Estrogen and 2-methoxyestradiol

Regulation of arthritis, inflammation and reactive oxygen species

Alexandra Stubelius

Institute of Medicine
at Sahlgrenska Academy
University of Gothenburg

UNIVERSITY OF GOTHENBURG

Alexand Stubelius

Estrogen and 2-methoxyestradiol – Regulation of arthritis, inflammation and reactive oxygen species

2014

The Sahlgrenska Academy

ISBN 978-91-628-8945-6
Printed by Kompendiet, Gothenburg

Estrogen and 2-methoxyestradiol

Regulation of arthritis, inflammation and reactive oxygen species

Alexandra Stubelius

Centre for Bone and Arthritis Research,
Department of Rheumatology and Inflammation Research
Sahlgrenska Academy at University of Gothenburg

UNIVERSITY OF GOTHENBURG

Gothenburg 2014

Estrogen and 2-methoxyestradiol

Alexandra.Stubelius@rheuma.gu.se

ISBN 978-91-628-8945-6
http://hdl.handle.net/2077/35197

Illustrations were produced using Servier medical art
Printed in Gothenburg, Sweden 2014
by Kompendiet, Aidla Trading AB, Göteborg

Estrogen and 2-methoxyestradiol
Regulation of arthritis, inflammation and reactive oxygen species

Alexandra Stubelius

Centre for Bone and Arthritis Research,
Department of Rheumatology and Inflammation Research,
Sahlgrenska Academy at University of Gothenburg
Göteborg, Sweden

ABSTRACT

Rheumatoid arthritis (RA) is characterized by severe synovial inflammation, cartilage destruction, and immune-mediated bone loss. Estrogen ameliorates experimental RA, reducing both inflammation and bone loss. The inflamed tissues are damaged partly by innate immune cells producing reactive oxygen species (ROS). ROS can also regulate the immune system. This thesis aimed to investigate the regulation of inflammation and joint destruction by 17β-estradiol (E2) and its metabolite 2-methoxyestradiol (2me2).

E2's and 2me2's immunomodulation were investigated both in experimental arthritis and in an unprovoked immune system. Both wild type (WT) mice and Catechol-*O*-methyltransferase (COMT)-deficient mice were used, as COMT metabolizes E2 into 2me2. Further, E2's regulatory role was investigated in WT mice or ROS-deficient mice (B10.Q.Ncf1*/*), in a model of osteoporosis and a local (LPS-induced) inflammation model.

2me2 ameliorated arthritis and bone mineral density (BMD), and regulated immune cells differently compared with E2. Treatment with high doses of 2me2 increased uteri weight, implying estrogen-receptor activation; 2me2 activated estrogen-response elements in a tissue-, and dose-dependent manner. Deficiency in the COMT enzyme only moderately affected the immune system, and males were more affected than females.

In ovx-induced bone loss, ROS-deficient mice displayed reduced osteoclastogenesis compared to controls, but similar bone mineral density and immunological profiles. In LPS-induced inflammation, E2 treatment in WT mice shifted neutrophil infiltration to macrophage infiltration, while in ROS-deficient mice E2 treatment induced neutrophil infiltration and reduced the macrophages.

In conclusion, E2's metabolite 2me2 can modulate arthritis and inflammation-triggered osteoporosis. At high doses 2me2 can induce estrogen receptor signaling. E2 together with ROS regulate inflammation and osteoclastogenesis. Understanding estrogenic cellular and molecular mechanisms are important for developing new arthritis and inflammation-treatments. Our results increase the understanding of estrogens' role in inflammation and motivate further investigations.

Keywords: Estrogen, arthritis, reactive oxygen species
ISBN: 978-91-628-8945-6

SAMMANFATTNING PÅ SVENSKA

De kvinnliga könshormonerna östrogener påverkar många processer i kroppen. Förutom deras effekter på de kvinnliga könsorganen kan de även påverka skelettet och immunsystemet. Immunsystemet utvecklas främst inuti skelettets benmärg. Aktivering av immunsystemet kan påverka skelettet, och vice versa. Hos kvinnor minskar produktionen av östrogen efter klimakteriet och detta leder till minskad bentäthet, bland annat via immunsystemet.

Ett sätt att studera kommunikationen mellan immunsystemet och skelettet är genom den autoimmuna ledsjukdomen reumatoid artrit (RA). RA är en sjukdom där inflammation i lederna orsakar brosk- och benförstörelse. Dessutom drabbas hälften av alla RA-patienter av generell benförlust (osteoporos). RA är tre gånger vanligare hos kvinnor än hos män och de flesta insjuknar i samband med eller under åren efter klimakteriet. Behandling med östrogen kan minska både inflammationen och benskörheten men långtidsbehandling med östrogen är inte längre rekommenderat på grund av riskerna för biverkningar. Man vill därför få fram alternativa läkemedel som behåller de positiva effekterna av östrogen men som saknar dess negativa effekter. Därför är det viktigt att studera hur immunsystemet, skelettet och östrogen påverkar varandra. I denna avhandling har vi både arbetat med ett nytt lovande östrogen-likt preparat kallat 2-metoxyöstradiol (2me2) och därefter har vi undersökt hur naturligt östrogent hormon kan påverka de signalmolekyler som kallas syreradikaler.

I en djurmodell av RA kunde vi visa att 2me2 förbättrade artriten, liksom både inflammation och benskörhet. Vi visar också att 2me2 påverkar andra immunceller än vad östrogen gör, men saknas 2me2 genom en mutation påverkas inte immunsystemet avsevärt. Våra resultat tyder på att högre 2me2 doser ger rent östrogen-lika effekter.

Därefter undersöktes och visades att östrogen tillsammans med syreradikaler påverkar celler som förstör ben, och att östrogen samverkar med syreradikaler i inflammation för att reglera immunsystemet.

Genom fördjupade kunskaper om östrogens roll när det gäller att styra immunsystemet kan specifika läkemedel mot inflammation och artrit utvecklas. Resultaten från denna avhandling ökar förståelsen för östrogens roll vid inflammation och inspirerar till fortsatt forskning.

LIST OF PAPERS

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

I. Alexandra Stubelius, Emil Andréasson, Anna Karlsson, Claes Ohlsson, Åsa Tivesten, Ulrika Islander, Hans Carlsten
Role of 2-methoxyestradiol as inhibitor of arthritis and osteoporosis in a model of postmenopausal rheumatoid arthritis
Clinical Immunology 2011: 140, 37-46.

II. Alexandra Stubelius, Malin C. Erlandsson, Ulrika Islander, Hans Carlsten.
Immunomodulation by the estrogen metabolite 2-methoxyestradiol
Clinical Immunology 2014, *in press*.

III. Alexandra Stubelius, Anna S. Wilhelmson, Joseph A. Gogos, Åsa Tivesten, Ulrika Islander, Hans Carlsten.
Sexual dimorphisms in the immune system of catechol-*O*-methyltransferase knockout mice.
Immunobiology 2012: 217, 751-760 .

IV. Alexandra Stubelius, Annica Andersson, Rikard Holmdahl, Claes Ohlsson, Ulrika Islander, Hans Carlsten
NADPH oxidase 2 influences osteoclast formation but is not critical for ovariectomy-induced bone loss
Manuscript in preparation

V. Alexandra Stubelius, Annica Andersson, Rikard Holmdahl, Ulrika Islander, Hans Carlsten
Role of estrogen in regulating LPS-induced inflammation in NADPH oxidase 2 deficient mice
Manuscript in preparation

CONTENT

ABBREVIATIONS

2me2	2-methoxyestradiol
APC	Antigen presenting cell
BMD	Bone mineral density
CD	Cluster of differentiation
cDC	Classical dendritic cell
CGD	Chronic granulomatous disease
CIA	Collagen induced arthrits
COMP	Cartilage oligomeric matrix protein
COMT	Catechol-*O*-methyltransferase
Con A	Concanavalin A
DAMP	Danger-associated molecular pattern
DC	Dendritic cell
DHEA	Dihydroepiandrosterone
DHR123	Dihydrorhodamine 123
Duox	Dual oxidase
E2	17β-Estradiol
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunosorbent spot assay
ER	Estrogen receptor
ERE	Estrogen response element
HIF-1α	Hypoxia inducible factor-1α
HRT	Hormone replacement therapy
IFN	Interferon
IL	Interleukin
ILC	Innate lymphoid cell
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide

M-CSF	Macrophage colony stimulating factor
MHC	Major histocompatibility complex
NADPH	Nicotinamide adenine dinucleotide phosphate
NCF1	Neutrophil cytosolic factor 1
NF-κB	Nuclear factor-κB
NK	Natural killer
NO	Nitric oxide
NOX	Nicotinamide adenine dinucleotide phosphate oxidase
OCL	Osteoclast
Ovx	Ovariectomy
PAMP	Pathogen-associated molecular pattern
phox	Phagocytic oxidase
pQCT	Peripheral quantitative computed tomography
RA	Rheumatoid arthritis
RANK	Receptor activator of nuclear factor κB
RANKL	Receptor activator of nuclear factor κB ligand
ROI	Reactive oxygen intermediates
ROS	Reactive oxygen species
SD	Standard deviation
SERM	Selective estrogen receptor modulator
SHBG	Sex hormone binding globulin
SLE	Systemic lupus erythematosus
TCR	T cell receptor
TEC	Thymic epithelial cells
TLR	Toll like receptor
TNF	Tumor necrosis factor
TRAP	Tartrate resistant acid phosphatase
WT	Wild type
uNK	Uterine Natural Killer

1 INTRODUCTION

Our immune system evolved and specialized during millions of years to defend us against invading pathogens and to detect and eliminate malignant cells (Fig. 1). An expert system developed employing cell-cell contacts, cytokines and chemokine to mount an appropriate defense.

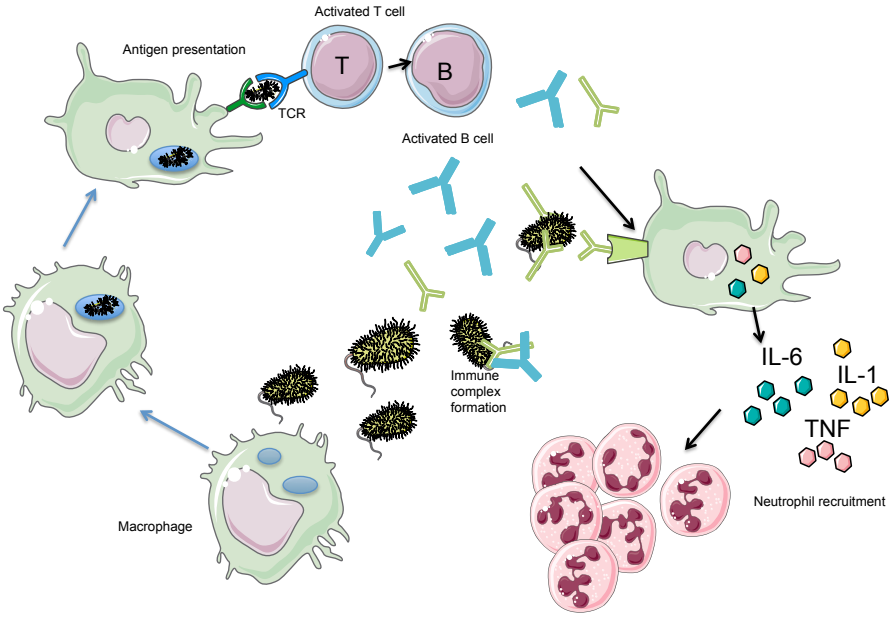


Figure 1. Macrophages phagocytize, degrade and present antigens such as peptides from bacteria in order to initiate an immune response. Specific T helper cells are presented these antigens via MHCII and TCR to become activated. They in turn trigger specific B cells to produce antibodies towards the antigen, facilitating phagocytosis.

Regulating this system became important, and cooperation with the endocrine system was one well-suited mechanism. Estrogen regulates the immune system; suppressing both B-, and T lymphopoiesis, stimulating immunoglobulin production, and downregulating natural killer (NK) cell cytotoxicity, and macrophage responses. The cooperation between the immune- and endocrine systems probably evolved for fetal implantation and growth, where the fetus should coexist with the mother during development. The endocrine system influences the general immune system, both in inflammation and autoimmune diseases.

The following sections aim at describing what is known today about the immune system, specifically the innate immune system, and its regulation by estrogens and its metabolites, contributing to find better therapies regulating both autoimmune diseases and inflammation.

1.1 Sex steroid hormones: estrogens

The gonads, the ovaries in women and the testes in men, produce sex steroid hormones. In humans, as opposed to rodents, the adrenal cortex also produce sex steroid hormones from sex steroid precursors dihydroepiandrosterone (DHEA) [1]. The sex steroid hormones include estrogens, androgens and progesterones. In this thesis, the focus lies on estradiol and estradiol metabolites. 17β-Estradiol (E2), the most potent form of estrogens, affects the skeleton, nervous, cardiovascular and immune system [1]. Serum levels varies from >50pg/ml in premenstrual girls; 27-460 pg/ml depending on menstrual phase during the fertile period; and after menopause descends to <27pg/ml. In men, E2 serum levels are under 54pg/ml. In female mice, the level varies from 50-400pg/ml in fertile mice depending on study, between 1000-2000pg/ml during pregnancy, descending to <30pg/ml after ovariectomy. In serum, E2 is bound to sex hormone binding globulin (SHBG) or albumin, leaving only the 2-3% of free hormone as biologically active.

In regard to sex steroid biology, mice differ from humans in lacking the protein SHBG, and the adult mouse does not produce the sex hormone precursor DHEA. In addition, testosterone levels in males depend on the rank in the hierarchy, where the dominant male has higher levels than other co-housed males [2]. SHBG and albumin carries sex steroid hormones in human plasma, where SHBG prolongs the half-life of bound hormones. In mice, this results in lower levels of hormone, but also higher intra-individual variation. As mentioned previously, only the gonads produce sex steroid hormones in mice, and removal of the gonads provides a simple tool for studying the role of hormones, both endogenous and sex-steroid signaling by exogenous agents.

1.1.1 Estrogen receptors and signaling

Sex steroid hormones bind members of the nuclear hormone receptor superfamily. E2 bind the estrogen receptor (ER) α, or β, to exert their effects (Fig. 2) [3]. The members of this superfamily are zinc-finger-containing transcription factors, with a central DNA-binding domain and a C-

terminal, ligand-binding domain. The ERs loosely bind receptor-associated proteins in the cytosol or nucleus (Fig. 2, [4]).

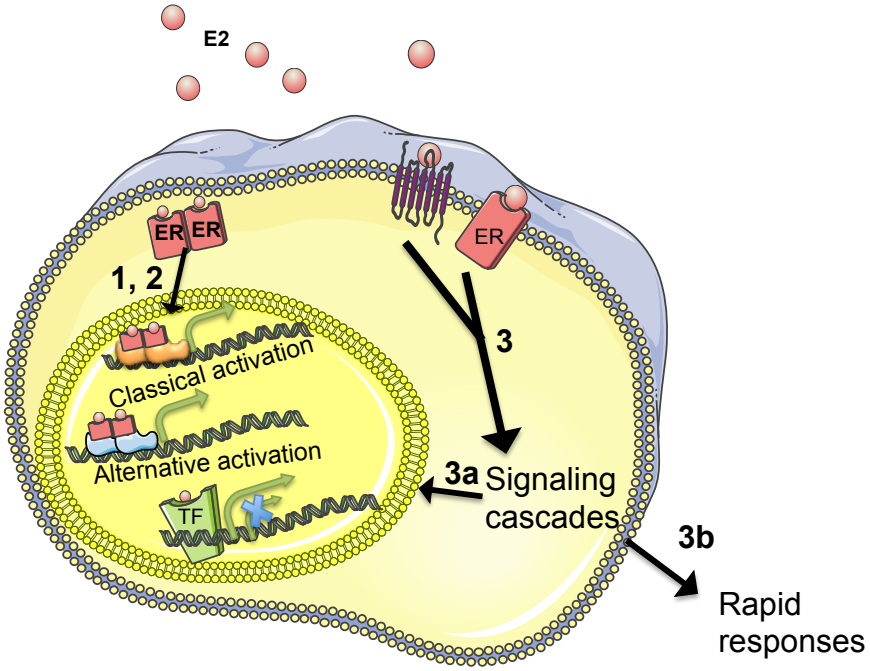


Figure 2. Estrogen receptor signaling: 1. Classical transcription: ER binds estrogen, forms a receptor dimer and translocate into the nucleus. Co-regulatory proteins are recruited and the complex binds to EREs to initiate transcription 2. Non-classical transcription pathway: the estrogen/ER-complex start transcription by binding alternative transcription factors such as AP-1, SP-1 and NF-κB. 3. Binding of membrane associated receptors such as ERs or GPR30 leads to rapid activation or repression of intracellular signaling pathways (calcium mobilization and PI3K activation) leading to 3a altered transcriptional activity via other transcription factors (TF):or 3b non-genomic signaling.

In the classical signaling pathway, estrogen binds the receptor, which forms a receptor dimer, and translocate to the nucleus [5]. The receptor dimer then binds co-regulatory proteins and attaches to estrogen response elements (ERE), and initiates transcription [6]. The EREs are located in the promoter regions of genes that are regulated by estrogens. In this thesis, we have used ERE-luciferase-coupled mice; enabling examination of this pathway, see the methods section for more details.

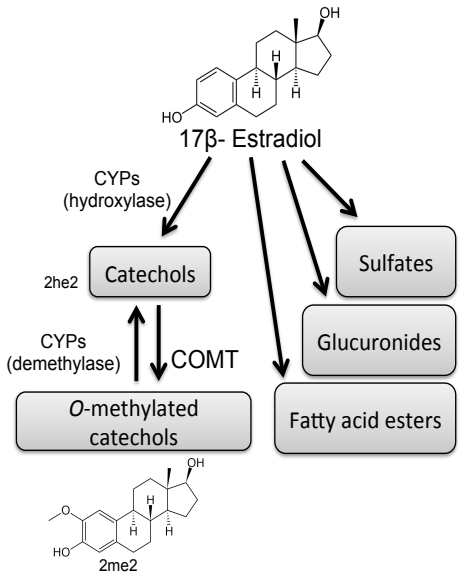
In the non-classical transcription pathway, the estrogen/ER-complex initiate transcription upon binding alternative transcription factors such as SP-1, AP-1, and NF-κB [7-9].

Rapid signaling initiates when estrogens bind ERs outside the nucleus, for example GPR30 in the plasma membrane. Other rapid signals include triggering the production of cyclic nucleotides, calcium influx, and activation of cytoplasmic kinases.

In addition to ligand-induced transcriptional activities of ER there are also ligand-independent pathways that can activate the ERs, such as mechanical loading.

The distribution of the ERs vary in different tissues, where high concentrations of ERα is expressed in the uterus, mammary gland, liver, and cardiovascular systems; whereas ERβ is highly expressed in the testis, ovaries, and the thyroid gland [3]. The receptors have low homology in the ligand-binding domain (55%), but high homology in the DNA-binding domain (97%), suggesting that they recognize similar DNA sequences but respond to different ligands [10]. Signaling through ERα is important in ameliorating arthritis, osteoporosis, and inducing uteri growth in female mice [11-13].

1.1.2 Estrogen metabolism



E2 – 17β-Estradiol – has a hydrophobic structure and is metabolized to increase hydrophilicity for elimination through the kidneys or the liver. As is denoted in Fig. 3, there are many fates of such a large compound where many possible side chains can be added, depending on availability of metabolizing enzymes [14].

