Osteoporosis in murine SLE

Treatment with a tissue-selective estrogen complex

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ABSTRACT

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease affecting mainly young women. Almost every organ in the body can be affected and SLE patients often suffer from co-morbidities such as cardiovascular disease and osteoporosis. Postmenopausal women with SLE have a three-fold increase in fractures compared with the healthy population. Treatment with bisphosphonates is associated with side effects and newer anti-osteoporotic treatments need to be tested in SLE patients.

MRL/*lpr* mice spontaneously develop lupus-like symptoms. We show that ovariectomized MRL/*lpr* mice also develop an osteoporotic bone phenotype and can be used as a model for osteoporosis in postmenopausal SLE.

Estrogen has been known to worsen SLE, however there are also contradictory findings available. A tissue-selective estrogen complex (TSEC) comprising estrogen and a selective estrogen receptor modulator (SERM) facilitates the positive effects by estrogen on for example bone, while the negative estrogenic effects on for example the endometrium is blocked by the SERM. The TSEC consisting of conjugated estrogens and the SERM Bazedoxifene (Bza) is approved for treatment of vasomotor symptoms and prevention of postmenopausal osteoporosis. In this thesis we show that treatment with TSEC and Bza protects from trabecular bone loss in ovariectomized MRL/*lpr* mice, without affecting uterus or lupus-associated disease parameters.

B lymphopoiesis and antibody production are regulated by estrogens. We show that TSEC share the estrogenic inhibitory effect on B lymphopoiesis and stimulatory effect on antibody production in healthy mice.

These findings have increased the knowledge regarding TSEC as a potential future drug for treating osteoporosis in postmenopausal SLE patients.

Keywords: Tissue-Selective Estrogen Complex, Osteoporosis, MRL/*lpr*, Systemic Lupus Erythematosus, Selective Estrogen Receptor Modulator.
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SAMMANFATTNING PÅ SVENSKA

Systemisk lupus erythematosus (SLE) är en kronisk autoimmun sjukdom som framförallt drabbar unga kvinnor. Nästan alla organ hos SLE patienter kan bli påverkade av sjukdomen, till exempel huden, leder och njurar. Sjukdomen går i skov som kan triggas igång utav till exempel UV-ljus. SLE-patienter drabbas ofta även av följdsjukdomar såsom hjärt- och kärlsjukdom och benskörhet.

Att SLE är en autoimmun sjukdom innebär att kroppens immunförsvar (som bland annat hjälper oss bekämpa infektioner) har fallerat och börjat attackera kroppens egna friska vävnader. Vårt immunförsvar kan delas in i två delar; det medfödda och det förvärvade. Det medfödda immunförsvaret är snabbt på plats för att bekämpa hot mot kroppen, medan det förvärvade immunförsvaret tar lite längre tid men är mer specialiserat. Båda två spelar stor roll vid SLE. B celler är en del av det förvärvade immunsystemet och av intresse i detta arbete på grund av sin funktion som antikroppsproducenter. Autoantikroppar, det vill säga antikroppar mot den egna kroppens delar, är en viktig del i sjukdomsmekanismen vid SLE.

Benskörhet uppstår när benet bryts ner i snabbare takt än det kan byggas upp. En av konsekvenserna av benskörhet är frakturer. Könshormonet östrogen har en skyddande effekt på ben, och vid klimakteriet (menopaus) minskar den naturliga östrogenproduktionen. Kvinnor med SLE som passerat klimakteriet (dvs postmenopausala) löper tre gånger så hög risk att drabbas av en fraktur jämfört med friska personer. Ett vanligt läkemedel för behandling av benskörhet är bisfosfonater, men det har bieffekter. Nya läkemedel mot benskörhet behöver tas fram och även testas systematiskt i just SLE patienter.

