B; resolution 70μm), with an interassay coefficient of variation of less than 2%, was used. The resolution of the pQCT is limited but adequate for the measurement of the arthritis-associated bone loss (paper I) and induction of bone mass after estrogen replacement (paper IV). Furthermore, pQCT measurements of BMD can be performed noninvasively, leaving the limbs intact for histological analysis in terms of microscopic arthritis (paper III).

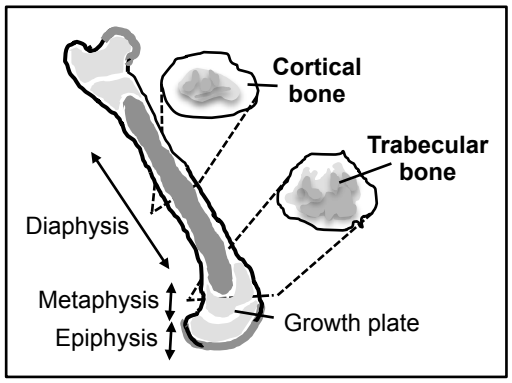

Figure 9. Schematic view of a longitudinal section through a long bone. The cortical bone parameters are measured in the diaphyseal section containing mainly cortical bone. The trabecular bone is measured in a metaphyseal section and defined by setting the inner threshold to 45% of the total area.

Immune assays

Serum IL-6 was measured, using a bioassay in order to determine the degree of ongoing inflammation. The level of collagen type II specific antibodies was determined using an ELISA to quantify the antibody response (paper I).

Flow cytometry

The ability to measure properties of particles and cells is fundamental in flow cytometry. This is achieved by injecting a single cell suspension

into a fluorescence-activated cell sorter (FACS), in which the cells, stained with antibody-conjugated fluorochromes directed to specific cell antigens, are ordered into a stream and fluorochromes are excited by lasers and emitted light is collected by detectors and presented on a plot. FACS is an excellent tool to characterize cell phenotypes, however scattered light from intracellular structures as well as debris can interfere with specific fluorescence emission and thereby give false positive results, so called autofluorescence. The FACS analyses in this thesis were mainly performed to evaluate the different immune cell frequencies, and calculations were done using FlowJo (version 8.5.2).

Single cell suspensions were made of BM, spleen, lymph nodes and synovial tissue. In paper I, cellularity and frequency of B cells were determined in BM. In paper II, cellularity and frequency of T_H and T_C cells were defined in splenocytes. Furthermore, synovial cell frequencies of monocytes, neutrophils, B cells and T cells were determined in paper II and III. Paper III investigated BM cells from the distal part of the femur, close to the inflamed knee joint, and BM cells from the proximal part of the femur. The frequency of preosteoclasts, as a reflection of potential osteoclastogenesis, and frequencies of immune cells including neutrophils, monocytes, T cells and B cells, as a reflection of ongoing inflammation, were investigated in BM cells. The reactive oxygen species (ROS) activation was measured with FACS in BM cells using a cell-permeable product that becomes fluorescent after oxidization with PMA (phorbol myristate acetate). Spleen and lymph nodes cells were also investigated to determine involvement of systemic immune respons.

Concanavalin A-induced cell proliferation

Proliferation of T cells was examined by *in vitro* cultures of splenocytes stimulated with the T cell mitogen concanavalin A. The entire spleen suspension proliferated after adding the mitogen and analyzed by addition of ^{3}H-tymidin using a β-counter (paper II). Cytokines were evaluated in the supernatants of the proliferation assay using a Flowcytomix kit. The Flowcytomix kit is a bead-based multiplex immunoassay for detection of several different components simultaneously using flow cytometry.

Real-time PCR

Real-time PCR is a sensitive method for the quantification of specific mRNAs. Isolated RNA is reversely transcribed into cDNA and mixed with specific primers and probes. The probe is attached with reporter and quenching dyes and when the probe is intact, no fluorescence is emitted. During replication, the probe is detached from the cDNA and

the quenching dye is cleaved off and separated from the reporter dye and fluorescence is emitted. This means that the fluorescence intensity is proportional to the amount of amplified product. Just as in FACS analyses, two different spectra are analyzed simultaneously in the StepOnePlus™ Real-time PCR system and thereby the gene of interest and an internal standard are analyzed simultaneously. In this thesis RNA was extracted from organs important in local and systemic immune mediated bone loss (paper III) and estrogen target cells (paper IV). The ribosomal RNA 18S was used as an internal standard. The mRNA expression of each gene was calculated using a standard curve in relation to the internal standard according to manufacturer's instruction.