Figure 3. Metabolism of estradiol. CYPs: Cytochrome P450 enzymes, COMT: catechol-O-methyltransferase, 2he2: 2-hydroxyestradiol, 2me2: 2-methoxyestradiol

1.1.3 Catechol-*O*-methyltransferase

The enzyme COMT recognizes a catechol group (Fig. 4), and introduces a methyl group on structures such as catecholamines and catecholestrogens. COMT converts 2-hydroxyestradiol into 2-methoxyestradiol, an important part of estradiol's metabolism [14]. COMT has been widely studied as it also metabolizes catecholamines such as dopamine, epinephrine, and norepinephrine, important in neurotransmission and defect in Parkinson's and some behavioral disorders [15-17]. Gogos *et al* [18] generated COMT homozygous knock out mice ($COMT^{-/-}$) in 1998, and demonstrated sexually dimorphic changes in catecholamine levels and behavior. Estrogen down-regulates COMT's promotor and consequently its activity, and the enzyme is more active in men than in women [19-21].

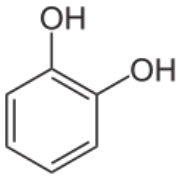


Figure 4. Catechol structure

Female $COMT^{-/-}$-mice develop preeclampsia [22]. Preeclampsia develops in 4-6% of all pregnancies and is characterized by hypertension, edema, and proteinuria after the 20th week of gestation [23-28]. The origin of the syndrome is not clearly defined, however impaired angiogenesis leads to hypoxia and inadequate fetal placentation. The immune system participates in this failed process. In the placenta of preeclamptic $COMT^{-/-}$ mice, uterine NK (uNK) cells – cells controlling neoangioegensis through the placenta – was dysregulated, resulting in an increased number of dead embryos compared to WT mice. When the $COMT^{-/-}$ mice were given 2me2, the number of dead embryos decreased and uNK numbers normalized. As the COMT enzyme participates in estradiol's metabolism, and they showed a dysfunctional immune system, we investigated how COMT-deletion would influence the general immune system in paper III.

1.1.4 2-methoxyestradiol

Endogenous 2me2 is formed during metabolism of E2. As E2, 2me2 can be found in both men and women, with the same principle metabolism pathways are followed [14, 29, 30]. Its physiological role is still unclear, however as stated previously, its levels has been found to be reduced in both mice and human preeclampsia [22, 31, 32]

2me2 gained interest due to its anticarcinogenic properties and possible cardiovascular benefits [33-35]. *In vitro*, 2me2 has a 500-fold lower affinity for ERα than E2 [36]. It has also shown antiproliferative, antiangiogenic, and

proapoptotic activity by binding the colchicine-binding site of tubulin leading to microtubule depolymerization, and downregulating transcription factors, including hypoxia inducible factor-1α (HIF-1α) [33, 37-40]. It has been tested in phase I and several clinical phase II studies of advanced stage cancers [41-45]. It showed amelioration of airway inflammation and RA models [46-50], and recently, amelioration of a multiple sclerosis model [51]. Some studies in inflammation focused on inhibiting angiogenesis [48, 49], but recently, more studies have been investigating the immunomodulatory role of 2me2 [51, 52]. In paper I, we investigated whether 2me2 could be beneficial in the treatment of postmenopausal arthritis, and in paper II we investigated the direct immunomodulatory properties of 2me2 and compared this to the established immunomodulatory effects of E2. We also investigated whether administration of 2me2 could lead to estrogen receptor signaling.

1.2 Autoimmune diseases

When the immune system reacts toward our own cells and tissues, the autoimmunity process has started (exemplified in Fig. 5). The breach of self-tolerance contributes to diseases such as rheumatoid arthritis (RA), celiac disease, systemic lupus erythematous (SLE), diabetes mellitus type 1, and many others. Activation of auto-reactive T cells, and B cells producing autoantibodies are hallmarks of autoimmunity. Autoimmune diseases are complex disorders of unknown etiology, involving genetic and environmental factors.

1.2.1 Rheumatoid arthritis

RA is an autoimmune, systemic, and inflammatory disorder, and if untreated, gravely disabling. The disease is characterized by morning stiffness, joint pain, and fatigue due to joint inflammation, articular destruction, bone loss, and generalized inflammation. 0.5-1% of the population develop RA, with 75% female predominance [53-57]. During recent years, research has unraveled parts of the disease pathology which has been translated into new therapies such as anti-TNF antibodies [58]. Unfortunately, not all patients respond to these new treatments, warranting further research. The pathogenesis of RA is complex, where several genetic, environmental-, and stochastic factors act to cause the pathological events. As new knowledge emerges, dividing RA patients into subsets potentially facilitates the choice of adequate treatments.

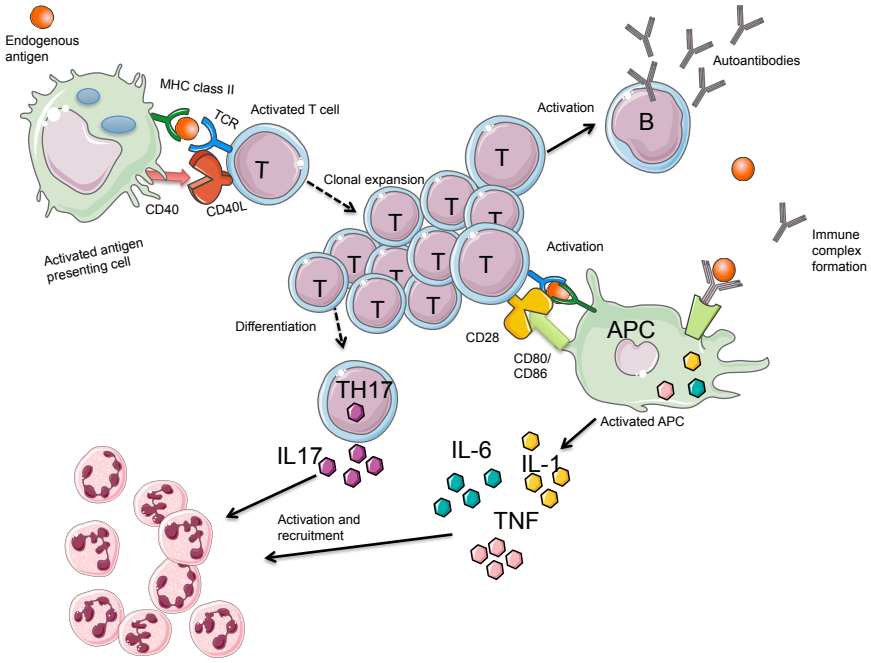


Figure 5. In autoimmunity such as RA, macrophages and other antigen presenting cells present auto-antigens to T cells. T cells are then activated and clonally expand. They can differentiate to T_H17 pro-inflammatory cells; they can activate B cells to produce autoantibodies towards the antigen; or activate other cytokine-producing cells.

The female incidence of RA coincides with the time after menopause, when estrogen levels decline [54, 57, 59]. In addition, 75 % of RA patients experience improvement during pregnancy with increased estrogen levels [60-62]. Estrogens' anti-arthritic effects are demonstrated in animal models [12, 63-67]. Clinical studies with estrogen-containing hormone replacement therapy (HRT) of postmenopausal RA patients however are inconclusive, but reduced disease activity has been reported [68, 69]. Long-term treatment with estrogens is not recommended due to side effects such as deep-vein thrombosis, and new therapies are needed [70].

1.3 The immune system

The immune system evolved in order to protect us against invading pathogens; the cells of the immune system recognize non-self structures such as pathogens and malignant cancer cells. The immune system can be divided into innate (i.e. naïve) and adaptive (i.e. acquired) immunity, where the innate immunity is traditionally viewed as the first line (hours) of defense

reacting in a non-specific manner, whereas the adaptive immunity is the second line (days) of defense, directed against specific pathogens and displays memory capacity [71]. More recently, with the discovery of the innate lymphoid cells − being of adaptive origin as lymphocytes but reacting in an "innate" fashion [72, 73] − and the discovery that innate cells show memory functions [74], the division between the innate and adaptive immunity has been debated.

Activation of the immune system can induce inflammation. Inflammation is traditionally defined from the Latin words calor, dolor, rubor, tumor, and functio laesa; meaning heat, pain, redness, swelling, and disturbance of function. These clinical signs reflect cytokines and immune cell activity in local blood vessels. Inflammation is a process common to many pathways activating the immune system, such as infections with bacteria and flares in rheumatoid arthritis. The underlying mechanisms and the degree of persistence can be very different, depending on type of trigger, for general activation see Fig. 1. The initiation of an immune response results in cell recruitment. Sentinel macrophages produce cytokines (small proteins involved in cell signaling) and chemokines (chemotactic cytokines), recruiting other inflammatory cells. The sentinels respond to signals they perceive as dangerous.

1.3.1 The innate immune system

The innate immune cells are the first cells at an infection site. The system discriminates "good" from "bad", generating a potent first line of defense against the invader. When a pathogen is recognized, neutrophils and monocytes patrolling the circulation are easily mobilized and can migrate and defend against the microbe. Macrophages and dendritic cells can also reside as specialized cells in tissues, first to recognize invading pathogens in tissues such as the skin, the mucosa, and the gut. The innate immune system reacts in the same way each time it encounters a certain microbe and its products, however different pathogens leads to different signals and responses.

1.3.1.1 Neutrophils

Neutrophils are the most frequent cell in the human circulation and their main task is to patrol the body searching for pathogens [75] They are recruited to inflammatory sites where they phagocytize and digest microbes. When a pathogen invades tissues, the neutrophil precedes monocytes/macrophages, releasing a wide range of toxic agents designed to kill the pathogens. While combating infections, it can also damage surrounding tissues.

1.3.1.2 Monocytes

Monocytes are circulating cells in the blood stream, giving rise to either macrophages or dendritic cells when entering tissues. They originate from the bone marrow, circulate for several days, and finally enter tissues to replenish macrophages/DC populations [76, 77]. These circulating cells are thus a heterogeneous population, constituting 5-10% of peripheral blood leukocytes in humans [78]. Pro-inflammatory, metabolic, and immune stimuli all elicit recruitment of monocytes into peripheral sites, where they differentiate and contribute to host defense, tissue remodeling, and repair [79, 80].

1.3.1.3 Dendritic cells

Two main type of dendritic cells aid in the interplay between the innate and the adaptive immune system: Classical dendritic cells (cDCs) are professional presenters of antigens that can activate the adaptive immune system. When immature, the phagocytic activity is high, while they as mature cells they produce massive amounts of cytokines. Plasmacytoid DCs differ from cDCs, being specialized responders to viral infections, producing massive amount of interferons (IFN) and are long lived [81, 82]. Both DC-types originate from the bone marrow, circulate as monocytes, and while they are rare in mouse circulation, in humans, they are present to a substantial extent [83, 84]. DCs migrate easily from tissue to lymph nodes to be able to present antigens and activate B and T cells. CDCs regulate T cell responses both during an infection and at steady state.

1.3.1.4 Macrophages

Macrophages have diverse roles in the immune defense; both killing microbes and directing the subsequent immune response [78, 81, 85]. They are strategically located throughout the body, ingesting and processing foreign materials, dead cells, and debris, and recruiting additional macrophages in response to inflammatory signals. They are highly plastic, rapidly changing their function in response to local signals as the situation requires.

They reside as phagocytic cells in both lymphoid and non-lymphoid tissues, involved in tissue homeostasis by clearing apoptotic cells [79]. Just as DCs, they can process and present peptides for activating the adaptive immune system, however, they are in addition equipped with a broad range of pattern recognition receptors, able to efficiently induce an inflammatory response [86]. They direct this subsequent response by producing cytokines and chemokines.

As resident macrophages, they themselves respond to environmental signals such as cytokines, apoptotic cells, and infections, changing their physiology in response to these factors. They are highly plastic, participating in homeostatic processes such as tissue remodeling and wound healing, as well as in host defense by changing their physiology. Each of these different macrophages can be potentially dangerous if not appropriately regulated. Macrophage modulation is thus important in general inflammation and diseases such as RA, as they decide the fate of tissue destruction, infection, and inflammation resolution. As the subsets exist on a continuum, few known marker combinations can definitively segregate subtypes of macrophages, and it is also difficult to dissect macrophages from dendritic cells, as these cells also overlap in expression of receptors [80, 87]. Macrophages have been classified along the T-cell literature, viewed on a linear scale, where M1 macrophages represent one extreme and M2 macrophages represent the other. The M1 macrophages were reserved for classically activated macrophages, and M2 designated alternatively activated macrophages. The M2 designation has however expanded to include all other types of macrophages, and the M1/M2 classification scheme has become insufficient [88]. Unfortunately, no consensus has been reached regarding newer classifications.

1.3.2 Sensing and initiating an immune defense

Immune cells recognize both exogenous and endogenous signals by a variety of different receptors, either through pathogen-associated molecular pattern receptors (PAMPs-exogenous signals), or danger-associated molecular pattern receptors (DAMPs-endogenous signals) [89]. PAMP receptors recognizing invading pathogens include toll-like receptors (TLRs). TLRs recognize well-preserved patterns, where lipopolysaccharide (LPS) − a component of the outer membrane of gram-negative bacteria − is one of the most potent inflammatory agents. When a macrophage recognizes LPS through TLR4 [90], cell functions such as motility, morphology, and synthesis of inflammatory mediators change. The produced cytokines can orchestrate the appropriate inflammatory and acquired immune defense, and can also initiate much of the pathology of a disease. These signals include tumor necrosis factor α (TNFα), interleukin-1 (IL1), and IL-6. Estrogen has been shown to regulate TLR4 signaling, pro-inflammation or anti-inflammatory signals depend on length of treatment and model system [91-94].

1.3.3 Reactive oxygen species

Reactive oxygen species (ROS) are chemical compounds containing oxygen with a single unpaired electron in the outer orbital. They are short-lived and

highly electrophilic molecules, helping the immune system as a tool for killing microbes, Fig. 6. ROS produced for killing in a controlled manner by the nicotinamide adenine dinucleotide phosphate (NADPH) complex are confined to the phagolysosome. The NADPH oxidase (NOX; see below) can also produce extracellular ROS, not only killing bacteria but also damaging tissues. ROS are not only produced from NOX, but also as byproducts from the respiratory chain in mitochondria and during the metabolism of compounds such as estrogen. NOX-dependent ROS production has recently shown physiological functions in a variety of cells and inflammatory conditions [95, 96].

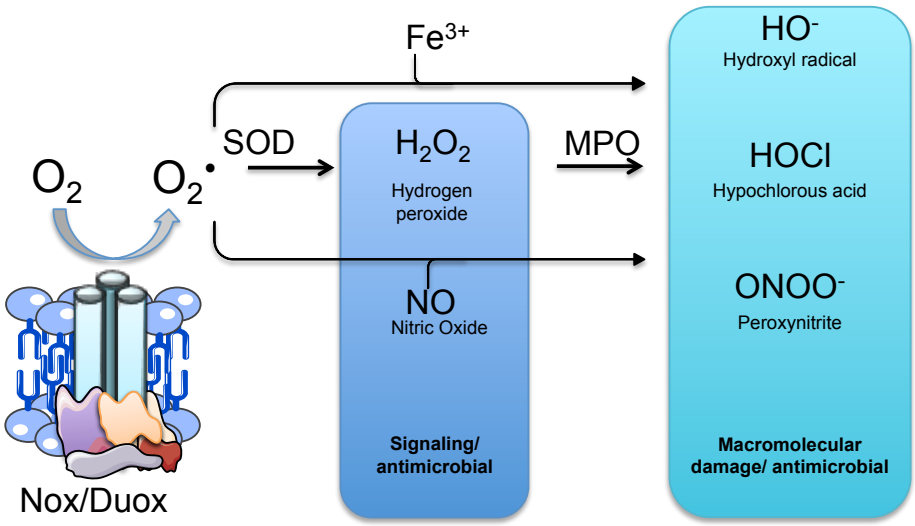


Figure 6. Formation of reactive oxygen intermediates and species. Adopted from Lambeth [96].

1.3.3.1 Defense against pathogens

The term oxidative stress refers to an overproduction of ROS. ROS include both radical and non-radical forms, where some species are highly oxidizing starting chain reactions; some mildly oxidative; and some can even acts as reductants (Table 1).

Table 1. Different biological situations create different mixtures of ROS, with its reactivity towards different biomolecules sometimes acting deleterious. Peroxidation of unsaturated lipids for example, can alter membrane structures and cell permeability leading to immune system recognition. It can also cause DNA strand breaks resulting in mutagenesis.

Molecular species	Properties	Molecular reactivity
Superoxide (O_2^-)	Weak oxidant, Weak reductant, Membrane impermeant	• Iron sulfur centers • Reacts with NO to form HONOO • Reacts with H_2O_2 to form OH
Hydrogen Peroxide (H_2O_2)	Moderate oxidant Membrane permeable	• Proteins with low –pKa cysteine residues • Reacts with CL^- to form HOCL (catalyzed by myeloperoxidase) • Peroxidases, unsaturated lipis
Hydroxyl radical (OH^-)	Highly reactive Produces secondary radicals	Protein, DNA, lipids
Peroxynitrite ($ONOO^-$)	Highly reactive Produces secondary radicals	Protein, DNA, lipids
Hypochlorous acid (HOCl)	Highly reactive Produced in areas of inflammation by reaction of CL^- and H_2O_2 with myeloperoxidase	Chlorination of tyrosine Oxidation of thiols, methionine, amines

1.3.3.2 Signaling messengers

Low amounts of ROS are produced in response to a variety of cellular signals such as cytokines, G-protein coupled receptors, and growth factors [97]. Both hydrogen peroxide and superoxide participate in cellular signaling and transcriptional regulation [98]. Proteins containing cysteine with a low pKa

are direct targets of oxidation, where the cysteine residues usually are located at the enzyme's active sites [99]. ERK1, ERK2, c-Jun N-terminal kinase, NF-κB, focal adhesion kinase, AP-1, Akt, Ras, Rac, and JAK-STAT are all targets for ROS regulation. Like phosphorylation, oxidation is reversible, often involving thioredoxin or glutathione, enabling system control.

ROS can also disturb cell signals by forming peroxinitrite with nitric oxide (NO), a highly oxidative molecule that cause molecular damage. This reaction affects the concentrations of NO, which is important in many different physiological processes such as vascular tone.

1.3.3.3 The NADPH oxidase

The seven different transmembrane NOX or dual oxidase (Duox) isoforms are professional ROS producers. They mediate diverse biological functions in different cell types. The phagocyte NOX 2 is the principal source of ROS generation in both activated neutrophils and macrophages. NOX 2 produce superoxide anions (see Fig. 7) that are further transformed into hydrogen peroxide and hydroxyl radicals (Fig. 6) [100].

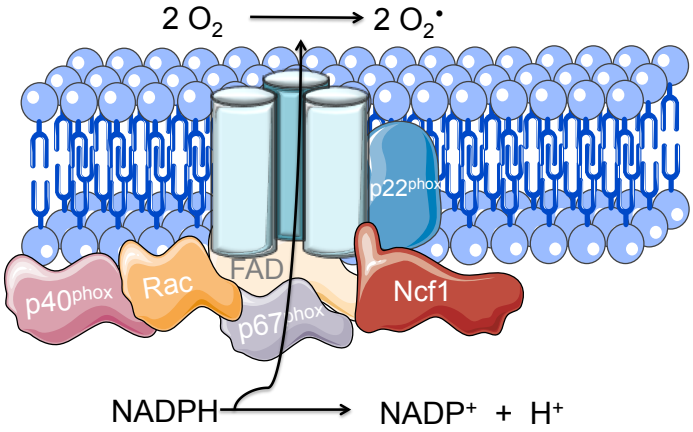


Figure 7. The NADPH oxidase 2 generates superoxide. The NADPH oxidases are electrogenic enzymes that can accept electrons from cytosolic NADPH, transport them through the membrane-embedded hemes, and donate single electrons to molecular oxygen.