Det är allmänt känt att östrogen förvärrar SLE-sjukdomen, men det finns också studier som inte visar detta. Östrogen har olika effekter i olika vävnader, såsom skyddande effekter på ben men bidrar till ökad risk för livmodercancer. Ett vävnadsspecifikt östrogent komplex (tissue-selective estrogen complex, TSEC) består utav både östrogen och en selektiv östrogenreceptor modulerare (selective estrogen receptor modulator, SERM). SERM är syntetiska läkemedel med syftet att fungera som östrogen i önskade vävnader, men inte i andra. TSEC bestående av

SERMet Bazedoxifene och östrogen är godkänt för behandling av klimakteriebesvär och skydda mot benskörhet. I ett TSEC utnyttjas östrogens positiva effekter på till exempel ben, medan de negativa effekterna på till exempel livmodern blockeras utav Bazedoxifene.

Syftet med detta arbete var dels att ta fram en experimentell metod för att kunna studera benskörhet vid SLE-sjukdom och dels att sedan använda modellen för att undersöka om benskörheten kunde behandlas med TSEC. Vi ville också studera effekterna utav TSEC på det friska immunsystemet med avseende på utvecklingen av B celler och deras förmåga att producera antikroppar.

Möss utav stammen MRL/*lpr* utvecklar spontant en SLE-liknande sjukdom med symptom såsom njursjukdom och produktion utav autoantikroppar. I detta arbete visar vi hur MRL/*lpr* möss som genomgått ovariektomi (borttagande av ovarierna och därigenom endogent östrogen) utvecklar benförlust. Därmed kan ovariektomerade MRL/*lpr* möss användas som experimentell modell för att studera benskörhet i postmenopausal SLE (**Paper I**).

Vi visar sedan hur behandling med TSEC eller Bazedoxifene skyddar mot benförlust i ovariektomerade MRL/*lpr* möss utan att påverka livmodern eller SLE-sjukdomen (**Paper II**).

Östrogen har hämmande effekter på utvecklingen utav B celler i benmärg och mjälte, samt ökar B cellers förmåga att producera antikroppar. Vi visar att behandling med TSEC i friska möss leder till samma hämmande effekter på utvecklingen av B celler och ökning i antalet antikroppsproducerande B celler som behandling med enbart östrogen (**Paper III**).

Sammanfattningsvis har detta arbete ökat kunskapen om TSEC som ett potentiellt framtida läkemedel för att behandla benskörhet i postmenopausala SLE-patienter.

LIST OF PAPERS

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

I. Nordqvist J, Lagerquist MK, Grahnemo L, Koskela A, Islander U, Carlsten H. **Osteoporosis in a murine model of postmenopausal lupus.** *Lupus,* 2020; 29(1): 58-66.

II. Nordqvist J*, Engdahl C*, Scheffler JM, Gupta P, Gustafsson KL, Lagerquist MK, Carlsten H, Islander U. **A tissue-selective estrogen complex as treatment of osteoporosis in experimental lupus.** *Manuscript in revision*.

III. Nordqvist J, Bernardi A, Islander U, Carlsten H. **Effects of a tissue-selective estrogen complex on B lymphopoiesis and B cell function.** *Immunobiology*, 2017; 222(8-9): 918-23.

*Authors contributed equally

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CONTENT

ABBREVIATIONS

aBMD	Areal bone mineral density
APC	Antigen presenting cell
BAFF	B cell activating factor
BMD	Bone mineral density
Bza	Bazedoxifene
CD	Cluster of differentiation
CE	Conjugated estrogens
CTX-1	C-terminal telopeptide of type I collagen
DAMP	Damage-associated molecular patterns
DC	Dendritic cell
DXA	Dual-energy X-ray absorptiometry
E1	Estrone
E2	Estradiol
E3	Estriol
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot
ER	Estrogen receptor
ERE	Estrogen response element
FACS	Fluorescence-activated cell sorting
FO	Follicular
HRT	Hormone replacement therapy
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
Lpr	Lymphoproliferation
MAC	Membrane-attack complex
M-CSF	Macrophage colony-stimulating factor
μCT	High-resolution micro-computed tomography
MHC	Major histocompatibility complex
MZ	Marginal zone
NET	Neutrophil extracellular trap
OCN	Osteocalcin
OPG	Osteoprotegerin
OVX	Ovariectomy
P1NP	N-terminal propeptide of type I procollagen
PAMP	Pathogen-associated molecular patterns