Statistics

All statistical calculations in this thesis were performed in GraphPad Prism 5.0d Macintosh version of Software MacKiev. The p-value is the probability of difference between observations. If the p-value is less than 0.05 the null hypothesis is rejected and the difference between the observations is assumed to be statistically significant.

Statistical calculations can be based on either parametric or non-parametric tests. The parametric method assumes that the data is normally distributed. If this assumption is correct, parametric methods produce more accurate and precise estimations. Non-parametric tests do not require any particular distribution of the data and are based on a ranking of individual observations.

In paper I, all calculations were performed with parametric tests. This was in relative large observation groups and normal probability distribution was assumed. The ordinary scale system in both arthritis development and histological examination does not provide normally distributed data and requires non-parametric statistical evaluation. This knowledge was acquired over time and therefore non-parametric statistical evaluation was not performed in paper I. In paper II and III, parametric calculations were performed with the exception of non-parametric calculation for the evaluation of data acquired from the ordinary scale system. The main findings in paper IV is on data from luciferase-reporter mice, which are not probability distributed and therefore non-parametric calculations were performed.

Results

Below is a brief description of the main results found in the four papers included in this thesis. For more details, see the full papers in the end of the thesis.

Paper I

To determine which estrogen receptor (ER) mediates the ameliorating effects of estrogen in collagen-induced arthritis (CIA) we investigated selective ER agonists.

Treatment with estradiol and the ERα agonist delayed the onset and reduced the severity of arthritis [184]. This was confirmed with histological examination, where estradiol and the ERα agonist reduced synovitis as well as bone erosions. The ERβ and GPR-30 agonists displayed no amelioration of either the macroscopic or microscopic signs of arthritis. The GPR-30 agonist rather tended to increase the severity, however after repeating the experiment, no inducing sign of the GPR-30 agonist was revealed. BM cellularity and B cell frequency were significantly decreased by estradiol, the ERα agonist and the ERβ agonist but not by the GPR-30 agonist. This confirms previous findings demonstrating the importance of both ERα and ERβ for BM cellularity and the use of sufficient doses of the selective ER agonists. The preservation of trabecular and cortical bone mineral density (BMD) following the arthritis development and was mediated via ERα as demonstrated by protective bone effects after treatment with estradiol and the ERα agonist.

In summary, the ERα agonist limited both arthritis development and had bone-preserving effects in CIA. Neither the ERβ nor the GPR-30 agonist had any effect on the arthritis or preservation of bone mass.

Paper II

To determine if estrogen ameliorates AIA and investigate the importance of total ERα expression as well as cartilage-specific ERα expression for the ameliorating effect of estrogen, we used total ERα KO mice and cartilage-specific ERα KO mice.

In paper II we determined that estradiol not only ameliorates CIA but also the monoarthritis induced by mBSA in the AIA mouse model. Estradiol ameliorated both synovitis and joint destruction in association with decreased frequencies of neutrophils and monocytes in synovial tissue and decreased splenocyte proliferation.

We also concluded that estradiol ameliorates AIA via ERα. Estradiol treatment in total ERα deficient mice showed no influence on arthritis development, splenocyte proliferation or splenic T cell frequency.

Finally, we determined that expression of ERα in chondrocytes is important for the ameliorating effect on joint inflammation but not joint destruction. In the cartilage-specific ERα deficient mice estradiol treatment resulted, as expected, in ameliorating effects on bone erosions and joint degradation. However, no significant effects on the local synovial inflammation were detected. Thus, deletion of ERα in chondrocytes affects only the local inflammation in the synovia, even though the systemic inflammation is intact, as reflected by a normal estrogenic response on splenocytes.

In summary, ERα is required for the protective effect of estrogen on AIA. Our data further suggest that ERα expression in chondrocytes is involved in the ameliorating effects of estrogen on synovitis but not joint destruction, suggesting different target cells and mechanisms for the estrogenic protection of synovitis and joint destruction.

Paper III

The aims of this paper were to characterize and establish AIA as a model to study periarticular bone loss and to determine the importance of NADPH oxidase 2 (NOX2) derived reactive oxygen species (ROS) for periarticular bone loss using the AIA model on Ncf1 deficient mice.