NOX 2 consists of the membrane embedded cytochrome component with the catalytic subunit NOX 2 ($gp91^{phox}$) and $p22^{phox}$, as well as the cytoplasmic subunits $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and rac. The cytoplasmic subunits translocate to the membrane upon activation, enabling oxidation of NADPH to $NADP^+$. Electrons are then transported down a reducing potential gradient that terminates when oxygen accepts an electron and is converted to

superoxide anion. NOX 1, 2, 3 and 5 transport electrons across membranes reducing oxygen to superoxide [100]. NOX 4, Duox1, and Duox2 produce H_2O_2 directly. The different isoforms use different cytoplasmic subunits for their ROS production.

Activation of the NADPH oxidase in neutrophils and macrophages occurs after stimuli such as formylated peptides, opsonized particles, integrin-dependent adhesion, and ligation of PAMPs. The produced ROS levels in neutrophils are generally much higher than in other cells. The different NOX isoforms are found in many different cells that are not classical immunological cells such as endothelial and vascular smooth muscle cells, or adventitial fibroblasts. Defects in NOX 2 cause chronic granulomatous disease (CGD; [101]). The defects lead to an inadequate bacterial killing and excess inflammatory response to bacteria, fungi, and yeast infections. The patients often contract diseases such as pneumonia and infectious dermatitis CGD can result from recessive mutations in any of the five NOX 2 subunits. The most common form is the X-linked CGD, caused by mutations in $gp91^{phox}$ accounting for 70 % of CGD cases. The most common form of autosomal recessive CGD results from mutations in the regulatory $NCF1/p47^{phox}$ protein, accounting for 20-30% of CGD cases.

NCF1-deficiency manifests in mice both as increased susceptibility to spontaneous infections, and a more severe inflammatory phenotype in experimental models of autoimmune chronic inflammation, such as arthritis and experimental autoimmune encephalomyelitis, a model for multiple sclerosis [102]. In these models, the mice showed defect T cell-dependent autoimmune responses. It was further shown that macrophages, with the highest burst capacity among antigen presenting cells, and macrophage-derived ROS dictated T cell selection, maturation, and differentiation, and also suppressed T-cell activation, and thereby mediating protection against the autoimmune diseases [102-104]. In addition, it was also demonstrated that it was NOX 2 restricted to monocytes/macrophages that protected against infections [105]. Interestingly, female *Ncf1*-mutated mice spontaneously developed severe arthritis during the postpartum period. In paper IV and V, we further explored the relationship between ROS and estrogen receptor signaling.

1.3.4 Innate lymphoid type I cells: NK cells

All human cells express self-peptides on the major histocompatibility complex (MHC) class I, in order to show they belong to the "self". Stressed cells that do not express the self-antigens are detected by natural killer cells,

such as tumor cells or virally infected cells [106-108]. Target cells are killed after triggering a variety of activating and co-activating receptors [109]. Engagement of these receptors can also trigger cytokine and chemokine secretion, such as IFN-γ and TNF, contributing to directing other components of the immune system. NK cells can also produce IFN-γ upon stimulation by combinations of cytokines such as IL-2, IL-12, IL-15, and IL-18.

After the NK cell has recognized a target cell, a lytic immunological synapse forms between the cells specifically killing the target cell [110]. The major cytotoxic proteins contained within secretory lysosomes are granzymes and perforin. Perforin facilitates the entry of granzymes into the target cell cytoplasm, where they cleave caspases resulting in cell death. To ensure non-aberrant killing there are subsequent stages, as depicted in Fig. 8. An immunological synapse forms after recognizing a target cell; the lysosomes becomes polarized towards the synapse, the lysozymes move into close apposition with the plasma membrane; and finally, the secretory lysosomes fuse with the plasma membrane and release their cytotoxic content. On average, one NK cell can kill four target cells each, thereafter exhausting their granzyme B and perforin levels. [111]

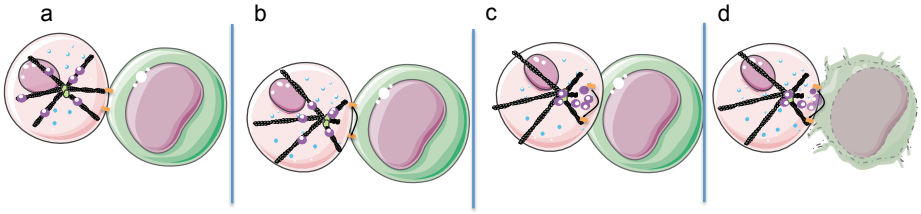


Figure 8. NK cell killing by secretory lysosyme exocytosis. (a) Stage 1: on recognition of a target cell, a lytic immunological synapse forms at contact point, and the cytoskeleton is reorganized. (b) Stage 2: secretory lysosomes polarize towards the lytic synapse. (c) Stage 3: secretory lysosomes move towards the plasma membrane. (d) Stage 4: the secretory lysosomes fuse with the plasma membrane, releasing their cytotoxic contents towards the target cell plasma membrane. Adopted from Topham et al *[110].*

In C57bl/6 mice, NK cells are defined as primarily being T cell receptor negative ($CD3^-$) and NK1.1-receptor positive. NK cell development is IL-15 dependent, and mice lacking IL-15 receptor lack NK cells. NK cell maturation involves sequential acquisition of multiple cell surface receptors such as NKG2A and Ly49 (recognizing MHC class I molecules), the TNF-receptor CD27, and a final maturation associated with increased expression of CD11b and CD43 [112, 113]. NK cell precursors originate from the bone marrow, mature, and migrate to different tissues, acquiring different effector

functions [114, 115]. Mature NK cells leave the bone marrow, migrate to the periphery and accumulate in the spleen, blood, lungs and lymph nodes before re-circulating through the bone marrow [112]. The unique microenvironments of each tissue influence the developmental program of NK cells, leading to heterogeneity and plasticity [116]. In the circulation and liver, they participate in immunosurveillance, whereas in the uterus; they play important roles in tissue remodeling and vascularization during pregnancy. The decidua and uterine NK cells have a unique functional profile, particularly with regards to chemokine and cytokine production.

NK cells stem from the common lymphoid progenitor cell also generating B-, and T cells but they are distinct from lymphoid cells, as they do not undergo gene rearrangements. Instead, they are defined as innate lymphoid cells, distinguished from other innate lymphoid cells (ILCs) by their cytotoxic capacity. In accordance to their lymphoid comrades, they can formulate antigen-specific immunological memory; and they are able expand, persists, and support strong secondary responses against previously encountered pathogens [117, 118]. NK cell dysfunction is implicated in many different diseases, including infection by viruses (especially the herpes virus family), autoimmunity, as well as reproductive failure [119-121]. They are also being exploited in cancer immunotherapies [122, 123]. In paper II, III, and in preliminary studies, we explored the regulation of NK cells by estrogens in both inflammatory and non-inflammatory settings.

1.3.5 The adaptive immune system

The adaptive immune system acts slower in response to different pathogens compared to the innate immune system. It takes several days to become active the first time it confronts a pathogen and then generates memory so next time it encounters the pathogen, it remembers and reacts faster and more powerful. The adaptive immune system consists of two parts: cell mediated immunity and humoral immunity. Humoral (antibody-mediated) is directed at extracellular microbes and antigens, and cell-mediated immunity is directed at intracellular microbes an antigens.

1.3.5.1 T cells

T cell progenitors originate in the bone marrow, but move at an early stage to the thymus for education. When the T cell arrives in the thymus, they express neither the T-cell receptor TCR nor the co-receptors CD4 and CD8, needed for immune-recognition. They are thus referred to as double-negative cells. Both negative and positive selection of T cells occur in the thymus, where the T cell is instructed not to react to self-antigens by thymic epithelial cells

(TECs) [124]. The selection is based on affinity by which the TCR can bind self-peptide/MHC complexes presented by the TEC. T cells that bind intermediately survive, whereas most of them will not recognize the self-peptide and will undergo apoptosis. Cells that have survived the thymic selection migrate to peripheral and secondary lymphoid organs such as spleen and lymph nodes. $CD8^+$ T cells participate in the killing of virally infected cells or cancer cells, and are referred to cytotoxic T cells. $CD4^+$ T cells recognize peptides presented on MHC class II, expressed on antigen presenting cells. CD4 cells are often called helper cells, as they can help other cells in the immune defense through producing various cytokines. The $CD4^+$ T cell can further develop into sub-populations, which is orchestrated by the cytokines in the microenvironment of the interaction. We have investigated T cells in arthritis, and how estrogens and estrogen metabolism can influence T-cell biology in papers I-III.

1.3.5.2 B cells

The main effector function of the B cell is to participate in the humoral defense by producing antibodies. These antibodies help to eliminate invading pathogens, for instance by enhancing targets for phagocytosis by macrophages. B cells are also potent antigen presenting cells, and they can produce many different cytokines. The B cell originates from the bone marrow, going through different developmental stages and then leaves in an immature state for further maturation in the spleen. Memory B cells, plasma cells, return later to reside in the bone marrow. In paper I we investigated B cell populations in different compartments, in paper II and III whether COMT deficiency or 2me2 influenced B-cell populations, and in paper IV whether B cells participate in ovx-induced bone loss.

1.4 Osteoimmunology

Osteoimmunology explores the reciprocal interactions between the immune and skeletal systems [125]. In autoimmune disorders such as RA, bone loss accompanies the inflammatory immune response. Clinical observations have described mechanisms by which immune cells and inflammatory cytokines drive bone catabolism (see Fig. 9). Bone degeneration is promoted by proinflammatory cytokines such as TNFα, acting via at least three different mechanisms: it can inhibit differentiation of osteoblasts; promote stromal cell expression of receptor activator of nuclear factor κB ligand (RANKL); and act directly on osteoclasts to potentiate their differentiation and increase their resorptive capacity [126]. Other cells involved in osteoimmunology are the T cells that are able to express RANKL. Lately, focus has been turned towards

the B cells, as they also have been found to be important in bone loss and can express RANKL [127, 128].

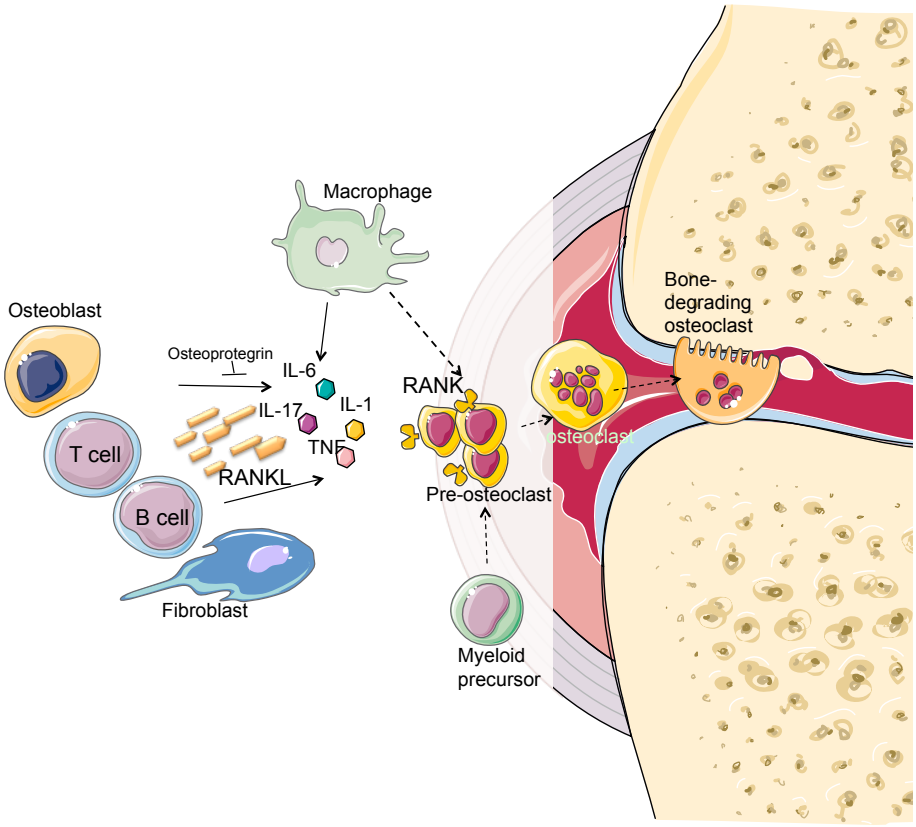


Figure 9. RANKL produced by osteoblasts and fibroblasts induce osteoclast-mediated bone loss. The process is further enhanced in inflammation by recruited immune cells producing amongst others, RANKL, IL-1, IL-17, and TNFα.

1.5 Bone

The skeleton supports the body by protecting inner organs, storing minerals (calcium and phosphate), and harboring hematopoiesis. It consists of inorganic matrix, hydroxyapatite, organic matrix, collagen I, osteocalcin, bone sialoprotein, and other bone proteins, but also bone cells: osteoblasts, osteocytes, and osteoclasts. These building blocks arrange themselves into two types of bone: trabecular (spongy/cancellous) bone and cortical bone (see Fig. 10). Trabecular bone comprises 20% of the total skeleton, but has 10 times the surface area compared to compact bone because of its porous appearance, enabling much more metabolic surface. The trabecular bone is

mainly found in the vertebrae, pelvis and in the ends of long bones. The cortical bone is predominantly found in the long bones of the extremities.

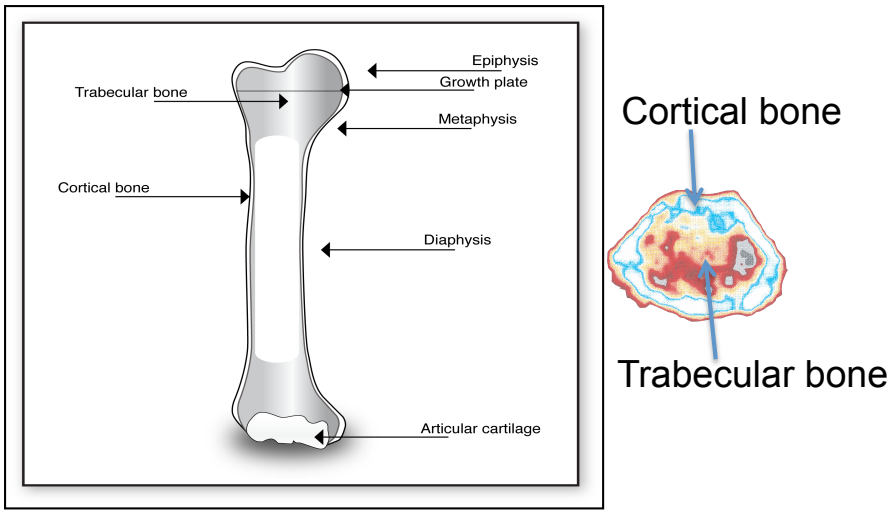


Figure 10. Schematic view of a longitudinal section through a long bone. The cortical bone parameters are measured in the diaphyseal section containing mainly cortical bone. The trabecular bone is measured in a metaphyseal section and defined by setting the inner threshold to 45% of the total area.

1.5.1 Bone cells

Osteoblasts originate from mesenchymal stem cells, and are responsible for bone formation, secreting bone matrix proteins including osteocalcin, collagen type I, and osteonectin. They are also responsible for the mineralization of the matrix, via alkaline phosphatase expressed on their surface. The matrix produced by the osteoblasts progressively hardens as calcium salts are deposited. Some osteoblasts become surrounded by the matrix and are trapped, subsequently developing into osteocytes. The osteocytes lie in lacunae, concentrically arranged around a Haversian canal with blood vessels, nerves and lymphatic tissue, communicating with each other and other cells via these canaliculi. They can sense loading of the bone, and are important in regulating bone remodeling, adjusting strength as appropriate.

Osteoclasts help in the remodeling process, as they are responsible for bone resorption. They develop from hematopoietic stem cells, the same route as macrophages. In the presence of macrophage-colony stimulating factor (M-CSF) and RANKL, pre-osteoclasts fuse to form multinucleated osteoclasts,

which can become activated, expressing tartrate-resistant acid phosphatase (TRAP) cathepsin K, and calcitonin receptors.

Biomarkers of the bone remodeling process can be measured in serum by osteocalcin (bone formation) and collagen type I (bone resorption) as they leak into serum during the process. These factors were measured in paper I.

1.5.2 Bone remodeling

Bone turnover in adult humans takes approximately 10 years. Bone-lining cells prepare the bone surface for degradation. Pre-osteoclasts are attracted to the site, fuse and mature into osteoclasts; attach to the bone with their ruffled border, seal off the area creating an acid microenvironment, ideal for bone resorption. Osteoblasts follow, producing new bone matrix to fill in the resulting gaps (Fig. 11). A whole bone turnover cycle takes 90 days and includes 10 days of resorption and 80 days of formation.

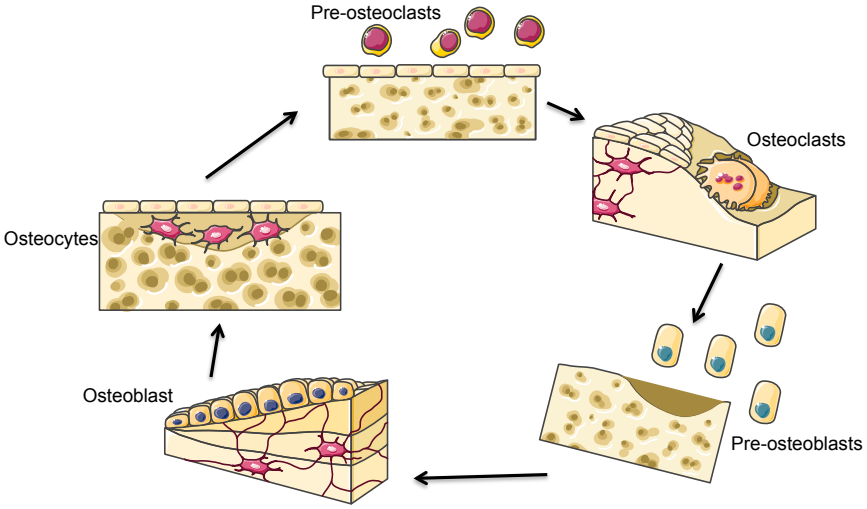


Figure 11. The bone remodeling cycle starts when pre-osteoclasts are recruited and differentiate into multinucleated osteoclasts in the presence of RANKL. RANKL binding its receptor RANK stimulates cell fusion and activates bone resorption. The osteoclast is then removed, followed by recruitment of pre-osteoblasts that differentiates into osteoblasts, subsequently forming bone. Some osteoblasts are trapped in the bone matrix and differentiate into osteocytes.

In adult individuals, bone resorption is balanced by bone formation, whereas in a growing individual there is a net increase in formation. A net decrease on the other hand, results in osteoporosis.

The rate of bone remodeling is controlled by several factors; loading, parathyroid hormone, sex steroid hormones, growth hormone, and different cytokines. Peak bone mass is reached around 30 years of age, albeit not uniformly at all skeletal sites; hip peak bone mass is reached earlier (20's) than spine (30's) [129, 130]. Men have a generally higher peak bone mass than females, and this difference persists as the bone mineral density (BMD) declines. After menopause when estrogen levels decline, all women experience a period of rapid bone loss, contributing to the development of osteoporosis.

1.5.3 Osteoporosis

Osteoporosis is characterized by a decrease in bone mass and density which can lead to an increased risk of fracture. Bone remodeling is essential for calcium homeostasis, however excessive bone resorption causes the bone loss observed in common diseases such as postmenopausal osteoporosis, rheumatoid arthritis and periodontitis. The prevalence of osteoporosis in Sweden is 2-3% among women in their 50's, and increases to approximately 50% in women over 80 [131]. General osteoporosis in RA is reported as over 50%, increasing the risk of fractures [132, 133]. The incidence is similar in the whole European Union. Age-related osteoporosis is due to decreased production of vitamin D, decreased uptake of calcium, and decreased concentrations of sex hormones and growth factors [134]. That estrogen deficiency contributes to bone loss has been known for over 70 years [135].