pQCT	Peripheral quantitative computed tomography
Pre-B	Precursor B
Pre-BCR	Precursor B cell receptor
Pro-B	Progenitor B
PRR	Pattern recognition receptors
qPCR	Quantitative polymerase chain reaction
RANK	Receptor activator of nuclear factor-κB
RANKL	Receptor activator of nuclear factor-κB ligand
RunX2	Runt-related transcription factor 2
SERM	Selective estrogen receptor modulator
SLE	Systemic lupus erythematosus
T1	Transitional 1
T2	Transitional 2
TCR	T cell receptor
Th	T helper cell
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TSEC	Tissue-selective estrogen complex
Veh	Vehicle
VMS	Vasomotor symptoms
WHI	Women's health initiative

1 BACKGROUND

1.1 SYSTEMIC LUPUS ERYTHEMATOSUS

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease that can affect almost every organ in the body. It often debuts at a young age, around 20 to 40 years, and is 9 times more common in women than men[1]. A study examining the Swedish national registers 2010 found a prevalence of 46-85/100 000 depending on the strictness of inclusion[2].

1.1.1 SLE DIAGNOSIS

The first clinical presentations of a patient that will later be diagnosed with SLE are heterogeneous. In 2019 the European League Against Rheumatism/ American College of Rheumatology presented new criteria for diagnosis of SLE[3]. The classification criteria are presented in detail in *Figure 1*.

1.1.2 SLE TREATMENT

Today, there is no cure available for SLE. However, with treatment care plans evolving over the last decades the survival time has increased. A recent study showed a 10-year survival rate of 88.8% in Finnish SLE patients[4]. SLE is a disease with a relapse/remitting pattern and patients need to be regularly monitored. Flares can appear at any time, but there are some known triggers; for example infections, ultraviolet radiation, and hormones[5]. Due to the heterogeneity of disease symptoms, individualized treatment plans are needed. Drugs commonly used to treat various symptoms in SLE patients are; antimalarials, glucocorticoids, immunosuppressive agents, and biological agents. The patient monitoring includes for example checking disease status, including organ damage as well as screening for co-morbidities, such as cardiovascular disease, osteoporosis, and certain malignancies. With the longer life expectancy, these co-morbidities are of increasing importance for the patient. Osteoporosis is the co-morbidity of special interest in this thesis.

Entery criterion

Presence of antinuclear antibodies (ANA) OR an earlier positive test (ever)

If absent, do not classify as SLE. If present, apply additive criteria

Additive criteria

Do not count a criterion if there is a more likely explanation than SLE
Occurrence of a criterion on at least one occasion is sufficient and they do not need to occur sumiltaneosly
Within each domain, only the highest weighed criterion is counted towards the total score

Clinical domains and criteria	Weight
Constitutional	
Fever	2
Hematologic	
Leukopenia	3
Thrombocytopenia	4
Autoimmune hemolysis	4
Neuropsychiatric	
Delirium	2
Psychosis	3
Seizure	5
Mucocutaneous	
Non-scarring alopecia	2
Oral ulcers	2
Subacute cutaneous ***OR***	
discoid lupus	4
Acute cutaneous lupus	6
Serosal	
Pleural or pericardial effusion	5
Acute pericarditits	6
Musculoskeletal	
Joint involvement	6
Renal	
Proteinuria >0.5g/24h	4
Renal biopsy Class II ***OR***	
V lupus nephritis	8
Renal biopsy Class III ***OR***	
IV lupus nephritis	10

Immunology domains and criteria	Weight
Antiphospholipid antibodies	
Anti-cardiolipin antibodies ***OR***	
Anti-β2GP1 antibodies ***OR***	
Lupus anticoagulant	2
Complement proteins	
Low C3 ***OR*** *low C4*	3
Low C3 ***AND*** *low C4*	4
SLE-specific antibodies	
Anti-dsDNA antibody ***OR***	
Anti-Smith antibody	6

Count the total score and classify as Systemic Lupus Erythematosus with a score of 10 or more if entry cirerion and at least one clinical criterion is fulfilled

Figure 1. Classification criteria for SLE, adapted from Aringer et al[3].