The AIA model results in a local arthritis development displaying synovitis and joint destruction in the joint challenged with mBSA. Periarticular bone loss was evaluated in the metaphyseal region surrounding the arthritic joint and a significant decrease in trabecular BMD was detected in the arthritic joint compared to the control joint injected with saline. Osteoclast number was induced in the epiphyseal regions close to the arthritic knee as compared to the non-arthritic knee indicating the importance of these cells in the immune-mediated bone loss. As expected, the inflammatory cells including T cells, monocytes and neutrophils, were recruited to the arthritic synovial tissue. The bone marrow cells were investigated in two separate compartments, the distal (close to the inflammation) and proximal part of the femur, to show if the effects were induced in a local manner. In the distal part of the bone marrow, cellularity was increased together with an expansion of preosteoclast, monocyte and neutrophil cell frequencies in the arthritic femur. The induction of monocytes and neutrophils in the distal part of the bone marrow was further accompanied with an improved capability of producing ROS.

The systemic immune system was investigated in spleen and draining lymph node and displayed no activation. Generalized bone loss was investigated in the diaphysis region of the femur displaying no differences in cortical BMD between the arthritic and the non-arthritic side. Furthermore, there was a strong local impact on the RNA expression of genes involved in bone resorption and inflammation. Hence these results imply that the AIA model can be used to investigate the pathogenesis of local immune-mediated bone loss.

To further evaluate the role of ROS production in local immune-mediated bone loss, Ncf1 deficient mice lacking NOX2-derived ROS, were subjected to the AIA model. These mice, with limited ROS production, showed similar periarticular bone loss and arthritis development as their WT control mice on B10.Q background. Therefore, ROS production derived from NOX2 is not involved in the periarticular bone loss.

In summary, AIA resulted in periarticular bone loss as well as joint destruction. This bone loss was associated with local effects on inflammatory cells and osteoclasts. Furthermore, using this model, we conclude that NOX2-derived ROS production is not essential for the immune-mediated periarticular bone loss.

Paper IV

To determine whether the selective estrogen receptor modulator (SERM) raloxifene can affect gene transcription via activation of the classical estrogen signaling pathway *in vivo*, we used estrogen response element (ERE)-reporter mice.

Raloxifene as well as estradiol increased BMD in OVX mice. This increased bone mass was associated with gene transcription via the classical estrogen signaling pathway, as reflected by increased luciferase activity, in bone [185]. Non-classical estrogen signaling was studied by examining expression of Igf1, reflecting non-classical estrogenic signaling and c-fos, reflecting transcription activated via phosphorylation cascades (pregenomic signaling). Expression of Igf1 was unaffected by both estradiol and raloxifene, while estradiol treatment upregulated c-fos mRNA in bone. These data indicate that the classical estrogen signaling pathway is the main pathway involved in the bone preserving effects of raloxifene *in vivo*.

The uterine weight as well as luciferase activation was affected by estradiol. In contrast, raloxifene had significantly less effects on both uterus weight and on the classical estrogen signaling pathway. Raloxifene and estradiol both induced expression of Igf1 in uterus as

an indication of activation of the non-classical estrogen signaling pathway. These data indicate that both classical and non-classical signaling may be involved in the regulation of uterine growth *in vivo*.

Thymus atrophy was induced by estradiol, while raloxifene did not alter thymus weight. However, both estradiol and raloxifene induced the classical signaling pathway similarly. Thus, there is a discrepancy between the classical estrogen signaling pathway and effects on thymus weight. Furthermore, estradiol but not raloxifene up-regulated Igf1 mRNA in thymus, indicating that the non-classical estrogen signaling pathway may be involved in thymus atrophy.

In summary, this investigation demonstrates that raloxifene can activate the classical estrogen signaling pathway *in vivo* in three estrogen-sensitive organs.

Discussion

Osteoporosis is a hallmark of postmenopausal arthritis and is caused by a complex pathophysiological process dependent on several factors including the immune as well as the hormonal status [77].