2 AIM

Rheumatoid arthritis is a systemic, inflammatory, and autoimmune disorder. Immunological research in the past decades has led to new biological agents targeting specific pathological molecules, however not all patients respond adequately to these treatments. In this thesis, the aim was to elucidate if 2me2 could be used as treatment for postmenopausal arthritis; if 2me2 modulates the immune system without estrogenic disadvantages; and to investigate the role of estradiol on the innate immune system.

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

I: To determine if 2me2 could ameliorate collagen induced arthritis and the associated bone loss.

II: To determine the immunomodulatory properties of 2me2 compared to those of E2.

III: To determine the role of the enzyme COMT for the development of the immune system.

IV: To investigate the role of reactive oxygen species in a model of postmenopausal bone loss.

V: To investigate the role of E2 and reactive oxygen species in initiating an immune response.

3 METHODOLOGICAL CONSIDERATIONS

The interaction between sex steroids and the immune system requires *in vivo* studies. The methods and materials employed in each study are described in detail in their respective papers. Here follows an overview of the most relevant aspects of methods in this thesis. The local ethics committee at University of Gothenburg approved all *in vivo* experiments.

3.1 Animal studies

3.1.1 Gonadectomy and hormone treatment

Ovariectomy (ovx) enables studies of sex-steroid deficiency in female mice, as the ovary is the only organ in female mice producing sex steroids. Estrogens from the ovaries induce uterus proliferation through ERα signaling [13], and measuring the uterus weight after ovx thus indicates a compounds ability to agonize ERα. All major characteristics of bone loss depending on deficiency of sex-steroids in humans can be mimicked in mice by gonadectomy [136, 137].

3.1.2 Hormone treatment

In this thesis, two different modes of administering substances have been used. Long-term treatments have been administered using subcutaneous slow release drug pellets. It minimizes handling the animals, as the pellets are implanted only once, and give a slow and continuous release of the drug. However, as unpublished data suggests, a more rapid release of the drug occurs during the first days after implantation before reaching steady state. This introduces the possibility of wrong dosage in short experiments, and the risk of insufficient drug amounts. To correct for these phenomena, we used 60-day pellets, ensuring sufficient amount of drug and sufficient amount of time to reach steady state (Papers I and II). As positive control, we used estradiol pellets, producing expected effects on arthritis amelioration and uteri growth, and as negative control we used placebo pellets.

Oil-based subcutaneous injections with the triglyceride Miglyol 812 was also used in these studies, as it is an inert, non-toxic oil dissolving estradiol. In the preparation of 2me2 – a drug with more hydrophobic properties than E2 based on the extra methyl group – both solubility and melting point (189-190°C) was accounted for when preparing the oil suspension, stirred on a

rotor with heating (60°C) for two hours. Before each administration, the suspension was mixed. The length of treatments for oil-based injections studies was between 3-5 days (Papers I and V).

3.1.3 Collagen-Induced Arthritis

The collagen-induced arthritis (CIA) model (Paper I) is the most widely used model, sharing pathology with human RA [138]. In the CIA model, mice are immunized with chicken type II collagen in an adjuvant (Freund's; Fig 12).

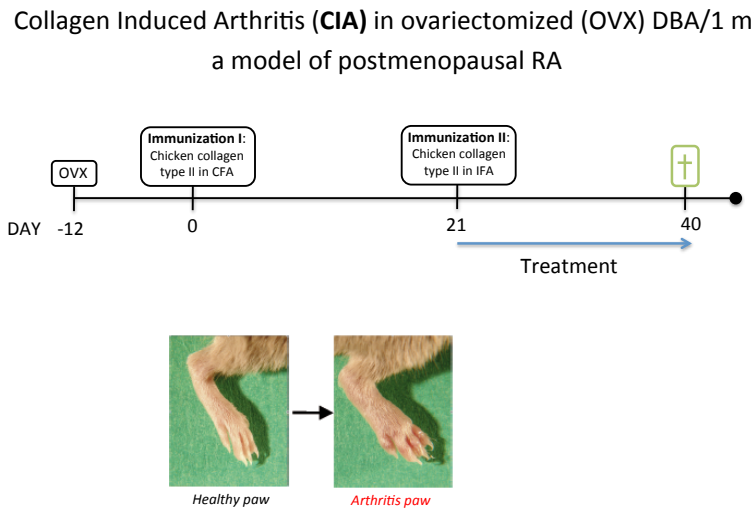


Figure 12. Time line of the collagen-induced arthritis model in ovx mice.

In both CIA and RA, APCs present peptides of collagen type II on MHC class II molecules breaching self-tolerance, activating the acquired immune system by inducing generation of autoantibodies toward cartilage in the joints. The pathological features of CIA include proliferative synovitis with infiltration of neutrophils and monocytes, pannus formation, cartilage degradation, bone erosions and fibrosis. In mice, disease development is restricted to the *H2q* haplotype of the MHC, found in e.g. the DBA/1 strain. Introducing ovx to CIA results in a model of postmenopausal RA, and in this thesis, it was used to evaluate 2me2 treatment (Paper I).

We have chosen to use the CIA model as mice develop a systemic polyarthritis. CIA in DBA/1 mice is not chronic, but the underlying mechanisms are similar to human RA. The disease progression was observed clinically and evaluated in a blinded fashion. The scale range from

2=swelling or erythema in 1 joint, 2= swelling in 2 joints, and 3=severe swelling of the entire paw or ankylosis. Even though this represents a rough grading system the effects of the treatments were clearly observed. The histological evaluation was also performed in a blinded fashion, using the entire paws and a 1-3 grading scale, separating synovitis from joint destruction.

CIA can also be induced in B10.Q mice, where they develop a chronic relapsing polyarthritis model most similar to human RA [102]. Another systemic arthritis model is the K/BxN model, where mice develop a robust spontaneous polyarthritis with synovitis and erosions at three weeks of age [139]. It is dependent on lymphocytes, but the mice have a reduced breeding capacity and are somewhat immune-compromised due to a limited diversity of the T-cell receptor. Passive transfer models are also available, with antibodies from both CIA and K/BxN mice that induce systemic arthritis in all recipient mice [139, 140]. The developed arthritis is only dependent on the response against the antibodies and leads to an acute systemic polyarthritis with both synovitis and erosions. Other systemic arthritis models include TNFα transgenic mice [141], adjuvant induced arthritis [142], septic arthritis [143], and monoarthritic models such as antigen-induced arthritis [144].

3.1.4 Air pouch model of inflammation

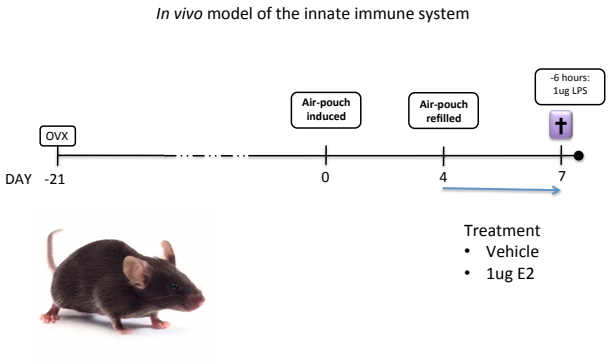


Figure 13. Time line of the air pouch model of inflammation.

The air pouch model (Paper V) allows quantification of leukocytes accumulating in the air pouch wall (tissues), cells migrating into the pouch (exudate), as well as characterization of chemokines and adhesion molecules responsible for diapedesis. Depending on stimuli and time point, different reactions can be studied. In paper V, we used the immune stimulant LPS, a

part of gram-negative bacteria, inducing an innate immune reaction, and a 6-hour time point (Fig. 13). By altering time points and stimuli, the model could be adapted to enable the study of other types of immune reactions. More relevant to the RA-disease progression would include stimulation with HMGB-1 or collagen type II, however this also requires adjusting the studied time points. The model was used to investigate how E2 influences the initiation of an innate immune defense.

2.1.5 Estrogen response element-luciferase coupled reporter mice

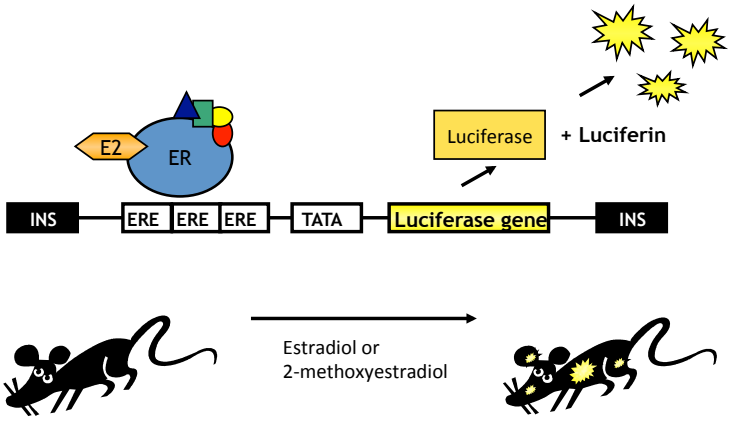


Figure 14. Scheme over the estrogen response element luciferase coupled reporter mouse.

Coupling the oxidative enzyme luciferase to different target genes enables bioluminescence technology to investigate transcription. Using luciferase coupled to ERE in mice enables investigation of *in vivo* signaling through the classical estrogen pathway, as it has a luciferase reporter gene under control of three consensus EREs coupled to a minimal TATA-box (Fig 14; [145]). Luciferase gene transcription starts when a ligand binds to ERs and activates the transcription complex. The amount of transcription can be estimated using an enzymatic reaction. These mice were used to determine the ability of 2me2 to activate the classical estrogen receptor signaling pathways in estrogen responsive tissues (Papers I and II).

3.2 Flow cytometry

Flow cytometry uses flow of cells, and laser excitation and emission of fluorophores to identify molecules and cells. Light scatter also enables discrimination between relative size and granularity of cells. The fluorophores can be conjugated to antibodies directed to surface epitopes of specific cells, or coupled to compounds that bind structures to identifying intracellular signaling pathways, intracellular cytokines, or free cytokines. In this thesis, flow cytometry was used for identifying cells (Papers I-V), surface antioxidant expression (Papers IV-V), reactive oxygen species (Paper IV), apoptosis and necrosis (Paper V), and measuring cytokines (Paper V). We used a FACS Canto II equipped with three lasers (red, blue, and violet) and eight filters. Seven different fluorophores were used as maximum in a combination. To ensure minimal spectral overlap, compensations were performed before each run. Single cell suspensions were prepared from isolated thymus, bone marrow, spleens, livers, lymph nodes and synovia. To avoid Fc-mediated adherence of antibodies, cells were blocked with anti-CD16/CD32 before staining. For analysis, fluorochrome-minus-one was used as controls, enabling a correct gating strategy.

3.3 Cellular functions

3.3.1 Dihydrorhodamine 123

DHR123 is converted to a green fluorescent compound after reaction with reactive oxygen intermediates (ROI; Paper IV). The fluorophore is stable for at least 30 minutes, which is good for analyzing multiple samples, however it does not identify a specific oxygen intermediate. The signal detected by DHR123 is due to an oxidation of the substrate, whereas other fluorophores detect substrate reductions, which might influence analysis. An advantage of this method is the simultaneous detection of ROI combined with the identification of specific cells using antibodies. It does not distinguish between intracellular and extracellular production, as it can diffuse between membranes.

3.3.2 Isoluminol-enhanced chemiluminescence

The isoluminol enhanced chemiluminescence technique was used to measure superoxide production (Papers I and III). Isoluminol is a chemical that exhibits chemiluminescence when mixed with an oxidizing agent, and is used as a substrate for extracellular reactive oxygen species. It reacts with oxygen species to produce an excited state intermediate that emits light upon

relaxation to the ground state. As it has a more hydrophilic profile than the most frequently used luminol, it does not transverse cell membranes and can only be excited by extracellularly produced ROS. The luminol enhanced assay works similarly; however it measures both extra-, and intracellular ROS production. The enzyme horseradish peroxidase catalyzes the reaction of ROS. The assay was performed in a six-channel Biolumat LB 9505 (Berthold Co.) A 500µl reaction mixture containing $5*10^5$ splenocytes, isoluminol, and horseradish peroxidase was put in 4 ml polypropylene tubes. The tubes were equilibrated for 10 minutes at 37°C before adding the antagonist: the hexapeptide Trp-Lys-Tyr-Met-Val-D-Met-NH2 (WKYMVm), and the light emission was recorded continuously. The peptide antagonizes formyl peptide receptors expressed on immune cells. It induces Ca^{2+} mobilization, superoxide production and chemotactic migration of monocytes and neutrophils. When measuring whole splenocyte suspensions (as in paper I), specific cells cannot be identified. The chemiluminescence method has great advantages; it is sensitive (as few as 250 neutrophils can be assayed), superoxide release over time can be analyzed, and it was found to specifically measure O_2^- [146]. In paper I, splenocytes from CIA mice was used to assess ROS production by isoluminol and WKYMVm.

3.3.3 The CytoTox Non-radioactive cytotoxicity assay

The golden standard for measuring NK cell cytotoxicity is a radioactive assay using ^{51}Cr. This assay has multiple disadvantages; low sensitivity; poor labeling; high spontaneous isotope release, it is bio hazardous, and there are issues with disposability. We set up another assay based on the same principle as the ^{51}Cr, however eliminating first and foremost the biohazard issues (Papers II and III). The CytoTox 96 Non-Radioactive Cytotoxicity Assay from Promega is a colorimetric assay, quantitatively measuring lactate dehydrogenase (LDH) − a stable cytosolic enzyme released upon cell lysis. This is measured by the conversion of a tetrazolium salt into a red formazan product. The amount of color formed is proportional to the number of lysed cells and can be read by a spectrophotometer. As controls, spontaneous release of LDH from pure NK cell, and Yac-1 cell cultures were deducted.

Other cytotoxicity assays can be flow-cytometry based, with the advantage of simultaneous cell identification and purification. However, multiple issues were encountered during method setup, such as poor labeling of target cells and fluorochrome spectral overlap.

In this assay, NK cells were first purified by negative selection using MACS beads, and co-cultured with YAC-1 cells for 4 hours. Negative selection does not provide the best purity, however, positive selection impairs NK cell cytotoxic functions, and is thus unsuitable for these types of assays.

3.3.4 Bone marrow-derived osteoclast formtion

The OCL is the cell responsible for degrading bone. These cells can be induced *in vitro* by culturing bone marrow cells, driving them first into macrophages by M-CSF, and then into OCL by RANKL. In paper IV, this method was used for evaluating the effects of the NCF1-complex on osteoclast formation. Mice were either sham or ovx-operated, and left for four weeks. Bone marrow derived cells were cultured on plates for two days with M-CSF and OCL were induced after another three days with RANKL. To identify OCL from the cultures, cells were stained for the enzyme TRAP, and counted as multinucleated cells with >3 cell nuclei. Both the number of weeks after ovx, and length of cell culture introduce time-dependent elements for the ability to form OCL, and in this assay they are not functionally tested (Paper IV).

3.3.5 [^{3}H]-Thymidine proliferation assay

The lectin concanavalin A (Con A) acts as a T-cell mitogen, stimulating energy metabolism [147]. The ability of T cells to proliferate was measured by stimulation of Con A and incorporation of ^3H-thymidine (Paper III). After 48h of stimulation, the radiolabeled nucleoside ^{3}H-thymidine was added to the cultures, and cells were left until next day. Cells were aspired, lysed by dH_2O, and further aspired through a 1.5 µm pore sized filter paper, enabling intact DNA fix on the filter. Radioactivity was measured by a liquid scintillation counter measuring β- radiation, where the amount of radioactivity corresponds to number of cells per well. Each assay was performed in triplicates, and radiation from non-stimulated samples was deducted. The Con A stimulation mostly stimulates T cells, however when culturing whole cell suspensions, there is always a possibility of inducing proliferation of other cells.

3.3.6 Enzyme-linked immunosorbent spot assay

The ELISPOT assay is a modified version of the ELISA assay (see below), enumerating cells secreting antigen-specific antibodies or cytokines. Cells were put into wells coated with a specific capture antibody, capturing secreted proteins during incubation. A biotinylated antibody detects the secreted proteins, the streptavidin conjugate visualizes proteins, spots are

developed, and one spot corresponds to one cell. The technique is easy to perform; however spots are manually counted, and therefore reproducibility is difficult to ensure. In this thesis, this technique was used together with flow cytometry data, enumerating B cells producing different classes of immunoglobulins (Paper III).

3.4 Cellular phenotypes and mechanisms

3.4.1 Histology and Immunohistochemistry

Histology is the study of cell and tissue anatomy, not using cell specific dyes. Immunohistochemistry instead uses antibodies to identify specific cells in tissues. Tissues and cells are usually sectioned before staining, followed by examination under a light microscope or fluorescent microscope. A confocal microscope is a fluorescent microscope that uses point illumination and a spatial pinhole to eliminate out-of-focus light in specimens that are thicker than the focal plane. This reduces the need for sectioning tissues and cells, and can enable a 3D image or analysis of a cell. In this thesis, histology was used to visualize pathology of joints in paper I, and NK cell cytotoxicity in paper II. In paper I, the dye hematoxylin with affinity for nucleic acids stained the nuclei; and eosin stained the cytoplasm pink. Tissue sections were then scored for synovitis and erosions. In paper II, we used fluorescent probes functioning the same way as described in flow cytometry to visualize cells. Cells from the cytotoxicity assay were stained with biotin anti-mouse NK 1.1 antibodies and streptavidin Alexa Fluor 488 to identify NK cells, and other cells were only stained with the nuclear dye DAPI. As controls, cells were stained with only the secondary antibody and DAPI, ensuring that the secondary antibody in itself did not stain cells. The cells were analyzed using an LSM confocal microscope and a 40x objective.

3.4.2 Peripheral quantitative computed tomography

Bone compartments in both humans and animals can be measured using peripheral quantitative computed tomography (pQCT). A rotating x-ray device provides a three dimensional measurement of the bone. The classical pQCT measurement of cortical thickness is done in the mid-diaphyseal section of the long bones, and the trabecular bone mineral density of the femur is measured in the metaphyseal section of the inner 45% of the total area of long bones, see Fig. 10. In this thesis, the Stratec pQCT XCT Research M was used, specifically modified for use on small bone specimens (version 5.4B; resolution 70 μm), with an interassay variation coefficient of

less than 2% (Papers I and IV). The resolution of the pQCT technique is enough to quantify total bone mineral density and trabecular bone mineral density, however cortical bone mineral density cannot be measured by this technique. At a cortical thickness of 200 μm, a pQCT resolution of 70 μm clearly influences the result (a phenomenon called partial volume effect). Instead, a resolution of 5μm using μCT should be used when measuring cortical bone mineral density. When studying the whole bone however, this partial volume effect is neglectable.

3.4.3 Enzyme-linked immunosorbent assay (ELISA)

The ELISA technique measures proteins in serum and supernatants from cell cultures. It is performed as the ELISPOT technique, with the difference that concentrations instead of specific cells are measured. In this thesis, we used commercially available kits for measuring type I collagen fibers from Nordic Bioscience (Paper I), cartilage oligomeric matrix proteins from AnaMar (Paper I), IL-1β from Biolegend (Paper V), and an in-house ELISA system for detecting anti-collagen type II antibodies (Paper I). An issue with the method is its sensitivity.

3.5 Statistics and calculations

In paper I and III, statistical evaluations were performed using GraphPad Prism version 5.0b. In paper III, the nonparametric Mann–Whitney test was used for statistical pair-wise comparisons between WT and $COMT^{-/-}$-female and male mice respectively. The nonparametric methods are distribution-free methods, requiring no or very limited assumptions to be made about the format of the data. They are useful for dealing with unexpected, outlying observations that might be problematic with a parametric method. Lacking power is the main disadvantage of the nonparametric methods. This is particularly true for small sample sizes, or if the assumptions for the corresponding parametric methods hold (e.g. normality of the data) [148]. Nonparametric methods are more geared toward hypothesis testing instead of estimation of effects. In addition, tied values can be problematic in nonparametric tests. Data from luciferase reporter mice are not normally distributed and thus require nonparametric statistical calculations. In paper I, a 2-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test was used for repeated measurements (arthritis incidence and severity and histopathological index). Data generated from such evaluations are based on ordinal scales, and these data should have been analyzed by nonparametric methods [148].