1.1.3 SLE ETIOLOGY

The etiology of SLE is multifactorial, including hormonal, genetic, and environmental factors. The female predisposition for SLE suggests sex hormones to be of importance. The effects of estrogen and similar components have shown mixed effects on the SLE disease and will be further discussed in section 1.4.8. There are differences in prevalence in ethnic populations, where Black people have a higher incidence of SLE than Caucasians, reviewed by Rees et al[6]. Genome-wide association studies have revealed 84 loci associated with SLE susceptibility, reviewed by Chen et al[7]. A majority of the genes associated with SLE are involved in innate or adaptive immune system pathways and there is consensus that the major histocompatibility complex region is associated with susceptibility for SLE[8, 9]. A Taiwanese study, including 23 Million participants, whereof 18000 had SLE, showed a heritability of 43.9%[10]. The surrounding environment can also play a role in SLE, including sunlight exposure, smoking and certain chemicals or medications, which can trigger flares[11]. There are also discussions whether Epstein Barr Virus infections can cause development of SLE disease[12].

1.2 THE IMMUNE SYSTEM

This part of the thesis will give a very brief version of the function of the immune system. The B cell development will be described in more detail since it is of special interest for Paper III, and some focus will be on describing what happens with the immune system in an SLE patient.

1.2.1 THE HEALTHY IMMUNE SYSTEM

Our immune system can be divided into the innate and the adaptive immune system. The innate immune system is our first defense mechanism against different threats. When foreign invaders, such as bacteria, enter our body, pattern recognition receptors (PRRs) on tissue-resident dendritic cells (DCs) or macrophages will bind to pathogen-associated molecular patterns (PAMPs) on the foreign substance. Damage-associated molecular patterns (DAMPs) from destroyed tissues or dying cells can also interact with PRRs. Upon binding, the DC or macrophage starts to produce inflammatory mediators such as the cytokines tumor necrosis factor (TNF), interleukin (IL)-6, and IL-10. The inflammatory mediators will be expressed in seconds, hours, or days. The invaders will be effectively phagocytized by tissue resident macrophages and recruited neutrophils. The second line of defense against pathogenic substances is the adaptive immune system. In short, an antigen presenting cell (APC) (for example a DC or macrophage) migrates to the lymph node where it will express an antigen from the pathogen and activate antigen-specific T-lymphocytes (T cells) and B-lymphocytes (B cells). The time from the first encounter to a fully developed adaptive immune response can be 1-2 weeks.

1.2.1.1 B CELLS

The B cells develop in the bone marrow. Starting from hematopoietic stem cells, the first step according to the Basel nomenclature is the development into progenitor B cells (pro-B cells)[13]. During B cell development the different cell populations express distinctive surface markers, see *Figure 2*. Pro-B cells develop into precursor BI cells (pre-BI cells) and then into pre-BII cells[13]. These cell populations express gradually increasing levels of the pre-B cell receptor (pre-BCR)[14]. The next stage in the B cell differentiation is the immature B cells, which are now ready to leave the bone marrow[15]. They express the BCR, which is an antibody of the IgM isotype. Immature B cells go through negative selection to eliminate cells with a BCR binding self-antigens[14], which is important for the central B cell tolerance.