Estrogen is a steroid hormone that is of indispensable importance for female development and maturation, but also in a variety of other biological systems. It is well known that estrogen has positive effects on bone metabolism and estrogen replacement in postmenopausal women with osteoporosis has proven to be beneficial. The role of estrogen in autoimmune diseases is more complex with conflicting results showing either positive, negative or no effects [151]. The importance of estrogens in postmenopausal RA is also contradictory with studies demonstrating beneficial as well as no effect of estrogen replacement [169-171]. Animal studies in OVX rodents have demonstrated that estrogen replacement can affect both the incidence and progression of arthritis in a favorable manner and protect against arthritis-related bone loss [172-174, 178, 179] and we determine that estrogen ameliorates disease activity in estrogen deficient mice in a monoarthritis mouse model.

In this thesis we show, using animal models, that estrogen may affect arthritis in different ways. Firstly, estrogen can attenuate the entire immune system as shown in CIA, via a decrease in BM cellularity and B cell frequency as well as a tendency to lower levels of both serum anti-collagen type II antibodies and IL-6. Furthermore, the splenic proliferation capacity after stimulation with the T cell mitogen conA as well as the frequency of splenic T_H cells was dampened by estrogen treatment in AIA. Secondly, estrogen reduces the synovitis displayed in both CIA and AIA and we propose that this is caused by a reduced infiltration of monocytes and neutrophils into the arthritic joints. Thirdly, estrogen inhibits the arthritis-associated bone loss, shown both for the generalized, systemic bone loss in CIA and for the local bone erosions and joint destruction in CIA and AIA.

Estrogen signaling is complex and estrogen exerts its physiological effects via the two classical nuclear ERs, ERα and ERβ as well as the transmembrane GPR30. The receptor or pathway via which estrogen influences autoimmune diseases is not completely established. However, estrogen receptors have been intensively studied in rodent models of several different autoimmune diseases. ERα is important for improvement of autoimmune diseases like Sjögrens syndrome and multiple sclerosis [186, 187]. In arthritis, the classical nuclear estrogen

receptors are important, as demonstrated by the fact that treatment with ICI 182,170, an agonist that specifically blocks ERα and ERβ, results in enhancement of CIA [177]. In line with this result we determined that ERα, but not ERβ or GPR-30, mediates the ameliorating effects of estrogen on ovariectomized CIA mice, both regarding arthritis and on immune-mediated bone loss, using receptor-selective agonists. Ameliorating effects of selective ERα agonists have been demonstrated in different models of arthritis [174, 188]. A selective ERβ agonist has been shown to have potential anti-inflammatory effects and thereby limit arthritis severity in a rat model of mycobacteria-induced arthritis [189]. The discrepancy between that study and ours may have several explanations including the use of different species, sexes and methods.

Furthermore, we show in a monoarthritis model induced with mBSA, that total deletion of ERα expression completely hinders the protective effects of estrogen on joint inflammation and destruction. This further demonstrates the crucial role of ERα for the ameliorating effects of estrogen replacement in female estrogen deficient mice. The monoarthritis model was used because of its histological features resembling human RA including infiltration of monocytes and neutrophils into the joint and destruction including cartilage and bone erosions [96]. Furthermore the ERα knockout mice have a genetic background that renders them less susceptible to development of CIA. The importance of using several different appropriate arthritis animal models and also different experimental setups is essential for understanding the complexity of RA.

The importance of ERα for the protection against ovariectomy-induced bone loss was previously determined in ERα knockout mouse models [190-193]. However, we are the first to show positive effects of estrogen via ERα on immune-mediated bone loss. In autoimmune arthritis like RA there are three different forms of bone loss (i) generalized osteoporosis, mainly mediated via an altered cytokine balance that influences the osteoclast maturation properties [55], (ii) periarticular bone loss, with accumulation of immune cells around the joint and (iii) focal bone erosions at the inflammation site [73, 74]. The exact mechanism behind periarticular bone loss is not well understood. Therefore we propose that the AIA model is an excellent model for increasing our knowledge about the periarticular bone loss. We demonstrated that the immune-meditated bone loss in AIA was associated with a local induction of osteoclasts and the BM only displayed an induction of osteoclast precursors close to the inflamed joint. A previous finding suggested that arthritic joints affect