In papers II, IV, and V, parametric tests were used to compare groups using IBM SPSS Statistics software 22.0.0.0 and GraphPad Prism version 6.0c. Student's t-test compared two independent groups. If Levene's test suggested unequal variances between the groups, Welch's t-test was used instead. When three or more independent groups were compared, one-way ANOVA with Tukey's post hoc test was used. If Levene's test suggested unequal variances between the groups, Dunnett's T3 post-hoc test was used instead. Logarithmic transformations were used to ensure normal distribution of data when appropriate. When adjustments for covariates were needed, i.e. when two experiments were pooled, ANCOVA was used.

The unpaired parametric tests assume that the data from the samples are both normally distributed, and that the variances from the samples are approximately equal [149]. Formal statistical tests examine whether a set of data are normal, or whether the two SDs (or, equivalently, two variances) are equal. In papers II, IV, and V, Levene's test of unequal variances was used. Results from these tests should always be interpreted in the context of sample size, and for small sample sizes, normality and assumptions can be assessed by looking at normality plots of the residuals between observed and fitted values. If the assumptions are not met, it may be possible to transform the data, such as by logarithmic transformations used in paper II, IV, and V.

More statistically advanced calculations were learned over time, and thus employed as appropriate. All analyses were two-tailed, and p-values < 0.05 were considered statistically significant.

4 RESULTS

Below is a brief description of the main results and conclusions from each paper included in the thesis.

4.1 Paper I

Role of 2-methoxyestradiol as inhibitor of arthritis and osteoporosis in a model of postmenopausal rheumatoid arthritis

In this paper, we evaluated 2me2 and E2 treatment in experimental postmenopausal arthritis. Female mice were ovariectomized and subjected to collagen-induced arthritis. At time of booster dose, mice were treated with estradiol, a low (6.6 µg/day), or a high (66.6 µg/day) dose of 2me2, and placebo either for the duration of arthritis, or during three days in a short-term study. Arthritis development was monitored in the long-term study during the three weeks treatment.

E2 and both doses 2me2 ameliorated clinical and histopathological arthritis, and preserved bone mineral density after therapeutic administration. E2 was more potent than 2me2 in ameliorating the disease, measured clinically by reduced incidence and severity, and by histology for reduced synovitis and erosivity. E2 treatment also resulted in higher trabecular bone mineral density compared to 2me2.

Locally in the joints, E2 reduced neutrophils already early in the disease progression, which was reflected by delayed clinical onset of the disease. After three weeks treatment, the two treatments did not affect the same leukocyte populations in different tissues. In the spleen, E2 expanded the macrophage population whereas 2me2 expanded T cells; both diminished neutrophils. In the lymph nodes E2 expanded the macrophage and B cell population, while 2me2 expanded T cells and diminished macrophages. In the joints, E2 diminished neutrophils and 2me2 diminished macrophages. Both treatments reduced extracellular ROS.

We also found a dose dependent increase of uteri weight after three weeks of treatment with 2me2, indicating that administration of 2me2 activates estrogen receptors. The highest dose of 2me2 induced uteri weight to the same extent as E2, but not the low dose of 2me2. We investigated whether 2me2 signals through ERE in the uterus using luciferase mice, and found that

low dose 2me2 did not induce ERE activation after 4 hours.

We conclude that 2me2 exerts positive effects on arthritis and bone parameters. We cannot exclude that administration of 2me2 leads to ER-mediated effects.

4.2 Paper II

Immunomodulation by the estrogen metabolite 2-methoxyestradiol

In this paper, we investigated if 2me2 could regulate the immune system and its possible ER-mediated effects. Female mice were ovx or sham operated, and treated for three weeks with two doses of 2me2, E2, or vehicle.

We found a dose-dependent estrogenic effect after 2me2 administration: the higher the dose of 2me2, the more E2-like effects both on the immune system and on reproductive organs. However, we also demonstrate that the lower dose 2me2 induce different immunomodulatory effects compared to E2:

- E2 and the high dose 2me2 diminished B cells in the bone marrow and spleen, but not the low dose 2me2.
- T cells expanded in the bone marrow and liver by both estrogens, but only the low dose 2me2 expanded T cells in the spleen.
- Both hormones expanded NK cells in the bone marrow, but only the low dose of 2me2 expanded the NK cells in spleen and liver.

As in paper I, we found that both doses 2me2 induced uterus proliferation in ovx mice. The low dose 2me2 induced uteri proliferation to similar weights as sham-operated animals, and the high dose to similar weights as E2. We further investigated its competitive antagonistic effects on ERs in the presence of endogenous estrogens, but found no reduction of uteri growth. In ERE-luciferase coupled mice, the high dose 2me2 induced estrogen receptor signaling in bone marrow, spleen and liver, and the low dose induced signaling only in bone marrow.

We conclude that 2me2 has immunomodulatory properties; however the higher doses reproduce effects similar to that of E2. 2me2 can activate estrogen receptor signaling, dependent on tissue and dose administered.

4.3 Paper III

Sexual dimorphisms in the immune system of catechol-*O*-methyltransferase knockout mice

COMT is part of the metabolism by which E2 is converted to 2me2. In this paper, we investigated the impact of COMT deficiency on the development and function of the immune system in both male and female mice.

Males lacking COMT were more affected than females: splenic T-, and B cells expanded, but the T-cell proliferative capacity was reduced. Immunoglobulin production and NK cell cytotoxicity was unaffected, but the liver NK cell population decreased. In females, the neutrophil population expanded in the bone marrow, but the extracellular ROS production was not affected. In female COMT deficient mice, we found an increase in uterine weight, indicating an altered estrogen metabolism.

We conclude that COMT deficiency has minor impact on the development and function of the resting immune system. In females, estrogen metabolism seems to be affected.

4.4 Paper IV

NADPH oxidase 2 influences osteoclast formation but is not critical for ovariectomy-induced bone loss

In this study, we investigated the regulatory role of NCF1 and NOX 2 in ovx-induced bone loss. Female B10.Q WT or B10.Q.Ncf1$^{*/*}$-mice were ovx and bone mineral density was investigated after two and after four weeks.

In both WT mice and ROS-deficient mice, ovx induced equal reduction in uterus weight. We found that ovx induces more osteoclastogenesis in WT mice compared to sham-operated mice, however ovx did not induce osteoclastogenesis after sham-operation in B10.Q.Ncf1*/* mice. The reduced capacity to generate the bone-degrading cells did not affect the trabecular bone mineral density, neither after two weeks nor after four weeks. Immunologically, we show in both genotypes similar B-cell population expansion after ovx, similar activation of DCs by CD80^{+} expression, and similar T-cell activation by CD69^{+} expression.

We conclude that ROS from the NCF1 complex is needed for the formation of osteoclasts after ovx *in vitro*, however reduced ROS production from the NCF1-complex does not affect the general bone loss seen after ovx *in vivo*.

4.5 Paper V

Role of estrogen in regulating LPS-induced inflammation in NADPH oxidase 2 deficient mice

In paper V, we investigated whether E2 and reactive oxygen species from the NCF1-dependent NOX 2 complex modulates the initiation of an immune response. Female B10.Q WT or B10.Q.Ncf1$^{*/*}$-mice were ovx or sham-operated, an air pouch was induced, and mice were treated with E2 or vehicle for three days before LPS was injected into the pouch. Six hours later cells from the pouch were purified and analyzed.

In sham-operated animals, we found that B10.Q and B10.Q.Ncf1$^{*/*}$-mice react equally to LPS. LPS induced more neutrophil and macrophage infiltration into the pouch.

In ovx mice, we found that treatment with E2 in WT mice reduced neutrophil infiltration and induced macrophage infiltration compared to vehicle. We detected an increase in percentage of CD200R^{+}-cells, and a decrease in Tim4^{+} and MHC class II^{+} cells. In B10Q.Ncf1$^{*/*}$-mice lacking ROS, E2 treatment induced neutrophil infiltration and reduced macrophage infiltration, and also induced Tim4^{+} macrophages. Measuring cytokines, we found that E2-treatment in B10Q.Ncf1$^{*/*}$-mice induced the production of pro-inflammatory cytokines such as MCP-1 and IL-6, but also IL-10. In B10.Q WT mice, E2 treatment instead reduced MCP-1 levels in the pouch.

We conclude that signaling through E2 together with ROS from the NOX 2-complex modulates the initiation of an immune defense.

5 DISCUSSION

It has long been noted that women respond more actively in both humoral and cell-mediated immune responses than men [150-152]. This dimorphism is possibly also the cause of the increased frequency of autoimmune diseases reported in females [94, 153, 154]. This thesis addresses the mechanisms of estrogens' immune regulation.

5.1 Estradiol, 2-methoxyestradiol, COMT and the immune system

We originally became interested in COMT and 2me2, as COMT deficiency resulted in preeclampsia [22]. In preeclampsia, specialized uterine NK cells (uNK) govern neoangiogenesis at the placental/decidual interface, and in $COMT^{-/-}$ mice, abnormally high numbers of uNK cells invaded the decidua. Angiogenesis was dysfunctional resulting in embryonic death, which indicates dysfunctional uNK cells. In parallel, chronic granulomatous disease (CGD) patients with dysfunctional ROS defense systems respond to an infection with increased leukocyte numbers into affected tissues. $COMT^{-/-}$-male mice showed an increased number of T cells with an impaired function (Paper III, figure 2). In paper III, we also investigated general NK cells. Neither numbers nor cytotoxic capacity was affected in splenic NK cells. The liver NK cell population decreased. In cancer, both cytotoxic NK cells and cytotoxic T cells can be considered malfunctioning and not recognizing malignant cells. Further, the Val158Met polymorphism in the COMT gene is associated with increased human cancers [155-157]. No mechanisms involving the immune system have been suggested. In paper III, we excluded that COMT regulates NK-cell cytotoxicity, and uNK cells might represent such a diverse NK cell species that other NK cells are able to compensate for the loss of COMT in normal situations. In paper III, we did not measure serum levels of neither catecholamines nor catechol estrogens, and we cannot confirm which catechol-deficiency contributes the most to the results in paper III. The enzyme COMT is the only contributor to the formation of 2me2 *in vivo*. UNK cells are only present in pregnancy, when high levels of 2me2 are needed, and there might be a role for COMT's NK cell regulation in a provoked situation such as a viral infection. After 2me2 reconstitution in $COMT^{-/-}$ female mice, uterine NK cell numbers and embryonic death normalized [22]. This either suggests that 2me2 regulate the neoangiogeneis process in preeclampsia, possibly by regulating HIF-1α, or that 2me2

regulate NK cells. In paper II, we could show that both NK cells and T-cell populations were expanded by the lower dose of 2me2.

5.1.1 A role for 2me2 in regulating cell signaling

Estrogens are metabolized by the P450 enzymes – expressed in the mammary gland, uterus, brain, and other target tissues – resulting in hydroxylated estrogens [158]. When estrogen metabolites are formed at or near the ERs, they may either inactivate or alter ER-signaling, or act on their own biological targets. Consequently, some of the many actions attributed to E2 may not be caused by E2 *per se*, but might result from active metabolites. Estrogens potently activate mitogenic actions in hormone-sensitive tissues such as the uterus and breast. Excessive mitogenic stimulation has long been considered important in estrogen-associated cancers. This has not only been attributed to E2 signaling, and both the hydroxylated metabolites 2-hydroxyestradiol and 4-hydroxyestradiol are implicated in tumorogenesis. They can increase cell proliferation and form reactive oxygen species inducing DNA mutations [159, 160]. 2me2 formed downstream of 2-hydroxyestradiol might has evolved as a natural "break", as it induces apoptosis in transformed-, and tumor cells, exhibiting an antiproliferative effect on tumor growth [33, 34].

5.1.2 2me2 administration induces estrogen receptor signaling

Estrogens are eliminated from the body by conversion into more water-soluble metabolites. 2me2's chemical structure is very similar to E2's, however, the properties of this extra methoxy-group both renders 2me2 more water soluble, as well as providing a steric hindrance for antagonizing ERs. 2me2 was reported *in vitro* not to bind ERs, however some *in vivo* data indicates otherwise [65, 161, 162]. Many selective estrogen receptor modulators induce ER-signals even though they do not fit perfectly in the ligand-binding domain of the receptor [3, 163]

The beneficial effects of estradiol on arthritis and bone are mediated by signaling via ERα [12, 13]. In paper I, we investigated whether ER signaling was induced also after 2me2 administration. Employing ERE-coupled luciferase mice, we administered 2me2 and analyzed signaling after 4 hours. The half-life of 2me2 is 20 minutes (whereas E2's half-life is between 1-2 hours), and the 4-hour time-point was chosen in order to detect whether the metabolite itself could activate ERE elements, or if the effects we detected was instead caused by a possible conversion back into E2. In paper I, we did not detect an activation of ERE's in uteri by the low 2me2 dose at this time-

point, indicating that it is not through activating ERE's that 2me2 mediates its effects. In paper II, we continued to investigate whether this was a tissue-dependent effect by investigating ERE signaling in other estrogen-sensitive tissues. Low dose of 2me2 activated EREs in the bone marrow, but not in the spleen and liver. This difference could be dependent on local availability of enzymes such as COMT, hydroxylases or demethylases. The ER antagonist ICI 182,780 blocks ER signaling, and simultaneous administrations of both ICI and 2me2 would be one way to delineate a possible 2me2 mediated ER signaling effect. Simultaneous administration of ICI and 2me2 to a macrophage cell line revealed no estrogenic effects, indicating that in macrophages, 2me2 does not act through ER signaling [52].

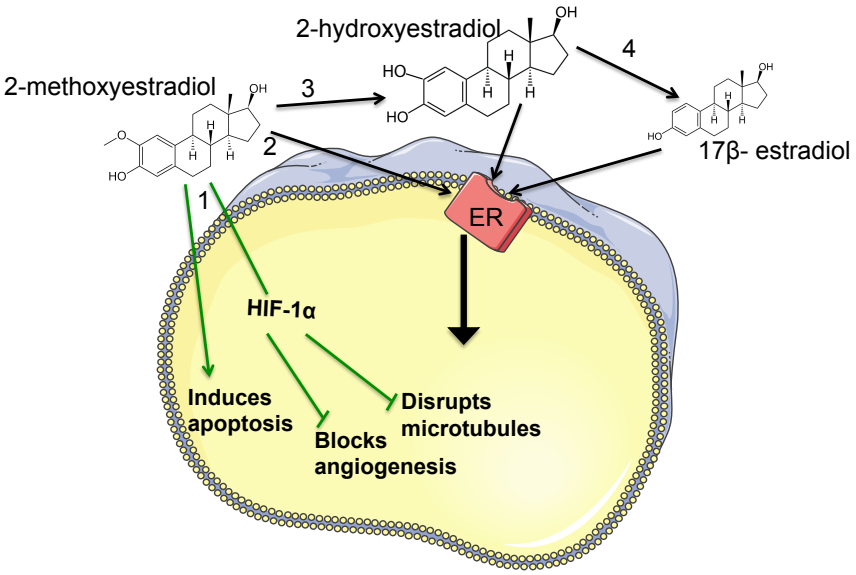


Figure 15. Possible signaling by 2me2. 1: Green arrows represent pathways previously reported. Black arrows represent possible other pathways affected by 2me2: 2: ER-activation 3: demethylation into 2-hydroxyestradiol and subsequent transformaton into 17β-estradiol (4) both with high affinity for the ERs.

Another way to investigate 2me2's possible ER-signaling would be to administer 2me2 to ER-knockout mice (ERKO). In a neointimal hyperplasia model in ERαKO male mice, our preliminary data indicate that T cells expand in both wild type mice and ERαKO by 2me2. The results indicate 2me2's effect on T cells is not ERα-mediated (*unpublished data*).

5.1.3 Immune regulation by 2me2

In paper I, we show amelioration of arthritis by both E2 and by 2me2. E2 is

known to have multiple effects on different immune cells. 2me2 seem to have a more limited effect as regards to which cells it can modulate, and the findings support a differential immunomodulatory role for 2me2 and E2. In paper II, we demonstrate that E2 regulates B cells but not the low dose 2me2, and direct modulation of neutrophils by 2me2 seems limited. Others have shown limited regulation in a neutrophil-dependent inflammatory model [46], and in our LPS-induced air pouch model, we detect no change in cellular components by 2me2 compared to vehicle *(unpublished data)*. 2me2 ameliorated the T-cell dependent delayed type hypersensitivity model [46], and in a model of multiple sclerosis, T-cell activation was diminished by 2me2 [51]. These data support a specific regulation of certain immune cells by 2me2, where our results indicate that 2me2 regulates NK cells, T cells, and possibly macrophages; whereas B cells and neutrophils are not affected. Why these cells are more sensitive to 2me2 regulation is unclear; Do they express an unknown 2me2 receptor? Are they more sensitive to HIF-1α, or to apoptosis? Are they predisposed to 2me2 regulation as they have a potential role in carcinogenesis and preeclampsia? These are questions for future investigations.

5.1.4 Future perspectives: 2me2 in the clinic

Whether or not 2me2 induce estrogen-signaling remains contradictory. It showed low affinity for the ERs *in vitro* [36], however such isolated systems might not represent the more complex *in vivo* situation. 2me2 administration has in some studies induced uteri proliferation [161, 162], however not in others [46]. Studies differ in administration-route and dose. Paper II indicates that the administered dose of 2me2 dictates its estrogenic activity.

2me2 display poor bioavailability in clinical trials, accounting for poor outcomes in cancer trials [42, 43, 164, 165]. Recently, structure/activity investigations and new formulations of 2me2 have revealed more positive results in cancer models and revived the interest for 2me2 in the clinic [41, 45, 166, 167]. Both modifying the structure and new formulations might diverge the molecule from being a substrate for E2-converting enzymes, and thus at the same time eliminating the problem of ER-related effects and increasing bioavailability.

5.2 Autoimmunity: role for innate cells

In paper I, E2 and 2me2 affected different cell types but both ameliorated arthritis, demonstrating that many cells are involved in the pathogenesis of autoimmune disorders. Innate cells are involved in both the initiation and

propagation of the disease. Macrophages and dendritic cells contribute to the onset of the autoimmune and inflammatory processes by presenting antigens, but macrophages also contribute to the pathology and clinical manifestations together with neutrophils, as they produce factors that damage tissues. The role for NK cells and other innate lymphoid cells in autoimmunity remains an open field for research.

5.2.1 Regulation of NK cells in arthritis and osteoimmunology

In paper II we show that both 2me2 and E2 regulated NK cell populations. NK cells' contribution in RA remains unclear, but both clinical and animal models have demonstrated their presence in joints and that they are crucial to disease progression [168-171]. In other autoimmune diseases, activated NK cells inhibit expansion of auto-reactive T cells, a mechanism that may also be important in RA pathogenesis [172, 173]. We analyzed NK cell populations from lymph nodes draining arthritic joints in paper I, and found that E2, but not 2me2, reduced the NK cell population. We further examined lymph nodes from arthritic mice treated with E2 from time of ovx instead of from arthritis onset, and detected no difference in NK cell populations, neither in lymph nodes nor in the joints. Neither did the cells differ in their expression of granzyme B or perforin. We concluded that NK cells are not direct targets for E2's amelioration in CIA development.

NK cells can however trigger osteoclastogenesis and bone destruction in arthritis models, where RANKL-expressing NK cells induced the differentiation of monocytes into osteoclasts [169]. The NK cell cytokine IL-15 was suggested to mediate joint destruction in septic arthritis [174]. We investigated the role of NK cells in ovx-induced bone loss, by removing NK cells through anti-asialo antibodies. Our preliminary results suggest that NK cells are involved in ovx-induced bone loss, but the mechanism behind this effect still remains unclear *(unpublished data)*.