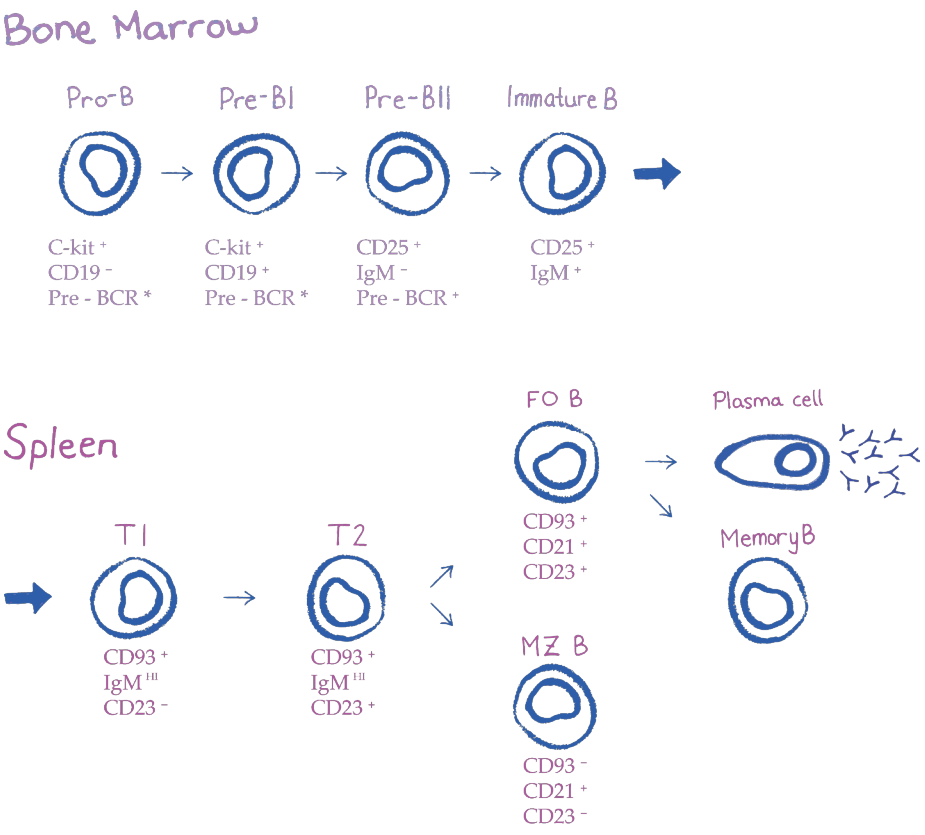

*Figure 2. Stages of B cell development in bone marrow and spleen. Surface markers for the different cell types are shown below each cell. All cells from Pro-B until FO B and MZ B are positive for B220. *Pre-BCR expressed to some extent in certain fractions of these populations. Pro-B; progenitor B cell, Pre-BI; precursor BI, Pre-BII; precursor BII, T1; transitional 1 B cell, T2; transitional 2 B cell, MZ; marginal zone, FO; follicular.*

The immature B cells then migrate to the spleen where they are called transitional B cells. First they are transitional 1 (T1) B cells before they go through a negative selection step and develop into transitional 2 (T2) B cells. The negative selection is mediated via the BCR[16]. The T2 B cells are the precursors for mature B cells. The T2 B cells then differentiate into marginal zone (MZ) B cells or follicular (FO) B cells. B cell activating factor (BAFF) is required in order for T2 B cells to develop into MZ B cell, and this cell fate

is correlated with a weak BCR signal[17]. The FO B cell fate, however, does not require BAFF, and it is associated with a strong BCR signal[17]. With the help from T follicular helper cells, FO B cells then mature into memory B cells or plasma cells. Plasma cells are fully developed B cells and our bodies' big machinery for producing antibodies[15].

As newly developed, the inactivated B cells express IgM or IgD isotype antibodies. When the B cells become activated, they can undergo class switch recombination into IgG, IgE, or IgA isotypes upon different stimuli. Typically IgM antibodies form pentamers, are found in the blood, are the first antibodies on site upon infection, and can activate the complement system[15]. IgG antibodies are monomers acting in blood and extracellular fluid that opsonizes pathogens and activate the complement system. IgA antibodies can form dimers and are important in for example the mucus epithelium. IgE antibodies are important in allergies[18].