osteoclasts via a paracrine mechanism since the number of osteoclasts dramatically decreases with the distance from the affected joint [194]. In this monoarthritis model, inflammation was induced in a local manner with a strong increase of inflammatory cells in the arthritic joint and in BM close to the inflammation site. This shows that the BM is not a homogenous structure, but that there are site-specific differences within the bone marrow compartment. ROS seemed to be important in immune-mediated bone loss since we demonstrated a local induced capability of ROS production in BM close to the inflammation site. ROS are widely considered to be involved in several destructive conditions, like osteoclast destruction of skeletal tissue and joint destruction in RA [195, 196]. It has also been shown that limited ROS production in mice with impaired NOX2 function results in enhanced disease severity in several different arthritis models [197, 198]. This suggests a dual role of ROS production in arthritis. However, we determined that NOX2 derived ROS was not essential for immune-mediated bone loss or arthritis development in AIA. NOX2 derived ROS production stands for a majority of the reactive oxygen species, however other forms of oxidases exist and therefore some ROS may still be present in the NOX2 deficient mice.

The articular chondrocyte has recently gained increased interest in the pathogenesis of arthritis. Synovial fluid from RA patients can trigger chondrocytes to secrete cytokines [199]. This suggests that these cells can actively take part in the inflammatory process and not just be passive participators. Estrogen affects protein production from articular chondrocytes and they express both forms of ERs [200]. We therefore further investigated the importance of chondrocyte ERα expression for the ameliorating effects of estrogen in AIA. We found that estrogen treatment of the cartilage-specific ERα deficient mice resulted in reduced joint destruction while no effect on the synovial inflammation was observed. These results suggest that there are different target cells for estrogens protective effects on synovitis and joint destruction in AIA. The cartilage-specific ERα deficient mice were generated using the Cre-loxP system. Insufficient exclusion of a gene or nonspecific deletion is a problem using the Cre-loxP system. The cartilage-specific ERα deficient mice have a 62% reduction of ERα protein in chondrocytes, but no reduction in other organs [182]. However, these mice display a phenotype in growth plate closure [182] and we show a reduction in estrogenic amelioration of synovitis, demonstrating sufficient exclusion of the ERα gene to result in physiological effects. The Cre-loxP technique provides a better understanding of the cell specific estrogen signaling mechanisms and

may result in improved knowledge for future development of estrogen replacement therapies in diseases like osteoporosis and RA.

In summary, these results from animal arthritis models demonstrate the positive effects of estrogen. Estrogen is, however, not considered a suitable long-term treatment because of its negative side effects. Therefore, it is important to determine the mechanism behind the protective effects of estrogens in arthritis to develop new treatment strategies that maintain estrogen's positive effects but avoid the negative effects.

Raloxifene is a SERM approved to treat postmenopausal osteoporosis and used as prophylaxis against invasive breast cancer [124, 125]. Raloxifene still has side effects including increased risk of coronary heart failure and thrombosis. In CIA, raloxifene ameliorates arthritis development as well as immune-mediated bone loss [178]. It displays superior affinity to ERα over ERβ [128, 129]. The binding of estrogen or raloxifene to the ER alters the structure complex and thereby diversifies the constellation of co-regulators that have the opportunity to bind the complex. This leads to cell type and cell context specific differences in the activation of ERs depending on the ligand and it is therefore necessary to study the signaling effects *in vivo*. Estrogen or SERMs mainly affect gene transcription via the classical signaling pathway, involving binding of the ligand-ER complex to EREs or the non-classical signaling pathway, involving binding of the ligand-ER complex to other transcription factors such as AP1, SP1 or NFκB. We found, in ERE-luciferase reporter mice, that raloxifene potently increased BMD accompanied by an increase in luciferase activation, demonstrating that raloxifene can induce the classical signaling pathway, suggesting that this pathway is involved in the bone-protective effects of raloxifene. This *in vivo* finding is in contrast to a previous *in vitro* finding of cultured osteoblasts, where raloxifene showed no or limited classical activation but instead non-classical activation [201]. The non-classical pathway, as we investigated by transcription of Igf1, was unaffected by raloxifene. However, our results do not rule out the possibility of non-classical signaling involvement in the regulation of bone mass. We further showed that raloxifene only had a mild agonistic effect on uterus accretion and on initiation of the classical signaling pathways in uterus. Classical, non-classical and phosphorylation cascades may all be involved in estrogenic regulation of uterine growth *in vivo*, however, the relative importance of these signaling pathways for the *in vivo* effects in uterus needs further investigation. Finally, we demonstrated that estradiol, but not raloxifene, induced thymic atrophy, but both substances

activated the classical signaling pathway to the same degree. This indicates that the classical signaling pathway in thymus is not essential for thymic atrophy but may be involved in T cell differentiation. Taken together, these results demonstrate that raloxifene can activate the classical signaling pathways *in vivo,* and increased knowledge about the differences in intracellular signaling between SERMs and estrogen is of importance for developing new and better SERMs.