5.2.2 The role of macrophages in inflammation and autoimmunity

Macrophages patrol tissues, recognizing foreign pathogens and tissue injury, but also eliminate toxic molecules and reconstitute tissue integrity. In inflammation, they can dictate the subsequent immune response by producing different mediators such as TNFα and ROS. In RA, macrophages are found in prominent numbers in the inflamed synovia membrane and at the cartilage-pannus junction [58, 175-177]. There, they can develop into osteoclasts to

degrade bone (see Fig. 9), secrete cytokines inducing inflammation, or propagate auto-reactive T cells. Controlling macrophages and their biology could impact not only RA, but also inflammation in general. During the inflammatory process, one macrophage can differentiate from a sentinel cell to a pro-inflammatory cytokine-producing and antigen-presenting cell, into an anti-inflammatory cell, and finally ending up as a tissue-healing macrophage [79]. Much of the heterogeneity could reflect a different stage in life – in development and function – responding to an infinite combination of signals. The full set of known and unknown markers and receptors macrophages display either represent an impressive array of host defense molecules, or dictate and determine a macrophage's potential function. There are also a number of mechanisms by which macrophages may limit inflammation, including internalization of apoptotic neutrophils [178-180]; modulating transcriptional factors such as NF-κB and Nrf2 [181, 182]; modifying the antigen processing [183]; or regulating T cells during antigen-presentation [184, 185].

In paper V, we used an inflammatory model and LPS, a well-known macrophage activator, in order to approach macrophage biology in inflammation. We investigated an array of receptors previously implicated in different inflammatory functions. Some were not involved in the process, however as stated previously, this outcome could reflect the chosen time-point for analysis. Regarding macrophage biology, a consensus is yet to be reached regarding receptor terminology. Their function is currently dictated by which effector molecules they produce. The model used in paper V offers a tool to study macrophage biology and their involvement in inflammation, modifiable both according to activation-molecule of choice and stage in inflammation. According to the detected cytokines, E2 treatment in ROS-deficient mice leads to macrophages with a more pro-inflammatory role, as MCP-1 and IL-6 was detected. However, the anti-inflammatory cytokine IL-10 was also increased. Further studies are needed to identify subsets of tissue-resident macrophages and what they locally produce in order to induce the difference in inflammatory response between E2 treatment in B10.Q and B10.Q.Ncf1$^{*/*}$-mice.

Whereas *in vitro* experiments have suggested that E2 exerts anti-inflammatory properties on monocyte/macrophage cell lines or microglial cells following activation by LPS, opposite results have been independently reported by analyzing *in vivo* effects. The effects of estrogens on macrophages must be considered in the context of both duration and level of estrogen exposure.

5.3 ROS and E2 in inflammation

In paper I, we investigated E2's amelioration of arthritis. Innate immune cells cause much of the tissue destruction in arthritis as they release pro-inflammatory products. ROS are such products, released by neutrophils and macrophages. In a set of experiments, we investigated whether *in vivo* E2 treatment influenced neutrophils' ROS production, specifically superoxide, i.e. $O_2^{\bullet}$. As in the arthritis study (Paper I), we administered mice E2 at 1 µg/day, but for only 5 days. After purifying neutrophils from the bone marrow they were primed with TNFα, inducing an inflammatory state and represents the maximum ROS capacity the cells can produce. E2 treatment diminished the total amount extracellular $O_2^{\bullet}$, suggesting an anti-inflammatory effect. Interestingly, when measuring both extracellular and intracellular $O_2^{\bullet}$, E2 increased the total amount $O_2^{\bullet}$, indicating that E2 increases intracellular $O_2^{\bullet}$ (*unpublished data).* This might point at a fundamental role for E2 in increasing oxidation of intracellular proteins, such as inducible nitric oxide synthase or general gene transcription, resulting in an anti-inflammatory defense.

In a scientific collaboration with Professor Rikard Holmdahl, we turned to NCF1-deficient mice, having a defect ROS production. The truncated *Ncf1*-gene leads to a defect in ROS derived from the NOX 2-complex and a mouse displaying a CGD-like phenotype, i.e. more susceptible to infections. More specifically, it was the defect in NCF1 in macrophages that were important in protecting from infections. In macrophage-specific NCF1-deficient mice, septic arthritis caused by *S. aureus* infection was decreased, in contrast to autoimmune arthritis, which was increased [105]. This was also caused by the macrophages, where the macrophage–T-cell communication resulted in increased auto-reactive T cells and aggravated both arthritis and multiple sclerosis [102-104, 186]. NOX 2 and NCF1 seem to have distinct roles in modulating the inflammatory response in autoimmune and septic arthritis.

In order to investigate whether E2 regulates NCF1-dependent inflammation, we used different models of inflammation; one that can be considered a chronic inflammation (Paper IV), and one that can be considered an acute inflammation (Paper V). The B10Q.Ncf1$^{*/*}$-females developed arthritis spontaneously post-partum, when the estrogen levels decline. In a preliminary experiment, we could confirm a role for sex steroids in NCF1-dependent CIA, where B10.Q mice developed less arthritis after sham operation as compared to ovx controls, whereas sham-operated B10Q.Ncf1$^{*/*}$-mice developed arthritis to the same extent as ovx mice *(unpublished data).* The data indicates that sex steroids and ROS-signaling

interact in the development of T cell/macrophage dependent immunity. Interestingly, septic arthritis in NCF1-deficient mice is ameliorated, and whether this depends on a defect defense against pathogens remains to be elucidated.

5.3.1 Bone loss in NCF1-deficient mice

An inflammatory mechanism has been suggested in ovx-induced osteoporosis, where activated DCs malfunction in their communication with T cells, increasing production of pro-inflammatory cytokines such as TNFα, which in turn induce osteoclastogenesis [187, 188]. As ROS-production has been suggested as an important proinflammatory factor in ovx-induced bone loss, we investigated this mechanism in paper IV. The *in vitro* data concerning osteoclastogenesis corroborated the theory, however *in vivo* data showed the opposite. This indicate a compensatory mechanism *in vivo*: ROS from another NOX, increased osteoblast activity, or another mechanism altogether. In a recent paper by Goettsch et al [189], NOX 4 producing H_2O_2 induced bone loss after ovx, and it has been suggested that RANKL induces a switch between NOX homologues, possibly accounting for the mechanism behind ovx-induced bone loss in NCF1-deficient mice [190].

5.3.2 Molecular pathways interconnecting E2 and ROS

Besides regulating macrophages and inflammation as stated above, NOX 2 derived ROS affects many different intracellular targets; thiols, histone modifications, NF-κB, Rac1, and NO (Fig. 16). Likewise, estrogen regulation also affects many different targets and cells [94]. Estrogen can exert its effects by inducing ERE transcription. Access to target genes require histone modifications and DNA breaks, which are regulated by ROS [191, 192]. Estrogen regulated genes might need ROS modifications for access to DNA in order to be transcribed (Fig. 16, pathway 2).

Another possible target is the subunit of the NOX 2 Rac1, as it regulates growth and cell adhesion by modifying actin filaments. Rac1 also mediated sex differences in cardiac TNFα expression via NOX-ERK MAPK pathway in endtoxemia [193]. Further, in an *in vitro* model of the vasculature, E2 inhibited monocyte adhesion by downregulating Rac1 (Fig. 16, pathway 4) [194].

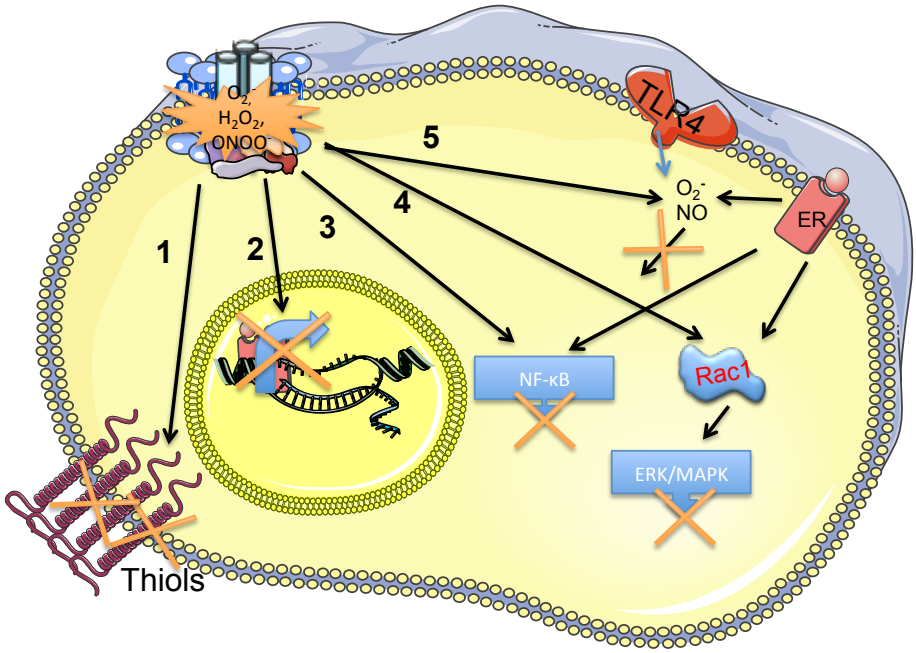


Figure 16. Scheme showing possible pathways interconnecting ROS and estrogen receptor signaling in an LPS model of inflammation. For details regarding the pathways, see the text. 1: affecting thiol expression, 2: affecting histone modifications, 3: disrupting NF-κB signaling, 4: disrupting Rac1 signaling, 5: disrupting nitric oxide regulated pathways.

As mentioned in the introduction, E2 controls vascular tone via NO, amongst others. This has been demonstrated in cardiovascular research and studies on the endothelium. In endothelial cells, E2 activates membrane ERs, which activates endothelial nitric oxide synthase [195]. In murine macrophages, E2 can also affect NO synthesis, and in addition, LPS binding to TLR4 induces inducible NOS, in turn producing NO. NO and O2• forms peroxynitrite (Fig 16 pathway 5, see introduction), forming protein radicals. As E2 regulates both TLR4 signaling and NO synthesis, this system might be affected in LPS-induced inflammation.

Other systems that E2/ROS regulates are antioxidant protein and enzyme expression in inflammation. In bone homeostasis, thiol expression increases by E2 (Fig. 16, pathway 1 [196]), and in myocardial cells, E2 increase Nrf2 translocation– a transcription factor responsible for cellular defense and redox status [197, 198]. As summarized in Fig. 16 and the introduction section, there are many possible mechanisms behind ROS/E2 regulated signaling, probably depending on target tissue and time-point.

5.3.3 Future perspectives: ROS as potential immunomodulators

Mice with a defect in their reactive oxygen species in the NOX2 complex develop more severe infections and autoimmune arthritis. It still remains unclear how inflammatory cytokines, TLR ligands, and inflammasomes trigger ROS production, and how the magnitude and duration of ROS production determines their functions. If one considers ROS regulation as diverse and useful as regulation by phosphorylation, many new questions arise. New tools needs to be developed for quantifying and identifying different ROS species and their subcellular localization- the challenges are considerable. Undeniably, considering ROS as only harmful species is obsolete, ROS should also be considered as regulators and preventers of autoimmune diseases.

5.4 Clinical relevance

Gene knockout animals represent models to study the specific role of a protein/gene and its effects on physiology, an artificial situation not seen in nature. Knockout mice most often lack the gene of interest throughout the lifetime, affecting all stages from embryology to death. As a gene is deleted during the development, compensatory mechanisms might be able to compensate for a loss-of-function of the protein. In the case of the $COMT^{-/-}$-mice, there seems to be compensatory mechanisms behind the differences seen between the genders. Most likely, E2 regulates COMT-gene expression, contributing to the minor effects seen in females [19, 199, 200]. As this also has been measured in human COMT, regulation of the gene by E2 appears to be preserved throughout mammalian species hierarchy [19]. COMT activity contributes to gender differences in behavioural and psychiatric disorders [16, 18]. As evident in the study by Kanasaki et al, COMT and 2me2 deficiencies in mice resulted in preeclampsia, and 2me2 levels was also decreased in women with preeclampsia [22]. Thus, the COMT gene might be able to regulate the immune defense in humans as well, possibly contributing to the general dimorphism seen in men and women.

Human CGD is mostly caused by single nucleotide mutations, resembling the B10Q.Ncf1$^{*/*}$ mice. The spontaneous single nucleotide mutation in *Ncf1* resulted in a truncated and nonfunctional $p47^{phox}$ protein. Consequently, the mice had no detectable NADPH oxidase function, measured by ROS response from neutrophils. There are several inherited forms of CGD, and the most frequent form is X-linked defect in the $gp91^{phox}$ subunit [201]. Mutations in the genes encoding for the subunits $p40^{phox}$, $p67^{phox}$, or $p47^{phox}$

are inherited in an autosomal recessive form, where the *Ncf1*-gene (encoding $p47^{phox}$) is the most common mutation, representing 20-30 % CGD cases [201-204]. The NCF1-deficient mice are also more susceptible to bacterial infections [105], representing an appropriate model to study the influence of NCF1 on LPS-infections. Arthritis in NCF1-deficient mice developed as a chronic relapsing disease, more accurately mimicking human RA.

6 CONCLUSION

Data generated in this thesis may have consequences for future treatment options in arthritis, inflammation, osteoporosis, and cancer.

In this thesis, we have investigated possible mechanisms behind estrogen and its metabolite 2me2 in the initiation and development of arthritis and inflammatory mechanisms in diseases predominantly affecting women.

In paper III, we exclude major effects on the immune system by the enzyme COMT and its catechol products. In female $COMT^{-/-}$-mice we detected increased uteri weight compared to WT mice, indicating that estrogen metabolism is disturbed. COMT is the only enzyme producing 2me2 *in vivo*, and $COMT^{-/-}$-mice develop preeclampsia. In patients with preeclampsia 2me2 levels were also found to be reduced [22, 31, 32]. Even though the immune system is involved in the underlying pathogenesis of preeclampsia, based on our results, preeclamptic patients with disturbed 2me2 levels would possibly benefit from therapies targeting other factors than the immune system for alleviation of their symptoms.

The results from papers I and II indicates that 2me2 at pharmacological doses may pose a possible modulator for treatments controlling the immune system such as cancer and inflammation. However, in the present formulation 2me2 induces unwanted estrogen receptor signaling. Derivates of 2me2 and newer formulations may result in more efficient therapies for inflammatory and autoimmune diseases as well as cancer.

In inflammation, reactive oxygen species protects against infections. However, this excess production was long seen as deleterious for endogenous tissues such as the joint in RA. The deleterious processes by which ROS contribute to mutagenesis, to protein modifications, and lipid modifications are all involved in the pathogenesis of different diseases such as RA. In both SLE and Sjögren's patients, oxidized autoantibodies possibly contribute to the disease development. These are all diseases with greater prevalence in women compared to men. In paper IV, we investigated the possible involvement of NOX2 and ROS in bone loss. Based on the result from paper IV, NOX 2 is not the major contributor to estrogen-deficiency induced bone loss, however it may still have a functional role in osteoporosis caused by inflammation.

In paper V, we investigated and confirmed that estrogen and ROS act in concert to balance inflammation. In NCF1-deficient males, septic arthritis caused by *S. aureus* infection decreases, while collagen-induced autoimmune arthritis increases. In both these processes, macrophages were essential– conveying protection against bacteria, but also inducing excessive auto-reactive T cells. How macrophage NOX 2 might protect or induce inflammation is still unknown, and a longstanding question has been whether this is secondary to the deficient protection against infections or vice versa, whether the lack of oxidative burst promotes inflammatory disorders. Based on the results from paper V, the NCF1-deficient system is regulated by estrogen.

The innate immune system and its production of ROS are gaining interest [95, 96, 183]. Not only are they regulators in autoimmune diseases, they are also involved in auto-inflammatory disorders: a report by Ferguson et al correlates the decreased intracellular ROS production with the incidence of the inflammatory disease SAPHO- synovitis, acne, pustulosis, hyperostosis, osteitis [205]. there are still many unknown factors controlling autoimmune-, and inflammatory disorders. We have shown in paper IV and V that estrogens have a role and participate in ROS regulation in general disease mechanisms.

In conclusion, autoimmune diseases and inflammation have a clear sexual dimorphism, where the female gender represents a risk factor for autoimmune development. Females in general generate a more substantial response against invading pathogens. In this thesis, we have shown that estradiol, estradiol metabolites, and reactive oxygen species all are involved in the process leading to regulating inflammation and arthritis. The long-term goal of research on sex steroids is not only to understand the hormonal regulation in disease, but also to understand underlying mechanisms between sex differences in disease prevalence and incidence. Future research will hopefully result in identifying novel targets for therapeutic purposes.

ACKNOWLEDGEMENT

Ett stort tack till alla ni som hjälpt till så att denna bok blev till:

Hans Carlsten, min huvudhandledare. Tack för att jag fick möjligheten att börja doktorera i din grupp. Tack för att du alltid tar dig tid att svara på frågor. Du har lärt mig så mycket om forskningsvärlden, om immunologi, och om att vara generös med sin kunskap. Tack för att du alltid skapar en så god och inspirerande miljö!

Ulrika Islander, min bihandledare. Tack för allt handfast stöd när jag började, och tack för all detaljsyn! Det är bra att kunna sakta ner sitt tempo ibland.

Tack till alla medförfattare som engagerat sig!

Mina rumskamrater och gruppmedlemmar, gamla som nya: Lucija, Cissi, Hardis, Annica, Lina, Anna S, och Maria (även fast du inte officiellt flyttade in i vårt rum...) Tänk att ni stått ut med allt mitt babbel! Det skulle varit så väldigt väldigt väldigt tråkigt utan er... Tack för alla glada dagar!

Tack alla i E2-gruppen, Marie, Caroline, Merja, Louise, Helena Catharina, Malin, Lennart och Martin. Tack för att ni lärt mig så mycket om reumatologi, immunologi, ben, klinisk forskning och olika smarta metoder, och tack för all hjälp vid avslut.

Tack till Anna och Åsa för all rolig och inspirerande samarbete vi har haft! Anna, det finns ju ingen som kan inspirera till gott arbete så bra som du!

Tack alla på reumatologiavdelningen! Främst för alla kaffepauser och roligheter under lunchen med djupa immunologiska samtal... Tack även till Mikael, Mattias och Nico för att ni hållit FACSen i ett sånt fint skick! Kristina, Harriet, Eva, Cathrine, Sofie: Tack för all hjälp med administrativt arbete, och tack Esbjörn för all hjälp med datorkrångel. Tack till alla fikaglada från andra våningen, jag har alltid undrat hur ni lyckas ta er ner så punktligt nästan varje dag! Tack för att jag fick komma och lära mej en massa spännande metoder hos er!

Tack till alla nuvarande och före detta doktorander vid avdelningen!!

Tack alla nere på CBAR och experterna på ben och benceller!

Hela min familj! Tack till min mamma: editor, inspiratör och barnvakt- det hade ju inte faktiskt inte blivit en bok utan dig! Till min pappa för att jag fått lov att göra som jag vill. Tack till min storasyster Petra, så att det alltid fanns någon att se upp till!

Tack till familjen Stubelius-/Ohlsson/Garnäs (-?-), för alla söndagsmiddagar, barnpassning, champagnefirande och att ni alltid serverar prinsesstårtor! Tack för att ni gett mej ett extra hem att komma till.

Tack till Fredrik, som låter mig vara den jag är och ändå inte tycker jag är hård och elak. Tack för allt vi gjort som varit roligt, för att allt är så roligt att göra med dej och att allt som kunde varit tråkigt blir roligt tillsammans med dej! Tack till Robert för att du är en sån himla rolig kille, det finns ingen som dansar som du! Tack för att jag får lov att ha det så fantastiskt roligt varje dag med er!