1.2.1.2 T CELLS

Common lymphoid progenitor cells from the bone marrow migrate to the thymus where they develop further[15]. First, the T cells express neither cluster of differentiation 4 (CD4) nor CD8 and are called double negative T cells. They then develop further into double positive T cells expressing CD4, CD8, and their antigen specific receptor, the T cell receptor (TCR). The T cells with a TCR that has a moderate binding affinity to major histocompatibility complex (MHC) class I or MHC class II will be positively selected and changed into being single positive for CD8 or CD4, respectively[19]. There is also a strong negative selection ensuring T cells are not specific against self, i.e. too strong TCR binding will lead to elimination of the T cell, which is an important mechanism in the central tolerance.

T cells expressing CD8 are called cytotoxic T cells and T cells expressing CD4 are called T helper cells. These naïve T cells are released into the circulation and enter the T cell zones of lymph nodes in order to find an APC expressing the correct antigen. For the T cell to become activated, the APC needs to have the MHC presenting the antigen specific for the T cell receptor as well as expression of the correct co-stimulatory molecules[20]. Depending on the cytokine milieu present, the T helper cells are further developed into different effector cells depending on their functions[21]. Th1 cells are active in the defense against intracellular pathogens. They induce cell-mediated immunity by for example IFNγ production, which activates macrophages[15].

Th2 cells produce IL-4 and IL-10 among many other cytokines and induces humoral immunity by for example affecting the class switching of B cells[22]. Th17 cells produce the pro-inflammatory cytokine IL-17 and are important in the defense against extracellular pathogens[23]. T follicular helper cells are found in the secondary lymphoid organs and aid B cells in their activation as well as participate in B cell affinity maturation in germinal centers[24]. T regulatory cells have importance for immunosuppressive functions and maintaining peripheral tolerance[25, 26]. Auto-reactive B cells in the periphery require auto-reactive T helper cells expressing a TCR specific for the same self-peptide in order to become activated. Therefore, by tightly regulating auto-reactive T helper cells, they indirectly help to maintain the peripheral B cell tolerance as well.

1.2.1.3 THE COMPLEMENT SYSTEM

One very important part of our body's defense against foreign substances includes the complement system. The cleavage of complement components creates active enzymes cleaving more components, and the response is largely amplified via this cascade. Activation of the complement system leads to opsonization, inflammation, or lysing of the pathogen[15]. The enzyme C3-convertase (consisting of C4b and C2a) cleaves the complement component C3 into C3b and C3a. C3b then coats the surface of the pathogen, encouraging recognition and phagocytosis by phagocytes, a process called opsonization. C3a is involved in inflammation where it activates neutrophils[27]. Lysing of the pathogen is performed by the membrane-attack complex (MAC), which consists of the complement factors C5-C9[15]. The complement system is activated in three different pathways; the lectin, the alternative, and the classical pathway, which all result in the formation of C3-convertase.

1.2.2 THE IMMUNE SYSTEM IN SLE

Loss of tolerance, sustained autoantibody production, and incomplete disposal of apoptotic material are important mechanisms for the pathogenesis of SLE[28]. Loss of tolerance is when the immune system reacts to substances that normally should not trigger an immune response. The tolerance towards self-antigens is important for the balance of the immune system, and when it fails autoimmunity occurs. In principle every organ can be affected by autoimmunity in SLE, which is why it has long been considered a prototypic disease for autoimmunity.

1.2.2.1 CLEARANCE OF APOPTOTIC MATERIAL

Apoptosis, programmed cell death, is a key feature in our body that provides packaging of cell material and making it available for phagocytosis. In SLE patients, the apoptosis mechanism is disturbed and apoptotic material, such as nuclear antigens, which are usually cleared rapidly, now becomes available to the cells of the immune system. The apoptotic material can for example originate from exposure to UV light or infections. One consequence is that immature B cells can mature to become plasma cells producing autoantibodies towards these materials[29].