Conclusion

Estrogen ameliorates arthritis and immune-mediated bone loss in animal models. Estrogen deficiency leads to a more severe arthritis development and negative effects in a wide variety of tissues such as bone, fat and heart. Long-term estrogen replacement is associated with side effects, such as increased risk of breast cancer and deep venous thrombosis. Knowledge about estrogen signaling in different tissues and in the development of arthritis is of importance to be able to design SERMs, which maintain the beneficial effects of estrogen but avoid the risks. This thesis has revealed that ERα is the estrogen receptor responsible for ameliorating arthritis and the associated immune-mediated bone loss. The dampening effect of estrogen on inflammation and joint destruction could be mediated independently of each other through signaling via different cell types. These results may facilitate the development of new treatment strategies for postmenopausal RA.

Osteoporosis is a major health problem in RA patients. Not only is the hormonal status involved in the bone loss, but also the systemic and local inflammation. The periarticular bone loss in RA is not well characterized. We demonstrate that the monoarthritis mouse model, with a local injection of mBSA, is an excellent model for investigation of this type of bone loss, since it results in profound local bone loss and inflammation, but no systemic bone loss or inflammation.

Raloxifene is a SERM approved for treatment of postmenopausal osteoporosis. By which signaling pathway raloxifene mediates its effects in various tissues is still not clearly understood. In this thesis, we demonstrate that raloxifene can activate the classical estrogen-signaling pathway in estrogen responsive tissues *in vivo*. This knowledge may help in the construction of new improved SERMs for patients with postmenopausal osteoporosis and RA.

Sammanfattning på svenska

Begreppet osteoimmunologi belyser sambanden mellan immunsystem och skelett. Kroppens immunsystem utvecklas främst inuti skelettets benmärg. Många processer som påverkar immunsystemet får därmed effekter på skelettet och vice versa. Att klargöra samverkan mellan skelett och immunsystem ökar förståelsen för sjukdomsprocesser där dessa påverkas, och detta ger nya möjligheter att utveckla effektiva läkemedel.

Ett tillstånd med tydliga osteoimmunologiska processer är reumatoid artrit (RA). RA karakteriseras av inflammation i ledslemhinnan vilket orsakar broskförstörelse samt generell och lednära benförlust. Frekvensen av benskörhet uppskattas till cirka 50 procent hos patienter med RA.

Vid klimakteriet minskar kroppens produktion av könshormonet östrogen vilket leder till ökad bennedbrytning. RA är tre gånger vanligare hos kvinnor än hos män och de flesta insjuknar i samband med eller åren efter klimakteriet. Vid RA efter klimakteriet påverkar både östrogenbristen och inflammationen i lederna benskörhetsutvecklingen. Behandling med östrogen förhindrar bennedbrytning samt mildrar artritutveckling. Flera studier har visat att långtidsbehandling med östrogen kan öka risken för allvarliga biverkningar. Därför arbetar man med att få fram alternativa läkemedel, som har östrogenets gynnsamma effekter men saknar dess biverkningar. Ett sådant läkemedel är raloxifen, som har östrogenlika effekter på ben och anti-östrogenlika effekter i livmodern. Raloxifen används sedan 1997 för att behandla benskörhet efter klimakteriet.

Östrogen påverkar cellerna i kroppen primärt genom att binda östrogenreceptorerna ERα och ERβ. Vi visar i två olika djurmodeller av artrit, att östrogen mildrar artrit samt dämpar benförlusten via ERα. Vidare visar vi att ERα i broskceller är viktigt för att dämpa inflammationen men påverkar ej den lednära benförlusten. Vi har dessutom beskrivit en ny modell för att studera den lednära benförlusten i en enstaka inflammerad led. Slutligen visar vi *in vivo* att raloxifen, kan aktivera klassisk östrogensignalering i ben.

Dessa fördjupade kunskaper om östrogenets effekter på artrit samt benförlust är viktiga pusselbitar i arbetet för utvecklingen av framtida specifika läkemedel mot artritsjukdomar.

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