REFERENCES

1. Melmed, S., P.M. Conn, and SpringerLink (Online service), *Endocrinology Basic and Clinical Principles*. 2005, Humana Press Inc.,: Totowa, NJ.
2. Van Loo, P.L., et al., *Modulation of aggression in male mice: influence of group size and cage size.* Physiol Behav, 2001. **72**(5): p. 675-83.
3. Beato, M. and J. Klug, *Steroid hormone receptors: an update.* Hum Reprod Update, 2000. **6**(3): p. 225-36.
4. Smith, D.F. and D.O. Toft, *Steroid receptors and their associated proteins.* Mol Endocrinol, 1993. **7**(1): p. 4-11.
5. Pettersson, K., et al., *Mouse estrogen receptor beta forms estrogen response element-binding heterodimers with estrogen receptor alpha.* Mol Endocrinol, 1997. **11**(10): p. 1486-96.
6. Heldring, N., et al., *Estrogen receptors: how do they signal and what are their targets.* Physiol Rev, 2007. **87**(3): p. 905-31.
7. Krishnan, V., X. Wang, and S. Safe, *Estrogen receptor-Sp1 complexes mediate estrogen-induced cathepsin D gene expression in MCF-7 human breast cancer cells.* J Biol Chem, 1994. **269**(22): p. 15912-7.
8. Kushner, P.J., et al., *Estrogen receptor pathways to AP-1.* J Steroid Biochem Mol Biol, 2000. **74**(5): p. 311-7.
9. Galien, R. and T. Garcia, *Estrogen receptor impairs interleukin-6 expression by preventing protein binding on the NF-kappaB site.* Nucleic Acids Res, 1997. **25**(12): p. 2424-9.
10. Kuiper, G.G., et al., *Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta.* Endocrinology, 1997. **138**(3): p. 863-70.
11. Borjesson, A.E., et al., *Roles of transactivating functions 1 and 2 of estrogen receptor-alpha in bone.* Proc Natl Acad Sci U S A, 2011. **108**(15): p. 6288-93.
12. Engdahl, C., et al., *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. **62**(2): p. 524-33.
13. Lindberg, M.K., et al., *Estrogen receptor specificity for the effects of estrogen in ovariectomized mice.* J Endocrinol, 2002. **174**(2): p. 167-78.
14. Martucci, C.P. and J. Fishman, *P450 enzymes of estrogen metabolism.* Pharmacol Ther, 1993. **57**(2-3): p. 237-57.
15. Bonifacio, M.J., et al., *Catechol-O-methyltransferase and its inhibitors in Parkinson's disease.* CNS drug reviews, 2007. **13**(3): p. 352-79.
16. Harrison, P.J. and E.M. Tunbridge, *Catechol-O-methyltransferase (COMT): a gene contributing to sex differences in brain function, and to sexual dimorphism in the predisposition to psychiatric disorders.* Neuropsychopharmacology, 2008. **33**(13): p. 3037-45.
17. Mannisto, P.T. and S. Kaakkola, *Catechol-O-methyltransferase (COMT): biochemistry, molecular biology, pharmacology, and clinical efficacy of the new selective COMT inhibitors.* Pharmacol Rev, 1999. **51**(4): p. 593-628.
18. Gogos, J.A., et al., *Catechol-O-methyltransferase-deficient mice exhibit sexually dimorphic changes in catecholamine levels and behavior.* Proc Natl Acad Sci U S A, 1998. **95**(17): p. 9991-6.
19. Jiang, H., et al., *Human catechol-O-methyltransferase down-regulation by estradiol.* Neuropharmacology, 2003. **45**(7): p. 1011-8.

20. Boudikova, B., et al., *Human liver catechol-O-methyltransferase pharmacogenetics.* Clin Pharmacol Ther, 1990. **48**(4): p. 381-9.
21. Cohn, C.K. and J. Axelrod, *The effect of estradiol on catechol-O-methyltransferase activity in rat liver.* Life Sci I, 1971. **10**(23): p. 1351-4.
22. Kanasaki, K., et al., *Deficiency in catechol-O-methyltransferase and 2-methoxyoestradiol is associated with pre-eclampsia.* Nature, 2008. **453**(7198): p. 1117-21.
23. Davey, D.A. and I. MacGillivray, *The classification and definition of the hypertensive disorders of pregnancy.* Am J Obstet Gynecol, 1988. **158**(4): p. 892-8.
24. Chappell, L.C., et al., *Adverse perinatal outcomes and risk factors for preeclampsia in women with chronic hypertension: a prospective study.* Hypertension, 2008. **51**(4): p. 1002-9.
25. Makkonen, N., S. Heinonen, and P. Kirkinen, *Obstetric prognosis in second pregnancy after preeclampsia in first pregnancy.* Hypertens Pregnancy, 2000. **19**(2): p. 173-81.
26. Hnat, M.D., et al., *Perinatal outcome in women with recurrent preeclampsia compared with women who develop preeclampsia as nulliparas.* Am J Obstet Gynecol, 2002. **186**(3): p. 422-6.
27. Hjartardottir, S., et al., *Recurrence of hypertensive disorder in second pregnancy.* Am J Obstet Gynecol, 2006. **194**(4): p. 916-20.
28. van Rijn, B.B., et al., *Outcomes of subsequent pregnancy after first pregnancy with early-onset preeclampsia.* Am J Obstet Gynecol, 2006. **195**(3): p. 723-8.
29. Berg, D., R. Sonsalla, and E. Kuss, *Concentrations of 2-methoxyoestrogens in human serum measured by a heterologous immunoassay with an 125I-labelled ligand.* Acta Endocrinol (Copenh), 1983. **103**(2): p. 282-8.
30. Berg, D., F. Thaler, and E. Kuss, *Concentrations of 2-hydroxyoestrogens in human sera measured by a heterologous immunoassay with an 125I-labelled ligand.* Acta Endocrinol (Copenh), 1982. **100**(1): p. 154-60.
31. Jobe, S.O., C.T. Tyler, and R.R. Magness, *Aberrant synthesis, metabolism, and plasma accumulation of circulating estrogens and estrogen metabolites in preeclampsia implications for vascular dysfunction.* Hypertension, 2013. **61**(2): p. 480-7.
32. Perez-Sepulveda, A., et al., *Low 2-methoxyestradiol levels at the first trimester of pregnancy are associated with the development of pre-eclampsia.* Prenat Diagn, 2012. **32**(11): p. 1053-8.
33. Mooberry, S.L., *Mechanism of action of 2-methoxyestradiol: new developments.* Drug Resist Updat, 2003. **6**(6): p. 355-61.
34. Mueck, A.O. and H. Seeger, *2-Methoxyestradiol--biology and mechanism of action.* Steroids, 2010. **75**(10): p. 625-31.
35. Verenich, S. and P.M. Gerk, *Therapeutic promises of 2-methoxyestradiol and its drug disposition challenges.* Mol Pharm, 2010. **7**(6): p. 2030-9.
36. LaVallee, T.M., et al., *2-Methoxyestradiol inhibits proliferation and induces apoptosis independently of estrogen receptors alpha and beta.* Cancer Res, 2002. **62**(13): p. 3691-7.
37. Ricker, J.L., et al., *2-methoxyestradiol inhibits hypoxia-inducible factor 1alpha, tumor growth, and angiogenesis and augments paclitaxel efficacy in head and neck squamous cell carcinoma.* Clin Cancer Res, 2004. **10**(24): p. 8665-73.
38. LaVallee, T.M., et al., *2-methoxyestradiol up-regulates death receptor 5 and induces apoptosis through activation of the extrinsic pathway.* Cancer research, 2003. **63**(2): p. 468-75.

39. Gao, N., et al., *2-Methoxyestradiol-induced apoptosis in human leukemia cells proceeds through a reactive oxygen species and Akt-dependent process.* Oncogene, 2005. **24**(23): p. 3797-809.
40. Fotsis, T., et al., *The endogenous oestrogen metabolite 2-methoxyoestradiol inhibits angiogenesis and suppresses tumour growth.* Nature, 1994. **368**(6468): p. 237-9.
41. Bruce, J.Y., et al., *A phase II study of 2-methoxyestradiol nanocrystal colloidal dispersion alone and in combination with sunitinib malate in patients with metastatic renal cell carcinoma progressing on sunitinib malate.* Invest New Drugs, 2012. **30**(2): p. 794-802.
42. Harrison, M.R., et al., *A phase II study of 2-methoxyestradiol (2ME2) NanoCrystal(R) dispersion (NCD) in patients with taxane-refractory, metastatic castrate-resistant prostate cancer (CRPC).* Investigational new drugs, 2011. **29**(6): p. 1465-74.
43. Kulke, M.H., et al., *A prospective phase II study of 2-methoxyestradiol administered in combination with bevacizumab in patients with metastatic carcinoid tumors.* Cancer chemotherapy and pharmacology, 2011. **68**(2): p. 293-300.
44. Matei, D., et al., *Activity of 2 methoxyestradiol (Panzem NCD) in advanced, platinum-resistant ovarian cancer and primary peritoneal carcinomatosis: a Hoosier Oncology Group trial.* Gynecol Oncol, 2009. **115**(1): p. 90-6.
45. Zhou, Q., et al., *A phase I dose-escalation, safety and pharmacokinetic study of the 2-methoxyestradiol analog ENMD-1198 administered orally to patients with advanced cancer.* Investigational new drugs, 2011. **29**(2): p. 340-6.
46. Josefsson, E. and A. Tarkowski, *Suppression of type II collagen-induced arthritis by the endogenous estrogen metabolite 2-methoxyestradiol.* Arthritis Rheum, 1997. **40**(1): p. 154-63.
47. Issekutz, A.C. and K. Sapru, *Modulation of adjuvant arthritis in the rat by 2-methoxyestradiol: an effect independent of an anti-angiogenic action.* Int Immunopharmacol, 2008. **8**(5): p. 708-16.
48. Brahn, E., et al., *An angiogenesis inhibitor, 2-methoxyestradiol, involutes rat collagen-induced arthritis and suppresses gene expression of synovial vascular endothelial growth factor and basic fibroblast growth factor.* J Rheumatol, 2008. **35**(11): p. 2119-28.
49. Plum, S.M., et al., *Disease modifying and antiangiogenic activity of 2-methoxyestradiol in a murine model of rheumatoid arthritis.* BMC Musculoskelet Disord, 2009. **10**: p. 46.
50. Huerta-Yepez, S., et al., *2-Methoxyestradiol (2-ME) reduces the airway inflammation and remodeling in an experimental mouse model.* Clin Immunol, 2008. **129**(2): p. 313-24.
51. Duncan, G.S., et al., *2-Methoxyestradiol inhibits experimental autoimmune encephalomyelitis through suppression of immune cell activation.* Proc Natl Acad Sci U S A, 2012. **109**(51): p. 21034-9.
52. Shand, F.H., et al., *In vitro and in vivo evidence for anti-inflammatory properties of 2-methoxyestradiol.* J Pharmacol Exp Ther, 2011. **336**(3): p. 962-72.
53. Doran, M.F., et al., *Trends in incidence and mortality in rheumatoid arthritis in Rochester, Minnesota, over a forty-year period.* Arthritis Rheum, 2002. **46**(3): p. 625-31.
54. Goemaere, S., et al., *Onset of symptoms of rheumatoid arthritis in relation to age, sex and menopausal transition.* J Rheumatol, 1990. **17**(12): p. 1620-2.
55. de Hair, M.J., et al., *The clinical picture of rheumatoid arthritis according to the 2010 American College of Rheumatology/European League Against Rheumatism criteria: is this still the same disease?* Arthritis Rheum, 2012. **64**(2): p. 389-93.

56. Barragan-Martinez, C., et al., *Gender differences in Latin-American patients with rheumatoid arthritis.* Gend Med, 2012. **9**(6): p. 490-510 e5.
57. Humphreys, J.H., et al., *The incidence of rheumatoid arthritis in the UK: comparisons using the 2010 ACR/EULAR classification criteria and the 1987 ACR classification criteria. Results from the Norfolk Arthritis Register.* Ann Rheum Dis, 2013. **72**(8): p. 1315-20.
58. Klareskog, L., A.I. Catrina, and S. Paget, *Rheumatoid arthritis.* Lancet, 2009. **373**(9664): p. 659-72.
59. Pikwer, M., et al., *Early menopause is an independent predictor of rheumatoid arthritis.* Ann Rheum Dis, 2012. **71**(3): p. 378-81.
60. Ostensen, M., P.M. Villiger, and F. Forger, *Interaction of pregnancy and autoimmune rheumatic disease.* Autoimmun Rev, 2012. **11**(6-7): p. A437-46.
61. de Man, Y.A., et al., *Disease activity of rheumatoid arthritis during pregnancy: results from a nationwide prospective study.* Arthritis Rheum, 2008. **59**(9): p. 1241-8.
62. Barrett, J.H., et al., *Does rheumatoid arthritis remit during pregnancy and relapse postpartum? Results from a nationwide study in the United Kingdom performed prospectively from late pregnancy.* Arthritis Rheum, 1999. **42**(6): p. 1219-27.
63. Holmdahl, R., et al., *Oestrogen is a potent immunomodulator of murine experimental rheumatoid disease.* Br J Rheumatol, 1989. **28 Suppl 1**: p. 54-8; discussion 69-71.
64. Ganesan, K., et al., *Comparative studies on the interplay of testosterone, estrogen and progesterone in collagen induced arthritis in rats.* Bone, 2008. **43**(4): p. 758-65.
65. Stubelius, A., et al., *Role of 2-methoxyestradiol as inhibitor of arthritis and osteoporosis in a model of postmenopausal rheumatoid arthritis.* Clin Immunol, 2011. **140**(1): p. 37-46.
66. Inoue, K., E. Inoue, and Y. Imai, *Female sex hormones ameliorate arthritis in SKG mice.* Biochem Biophys Res Commun, 2013. **434**(4): p. 740-5.
67. Jochems, C., et al., *Osteoporosis in experimental postmenopausal polyarthritis: the relative contributions of estrogen deficiency and inflammation.* Arthritis Res Ther, 2005. **7**(4): p. R837-43.
68. d'Elia, H.F. and H. Carlsten, *The impact of hormone replacement therapy on humoral and cell-mediated immune responses in vivo in post-menopausal women with rheumatoid arthritis.* Scand J Immunol, 2008. **68**(6): p. 661-7.
69. D'Elia, H.F., et al., *Influence of hormone replacement therapy on disease progression and bone mineral density in rheumatoid arthritis.* J Rheumatol, 2003. **30**(7): p. 1456-63.
70. Rossouw, J.E., et al., *Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial.* JAMA, 2002. **288**(3): p. 321-33.
71. Abbas, A.L., A., *Basic Immunology; Functions and Disorders of the Immune System*. 2009: Elsevier Inc., Saunders,.
72. Bjorkstrom, N.K., E. Kekalainen, and J. Mjosberg, *Tissue-specific effector functions of innate lymphoid cells.* Immunology, 2013. **139**(4): p. 416-27.
73. Sonnenberg, G.F., et al., *SnapShot: innate lymphoid cells.* Immunity, 2013. **39**(3): p. 622-622 e1.
74. Quintin, J., et al., *Innate immune memory: towards a better understanding of host defense mechanisms.* Curr Opin Immunol, 2014. **29C**: p. 1-7.
75. Mocsai, A., *Diverse novel functions of neutrophils in immunity, inflammation, and beyond.* J Exp Med, 2013. **210**(7): p. 1283-99.
76. Volkman, A. and J.L. Gowans, *The Origin of Macrophages from Bone Marrow in the Rat.* Br J Exp Pathol, 1965. **46**: p. 62-70.

77. Volkman, A. and J.L. Gowans, *The Production of Macrophages in the Rat.* Br J Exp Pathol, 1965. **46**: p. 50-61.
78. Gordon, S. and P.R. Taylor, *Monocyte and macrophage heterogeneity.* Nat Rev Immunol, 2005. **5**(12): p. 953-64.
79. Soehnlein, O. and L. Lindbom, *Phagocyte partnership during the onset and resolution of inflammation.* Nat Rev Immunol, 2010. **10**(6): p. 427-39.
80. Davies, L.C., et al., *Distinct bone marrow-derived and tissue-resident macrophage lineages proliferate at key stages during inflammation.* Nat Commun, 2013. **4**: p. 1886.
81. Geissmann, F., et al., *Development of monocytes, macrophages, and dendritic cells.* Science, 2010. **327**(5966): p. 656-61.
82. Colonna, M., G. Trinchieri, and Y.J. Liu, *Plasmacytoid dendritic cells in immunity.* Nat Immunol, 2004. **5**(12): p. 1219-26.
83. Waskow, C., et al., *The receptor tyrosine kinase Flt3 is required for dendritic cell development in peripheral lymphoid tissues.* Nat Immunol, 2008. **9**(6): p. 676-83.
84. Liu, K., et al., *In vivo analysis of dendritic cell development and homeostasis.* Science, 2009. **324**(5925): p. 392-7.
85. Murray, P.J. and T.A. Wynn, *Protective and pathogenic functions of macrophage subsets.* Nat Rev Immunol, 2011. **11**(11): p. 723-37.
86. Gordon, S., *Pattern recognition receptors: doubling up for the innate immune response.* Cell, 2002. **111**(7): p. 927-30.
87. Serbina, N.V., et al., *TNF/iNOS-producing dendritic cells mediate innate immune defense against bacterial infection.* Immunity, 2003. **19**(1): p. 59-70.
88. Mosser, D.M. and J.P. Edwards, *Exploring the full spectrum of macrophage activation.* Nat Rev Immunol, 2008. **8**(12): p. 958-69.
89. Janeway, C.A., Jr. and R. Medzhitov, *Innate immune recognition.* Annu Rev Immunol, 2002. **20**: p. 197-216.
90. Poltorak, A., et al., *Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene.* Science, 1998. **282**(5396): p. 2085-8.
91. Calippe, B., et al., *17Beta-estradiol promotes TLR4-triggered proinflammatory mediator production through direct estrogen receptor alpha signaling in macrophages in vivo.* J Immunol, 2010. **185**(2): p. 1169-76.
92. Calippe, B., et al., *Chronic estradiol administration in vivo promotes the proinflammatory response of macrophages to TLR4 activation: involvement of the phosphatidylinositol 3-kinase pathway.* J Immunol, 2008. **180**(12): p. 7980-8.
93. Rettew, J.A., Y.M. Huet, and I. Marriott, *Estrogens augment cell surface TLR4 expression on murine macrophages and regulate sepsis susceptibility in vivo.* Endocrinology, 2009. **150**(8): p. 3877-84.
94. Straub, R.H., *The complex role of estrogens in inflammation.* Endocr Rev, 2007. **28**(5): p. 521-74.
95. Lambeth, J.D., *NOX enzymes and the biology of reactive oxygen.* Nat Rev Immunol, 2004. **4**(3): p. 181-9.
96. Lambeth, J.D. and A.S. Neish, *Nox Enzymes and New Thinking on Reactive Oxygen: A Double-Edged Sword Revisited.* Annu Rev Pathol, 2013.
97. Finkel, T., *Oxygen radicals and signaling.* Curr Opin Cell Biol, 1998. **10**(2): p. 248-53.
98. Burdon, R.H., *Superoxide and hydrogen peroxide in relation to mammalian cell proliferation.* Free Radic Biol Med, 1995. **18**(4): p. 775-94.
99. Jones, D.P., *Redox sensing: orthogonal control in cell cycle and apoptosis signalling.* J Intern Med, 2010. **268**(5): p. 432-48.
100. Petry, A., M. Weitnauer, and A. Gorlach, *Receptor activation of NADPH oxidases.* Antioxid Redox Signal, 2010. **13**(4): p. 467-87.