1.2.2.2 NEUTROPHIL EXTRACELLULAR TRAPS (NETS)

As a part of the immune defense mechanism against foreign substances, extracellular fibers, called neutrophil extracellular traps (NETs)[30], are cast out from dying neutrophils in a process now called NETosis. Patients with SLE have large quantities of NETs present in for example the kidneys, skin, and blood and it is positively correlated with increased disease activity[31, 32]. The presence of NETs allows for prolonged exposure of e.g. nuclear DNA, which serves as self-antigens driving the autoimmunity and increases the type I IFN signature. The type I IFN signature is further described in section 1.2.2.4. DNAse I is the enzyme involved in the degradation of NETs, and SLE patients have lower DNAse I levels than healthy controls[32].

1.2.2.3 THE COMPLEMENT SYSTEM IN SLE

The complement system has both destructive and protective effects in SLE[33]. For example, it is involved in tissue injury. Lupus patients have been found to have low C3 and/or low C4[3]. These complement components are assumed to be consumed in the complement pathway, and without sufficient levels of components the positive effects of the complement system cannot be sustained. This can be exemplified by genetic defects resulting in lack of complement factors. In principle all patients with deficiency of complement factor C1q develop SLE[33].

1.2.2.4 B CELLS AND AUTOANTIBODIES IN SLE

B cells, or more specifically plasma cells, are the producers of autoantibodies and have long been considered to play a major role in SLE. In SLE patients, the B cell differentiation is disturbed and there is a loss of tolerance. For example, BCRs of B cells that have escaped the negative selection in the bone marrow can bind free DNA, which is then internalized and can bind to

toll-like receptor (TLR) 9 that is present on intracellular compartments[34]. This increases the differentiation, activation, and proliferation of auto-reactive B cells. Elevated levels of TLR9 expressing B cells have been found in SLE patients and correlated with levels of antibodies to anti-dsDNA antibodies[35]. B cell differentiation and loss of tolerance can also be promoted by type I interferons, typically IFNα and IFNβ, and SLE is strongly associated with type I interferon, reviewed by Rönnblom and Leonard[36]. Domeier et al showed that involvement of the receptor for IFNα promoted the expansion of auto-reactive B cells through regulation of the BCR-signaling[37]. The type I interferon signature is when there is a prominent expression of genes regulated by type I interferons and it can be seen as a persistent self-directed immune response mimicking an anti-viral response. Type I interferons are involved in multiple other cellular mechanisms, such as suppressing T regulatory cells and increasing differentiation of Th17 cells[36].

Overexpression of BAFF can also lead to a break in tolerance, where self-reactive B cells are rescued from deletion and allowed to mature and colonize B cell follicles[38]. In SLE patients with active disease, the B cells are antibody-producing plasma cells to a higher proportion compared with the patients with inactive disease[39]. Autoantibodies can, for example, form complexes with antigens (immune complexes), which in turn can activate complement and drive inflammation as well as cause tissue damage. Immune complexes are also implicated in glomerulonephritis, a common renal disease in SLE patients. TLR7 sense single-stranded RNA and is required for the activation of B cells expressing RNA-reactive autoantibodies and the development of glomerulonephritis[40].

1.2.2.5 T CELLS IN SLE

T cells are also highly involved in the pathogenesis of SLE. Many T cell subsets are altered in SLE. For example, defects in regulatory T cells have been seen in both mouse models of SLE and human SLE, as reviewed by Chavele and Ehrenstein[41]. Also, Th17 cells are increased as well as IL-17 producing double negative T cells. The increase in IL-17 has been shown to stimulate infiltration of T cells to the kidneys and aid in B-cell activation and production of antibodies[42].

1.2.2.6 CYTOKINES IN SLE

Apart from BAFF and type I interferons, such as IFNα, many other cytokines are elevated in SLE patients including TNF, IL-6, IL-10, and IFNγ, and these are involved in inflammation and the production of autoantibodies, reviewed by Tsokos et al[28]. For example, TNF sustains the type I interferon signature[43], both IL-6 and IL-10 increase B cell proliferation, and IFNγ increase the antigen presentation on DCs and B cells.