101. Kuijpers, T. and R. Lutter, *Inflammation and repeated infections in CGD: two sides of a coin.* Cell Mol Life Sci, 2012. **69**(1): p. 7-15.
102. Hultqvist, M., et al., *Enhanced autoimmunity, arthritis, and encephalomyelitis in mice with a reduced oxidative burst due to a mutation in the Ncf1 gene.* Proc Natl Acad Sci U S A, 2004. **101**(34): p. 12646-51.
103. Hultqvist, M., et al., *Lack of reactive oxygen species breaks T cell tolerance to collagen type II and allows development of arthritis in mice.* J Immunol, 2007. **179**(3): p. 1431-7.
104. Pizzolla, A., et al., *CD68-expressing cells can prime T cells and initiate autoimmune arthritis in the absence of reactive oxygen species.* Eur J Immunol, 2011. **41**(2): p. 403-12.
105. Pizzolla, A., et al., *Reactive oxygen species produced by the NADPH oxidase 2 complex in monocytes protect mice from bacterial infections.* J Immunol, 2012. **188**(10): p. 5003-11.
106. Karre, K., *Natural killer cell recognition of missing self.* Nat Immunol, 2008. **9**(5): p. 477-80.
107. Kiessling, R., et al., *"Natural" killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell.* Eur J Immunol, 1975. **5**(2): p. 117-21.
108. Kiessling, R., E. Klein, and H. Wigzell, *"Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype.* Eur J Immunol, 1975. **5**(2): p. 112-7.
109. Lanier, L.L., *Up on the tightrope: natural killer cell activation and inhibition.* Nat Immunol, 2008. **9**(5): p. 495-502.
110. Topham, N.J. and E.W. Hewitt, *Natural killer cell cytotoxicity: how do they pull the trigger?* Immunology, 2009. **128**(1): p. 7-15.
111. Bhat, R. and C. Watzl, *Serial killing of tumor cells by human natural killer cells--enhancement by therapeutic antibodies.* PLoS One, 2007. **2**(3): p. e326.
112. Gregoire, C., et al., *The trafficking of natural killer cells.* Immunol Rev, 2007. **220**: p. 169-82.
113. Chiossone, L., et al., *Maturation of mouse NK cells is a four-stage developmental program.* Blood, 2009.
114. Vivier, E., et al., *Functions of natural killer cells.* Nat Immunol, 2008. **9**(5): p. 503-10.
115. Yu, J., A.G. Freud, and M.A. Caligiuri, *Location and cellular stages of natural killer cell development.* Trends Immunol, 2013. **34**(12): p. 573-82.
116. Di Santo, J.P., *Functionally distinct NK-cell subsets: developmental origins and biological implications.* Eur J Immunol, 2008. **38**(11): p. 2948-51.
117. Sun, J.C., J.N. Beilke, and L.L. Lanier, *Adaptive immune features of natural killer cells.* Nature, 2009.
118. Sun, J.C. and L.L. Lanier, *Natural killer cells remember: an evolutionary bridge between innate and adaptive immunity?* Eur J Immunol, 2009. **39**(8): p. 2059-64.
119. Orange, J.S., *Natural killer cell deficiency.* J Allergy Clin Immunol, 2013. **132**(3): p. 515-25; quiz 526.
120. Parham, P. and A. Moffett, *Variable NK cell receptors and their MHC class I ligands in immunity, reproduction and human evolution.* Nat Rev Immunol, 2013. **13**(2): p. 133-44.
121. Flodstrom-Tullberg, M., et al., *Natural killer cells in human autoimmunity.* Curr Opin Immunol, 2009. **21**(6): p. 634-40.
122. Ljunggren, H.G. and K.J. Malmberg, *Prospects for the use of NK cells in immunotherapy of human cancer.* Nat Rev Immunol, 2007. **7**(5): p. 329-39.

123. Koepsell, S.A., J.S. Miller, and D.H. McKenna, Jr., *Natural killer cells: a review of manufacturing and clinical utility.* Transfusion, 2013. **53**(2): p. 404-10.
124. Germain, R.N., *T-cell development and the CD4-CD8 lineage decision.* Nat Rev Immunol, 2002. **2**(5): p. 309-22.
125. Arron, J.R. and Y. Choi, *Bone versus immune system.* Nature, 2000. **408**(6812): p. 535-6.
126. Karmakar, S., J. Kay, and E.M. Gravallese, *Bone damage in rheumatoid arthritis: mechanistic insights and approaches to prevention.* Rheum Dis Clin North Am, 2010. **36**(2): p. 385-404.
127. Manolagas, S.C., C.A. O'Brien, and M. Almeida, *The role of estrogen and androgen receptors in bone health and disease.* Nat Rev Endocrinol, 2013. **9**(12): p. 699-712.
128. Miyaura, C., et al., *Increased B-lymphopoiesis by interleukin 7 induces bone loss in mice with intact ovarian function: similarity to estrogen deficiency.* Proc Natl Acad Sci U S A, 1997. **94**(17): p. 9360-5.
129. Teegarden, D., et al., *Peak bone mass in young women.* J Bone Miner Res, 1995. **10**(5): p. 711-5.
130. Berger, C., et al., *Peak bone mass from longitudinal data: implications for the prevalence, pathophysiology, and diagnosis of osteoporosis.* J Bone Miner Res, 2010. **25**(9): p. 1948-57.
131. Hernlund, E., et al., *Osteoporosis in the European Union: medical management, epidemiology and economic burden. A report prepared in collaboration with the International Osteoporosis Foundation (IOF) and the European Federation of Pharmaceutical Industry Associations (EFPIA).* Arch Osteoporos, 2013. **8**(1-2): p. 136.
132. Wright, N.C., et al., *Arthritis increases the risk for fractures--results from the Women's Health Initiative.* J Rheumatol, 2011. **38**(8): p. 1680-8.
133. Forsblad D'Elia, H., et al., *Radiographic joint destruction in postmenopausal rheumatoid arthritis is strongly associated with generalised osteoporosis.* Ann Rheum Dis, 2003. **62**(7): p. 617-23.
134. Khosla, S., M.J. Oursler, and D.G. Monroe, *Estrogen and the skeleton.* Trends Endocrinol Metab, 2012. **23**(11): p. 576-81.
135. Albright, F., *Post-menopausal osteoporosis.* Trans Assoc Am Physicians, 1940. **55**: p. 298-305.
136. Jilka, R.L., et al., *Increased osteoclast development after estrogen loss: mediation by interleukin-6.* Science, 1992. **257**(5066): p. 88-91.
137. Kousteni, S., et al., *Reversal of bone loss in mice by nongenotropic signaling of sex steroids.* Science, 2002. **298**(5594): p. 843-6.
138. Myers, L.K., et al., *Collagen-induced arthritis, an animal model of autoimmunity.* Life Sci, 1997. **61**(19): p. 1861-78.
139. Monach, P.A., D. Mathis, and C. Benoist, *The K/BxN arthritis model.* Curr Protoc Immunol, 2008. **Chapter 15**: p. Unit 15 22.
140. Nandakumar, K.S., L. Svensson, and R. Holmdahl, *Collagen type II-specific monoclonal antibody-induced arthritis in mice: description of the disease and the influence of age, sex, and genes.* Am J Pathol, 2003. **163**(5): p. 1827-37.
141. Li, P. and E.M. Schwarz, *The TNF-alpha transgenic mouse model of inflammatory arthritis.* Springer Semin Immunopathol, 2003. **25**(1): p. 19-33.
142. Ward, J.R. and R.S. Jones, *Studies on adjuvant-induced polyarthritis in rats. I. Adjuvant composition, route of injection, and removal of depot site.* Arthritis Rheum, 1962. **5**: p. 557-64.
143. Tarkowski, A., et al., *Model systems: modeling human staphylococcal arthritis and sepsis in the mouse.* Trends Microbiol, 2001. **9**(7): p. 321-6.

144. Lawlor, K.E., et al., *Molecular and cellular mediators of interleukin-1-dependent acute inflammatory arthritis.* Arthritis Rheum, 2001. **44**(2): p. 442-50.
145. Lemmen, J.G., et al., *Tissue- and time-dependent estrogen receptor activation in estrogen reporter mice.* J Mol Endocrinol, 2004. **32**(3): p. 689-701.
146. Lundqvist, H. and C. Dahlgren, *Isoluminol-enhanced chemiluminescence: a sensitive method to study the release of superoxide anion from human neutrophils.* Free Radic Biol Med, 1996. **20**(6): p. 785-92.
147. Palacios, R., *Concanavalin A triggers T lymphocytes by directly interacting with their receptors for activation.* Journal of immunology, 1982. **128**(1): p. 337-42.
148. Whitley, E. and J. Ball, *Statistics review 6: Nonparametric methods.* Crit Care, 2002. **6**(6): p. 509-13.
149. Whitley, E. and J. Ball, *Statistics review 5: Comparison of means.* Crit Care, 2002. **6**(5): p. 424-8.
150. Grossman, C.J., *Interactions between the gonadal steroids and the immune system.* Science, 1985. **227**(4684): p. 257-61.
151. Grossman, C.J., *Regulation of the immune system by sex steroids.* Endocr Rev, 1984. **5**(3): p. 435-55.
152. Olsen, N.J. and W.J. Kovacs, *Gonadal steroids and immunity.* Endocr Rev, 1996. **17**(4): p. 369-84.
153. Oliver, J.E. and A.J. Silman, *Why are women predisposed to autoimmune rheumatic diseases?* Arthritis Res Ther, 2009. **11**(5): p. 252.
154. Fairweather, D., S. Frisancho-Kiss, and N.R. Rose, *Sex differences in autoimmune disease from a pathological perspective.* Am J Pathol, 2008. **173**(3): p. 600-9.
155. Lin, G., et al., *Contribution of catechol-O-methyltransferase Val158Met polymorphism to endometrial cancer risk in postmenopausal women: a meta-analysis.* Genet Mol Res, 2013. **12**(4): p. 6442-53.
156. Qin, X., et al., *Association of COMT Val158Met polymorphism and breast cancer risk: an updated meta-analysis.* Diagn Pathol, 2012. **7**: p. 136.
157. Tian, C., et al., *The Val158Met polymorphism in the COMT gene is associated with increased cancer risks in Chinese population.* Tumour Biol, 2013.
158. Zhu, B.T. and A.H. Conney, *Functional role of estrogen metabolism in target cells: review and perspectives.* Carcinogenesis, 1998. **19**(1): p. 1-27.
159. Okoh, V.O., et al., *Reactive oxygen species via redox signaling to PI3K/AKT pathway contribute to the malignant growth of 4-hydroxy estradiol-transformed mammary epithelial cells.* PLoS One, 2013. **8**(2): p. e54206.
160. Joubert, A., et al., *C2- and C4-position 17beta-estradiol metabolites and their relation to breast cancer.* Biocell, 2009. **33**(3): p. 137-40.
161. Sutherland, T.E., et al., *2-methoxyestradiol is an estrogen receptor agonist that supports tumor growth in murine xenograft models of breast cancer.* Clin Cancer Res, 2005. **11**(5): p. 1722-32.
162. Sibonga, J.D., et al., *Dose-response effects of 2-methoxyestradiol on estrogen target tissues in the ovariectomized rat.* Endocrinology, 2003. **144**(3): p. 785-92.
163. Brzozowski, A.M., et al., *Molecular basis of agonism and antagonism in the oestrogen receptor.* Nature, 1997. **389**(6652): p. 753-8.
164. LaVallee, T.M., et al., *Significant antitumor activity in vivo following treatment with the microtubule agent ENMD-1198.* Molecular cancer therapeutics, 2008. **7**(6): p. 1472-82.
165. Pasquier, E., et al., *ENMD-1198, a new analogue of 2-methoxyestradiol, displays both antiangiogenic and vascular-disrupting properties.* Molecular cancer therapeutics, 2010. **9**(5): p. 1408-18.
166. Chua, Y.S., Y.L. Chua, and T. Hagen, *Structure activity analysis of 2-methoxyestradiol analogues reveals targeting of microtubules as the major*

mechanism of antiproliferative and proapoptotic activity. Mol Cancer Ther, 2010. **9**(1): p. 224-35.
167. Guo, X., et al., *Inhalable microspheres embedding chitosan-coated PLGA nanoparticles for 2-methoxyestradiol.* J Drug Target, 2014.
168. Leavenworth, J.W., et al., *Mobilization of natural killer cells inhibits development of collagen-induced arthritis.* Proc Natl Acad Sci U S A, 2011. **108**(35): p. 14584-9.
169. Soderstrom, K., et al., *Natural killer cells trigger osteoclastogenesis and bone destruction in arthritis.* Proc Natl Acad Sci U S A, 2010. **107**(29): p. 13028-33.
170. de Matos, C.T., et al., *Activating and inhibitory receptors on synovial fluid natural killer cells of arthritis patients: role of CD94/NKG2A in control of cytokine secretion.* Immunology, 2007. **122**(2): p. 291-301.
171. Dalbeth, N. and M.F. Callan, *A subset of natural killer cells is greatly expanded within inflamed joints.* Arthritis Rheum, 2002. **46**(7): p. 1763-72.
172. Leavenworth, J.W., et al., *Analysis of the cellular mechanism underlying inhibition of EAE after treatment with anti-NKG2A F(ab')2.* Proc Natl Acad Sci U S A, 2010. **107**(6): p. 2562-7.
173. Lu, L., et al., *Regulation of activated CD4+ T cells by NK cells via the Qa-1-NKG2A inhibitory pathway.* Immunity, 2007. **26**(5): p. 593-604.
174. Henningsson, L., et al., *Interleukin 15 mediates joint destruction in Staphylococcus aureus arthritis.* J Infect Dis, 2012. **206**(5): p. 687-96.
175. McInnes, I.B. and G. Schett, *The pathogenesis of rheumatoid arthritis.* The New England journal of medicine, 2011. **365**(23): p. 2205-19.
176. Hollander, A.P., et al., *Expression of hypoxia-inducible factor 1alpha by macrophages in the rheumatoid synovium: implications for targeting of therapeutic genes to the inflamed joint.* Arthritis Rheum, 2001. **44**(7): p. 1540-4.
177. Joyce-Shaikh, B., et al., *Myeloid DAP12-associating lectin (MDL)-1 regulates synovial inflammation and bone erosion associated with autoimmune arthritis.* J Exp Med, 2010. **207**(3): p. 579-89.
178. Sanmun, D., et al., *Involvement of a functional NADPH oxidase in neutrophils and macrophages during programmed cell clearance: implications for chronic granulomatous disease.* Am J Physiol Cell Physiol, 2009. **297**(3): p. C621-31.
179. Hampton, M.B., et al., *Oxidant-mediated phosphatidylserine exposure and macrophage uptake of activated neutrophils: possible impairment in chronic granulomatous disease.* J Leukoc Biol, 2002. **71**(5): p. 775-81.
180. Fernandez-Boyanapalli, R.F., et al., *Impaired apoptotic cell clearance in CGD due to altered macrophage programming is reversed by phosphatidylserine-dependent production of IL-4.* Blood, 2009. **113**(9): p. 2047-55.
181. Han, W., et al., *NADPH oxidase limits lipopolysaccharide-induced lung inflammation and injury in mice through reduction-oxidation regulation of NF-kappaB activity.* J Immunol, 2013. **190**(9): p. 4786-94.
182. Segal, B.H., et al., *NADPH oxidase limits innate immune responses in the lungs in mice.* PLoS One, 2010. **5**(3): p. e9631.
183. Sareila, O., et al., *NOX2 complex-derived ROS as immune regulators.* Antioxid Redox Signal, 2011. **15**(8): p. 2197-208.
184. Mantegazza, A.R., et al., *NADPH oxidase controls phagosomal pH and antigen cross-presentation in human dendritic cells.* Blood, 2008. **112**(12): p. 4712-22.
185. Romani, L., et al., *Defective tryptophan catabolism underlies inflammation in mouse chronic granulomatous disease.* Nature, 2008. **451**(7175): p. 211-5.
186. Gelderman, K.A., et al., *Macrophages suppress T cell responses and arthritis development in mice by producing reactive oxygen species.* J Clin Invest, 2007. **117**(10): p. 3020-8.

187. Grassi, F., et al., *Oxidative stress causes bone loss in estrogen-deficient mice through enhanced bone marrow dendritic cell activation.* Proc Natl Acad Sci U S A, 2007. **104**(38): p. 15087-92.
188. Kimble, R.B., S. Bain, and R. Pacifici, *The functional block of TNF but not of IL-6 prevents bone loss in ovariectomized mice.* J Bone Miner Res, 1997. **12**(6): p. 935-41.
189. Goettsch, C., et al., *NADPH oxidase 4 limits bone mass by promoting osteoclastogenesis.* J Clin Invest, 2013.
190. Sasaki, H., et al., *Receptor activator of nuclear factor-kappaB ligand-induced mouse osteoclast differentiation is associated with switching between NADPH oxidase homologues.* Free Radic Biol Med, 2009. **47**(2): p. 189-99.
191. Ju, B.G., et al., *A topoisomerase IIbeta-mediated dsDNA break required for regulated transcription.* Science, 2006. **312**(5781): p. 1798-802.
192. Perillo, B., et al., *DNA oxidation as triggered by H3K9me2 demethylation drives estrogen-induced gene expression.* Science, 2008. **319**(5860): p. 202-6.
193. Zhu, H., L. Shan, and T. Peng, *Rac1 mediates sex difference in cardiac tumor necrosis factor-alpha expression via NADPH oxidase-ERK1/2/p38 MAPK pathway in endotoxemia.* J Mol Cell Cardiol, 2009. **47**(2): p. 264-74.
194. Friedrich, E.B., et al., *17Beta-estradiol inhibits monocyte adhesion via down-regulation of Rac1 GTPase.* J Mol Cell Cardiol, 2006. **40**(1): p. 87-95.
195. Chow, R.W., D.J. Handelsman, and M.K. Ng, *Minireview: rapid actions of sex steroids in the endothelium.* Endocrinology, 2010. **151**(6): p. 2411-22.
196. Lean, J.M., et al., *A crucial role for thiol antioxidants in estrogen-deficiency bone loss.* J Clin Invest, 2003. **112**(6): p. 915-23.
197. Yu, J., et al., *17beta-estradiol regulates the expression of antioxidant enzymes in myocardial cells by increasing Nrf2 translocation.* J Biochem Mol Toxicol, 2012. **26**(7): p. 264-9.
198. Hayes, J.D. and A.T. Dinkova-Kostova, *The Nrf2 regulatory network provides an interface between redox and intermediary metabolism.* Trends Biochem Sci, 2014.
199. Dawling, S., et al., *Catechol-O-methyltransferase (COMT)-mediated metabolism of catechol estrogens: comparison of wild-type and variant COMT isoforms.* Cancer Res, 2001. **61**(18): p. 6716-22.
200. Stubelius, A., et al., *Sexual dimorphisms in the immune system of catechol-O-methyltransferase knockout mice.* Immunobiology, 2012. **217**(8): p. 751-60.
201. Roos, D., et al., *Hematologically important mutations: the autosomal recessive forms of chronic granulomatous disease (second update).* Blood Cells Mol Dis, 2010. **44**(4): p. 291-9.
202. Hayrapetyan, A., et al., *Different unequal cross-over events between NCF1 and its pseudogenes in autosomal p47(phox)-deficient chronic granulomatous disease.* Biochim Biophys Acta, 2013. **1832**(10): p. 1662-72.
203. Gorlach, A., et al., *A p47-phox pseudogene carries the most common mutation causing p47-phox- deficient chronic granulomatous disease.* J Clin Invest, 1997. **100**(8): p. 1907-18.
204. Koker, M.Y., et al., *Clinical, functional, and genetic characterization of chronic granulomatous disease in 89 Turkish patients.* J Allergy Clin Immunol, 2013. **132**(5): p. 1156-1163 e5.
205. Ferguson, P.J., et al., *Neutrophil dysfunction in a family with a SAPHO syndrome-like phenotype.* Arthritis Rheum, 2008. **58**(10): p. 3264-9.