1.3 BONE

The skeleton is a rigid structure that provides support for our body and enables mobility. The bone can be divided into two types; the hard cortical bone and the cancellous trabecular bone. Cortical bone is found predominantly in the appendicular skeleton, while trabecular bone is primarily found in the axial skeleton.

1.3.1 BONE REMODELING

The whole skeleton is renewed during a period of 10 years[44]. The breakdown of old bone and building of new bone is called the bone remodeling process. When these are in balance there is a bone homeostasis. There are three cell types in the bone. Osteoclasts break down or absorb bone, osteoblasts build up new bone, and osteocytes reside in the bone matrix and regulate the bone turnover. Osteoclasts secrete hydrochloric acid and proteolytic enzymes to dissolve bone mineral and the bone matrix. The main task for osteoblasts is to secrete type I collagen and regulate the mineralization of the bone matrix.

Receptor activator of nuclear factor-κB (RANK) is expressed on osteoclastic precursor cells, and when RANK ligand (RANKL) binds to RANK, osteoclast differentiation and activation is facilitated[45]. The RANKL/RANK binding activates downstream signaling pathways that regulate the expression of osteoclast genes. Osteoprotegerin (OPG) is a decoy receptor for RANKL able to inhibit osteoclast differentiation by hindering RANKL to bind to RANK. The RANKL:OPG ratio is therefore an important variable in regulating bone resorption. Cytokines such as IL1, IL6, and TNFα stimulate osteoclastogenesis[46]. IL-17, induces the expression of RANKL, reviewed by Lee[47]. Osteocalcin (OCN) is produced by osteoblasts and is often used as a bone-remodeling marker for osteoblast activity. The runt-related transcription factor 2 (RunX2) is essential to initiate osteoblast differentiation[48].

1.3.2 OSTEOPOROSIS

Osteoporosis is estimated to affect 200 million people worldwide and is defined as a “systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture”[49].

The diagnostic criterion for osteoporosis is a bone mineral density (BMD) with a T-score of -2.5 or lower[50]. The T-score corresponds to the number of

standard deviations from the mean value of a healthy 30-year old individual. Further, a BMD T-score of -1 to -2.5 corresponds to osteopenia, which is a precursor to osteoporosis.

The pathophysiological mechanism for osteoporosis can be simplified as a disrupted balance in the bone remodeling cycle, where bone resorption is increased relative to bone formation. Trabecular bone has a greater surface area compared with cortical bone, and is therefore affected first during osteoporosis development[51]. However, also cortical bone is affected by osteoporosis. There are over 8.9 million osteoporotic fractures occurring in the world each year[52]. Treatment of osteoporotic fractures is expensive, and preventing osteoporotic fractures is therefore of big interest for both the health care systems and our society.

Due to menopause and loss of endogenous estradiol, women are more prone to osteoporosis than men. In Sweden 21% of women and 6% of men between the ages of 50 to 84 suffer from osteoporosis[53]. The consequence of estrogen deficiency is that the bone resorption is faster than the bone formation and a resulting net bone loss and susceptibility to fractures. After a few years, the bone loss occurs in a slower manner.

1.3.3 INFLAMMATION-INDUCED BONE LOSS

Many autoimmune diseases are associated with increased risk for osteopenia, osteoporosis, and fractures. Pro-inflammatory cytokines, such as IL-1, IL-6, and TNFα, are elevated during inflammation. Among many others, these cytokines are highly involved in the bone remodeling process, stimulating osteoclastogenesis, as previously mentioned. TNFα and IL-1 can synergistically upregulate RANKL expression, thereby affecting the OPG/RANKL ratio to promote osteoclastogenesis[54]. And a study with transgenic mice overexpressing IL-6 showed an increased osteoclasto-genesis[55].

The link between glucocorticoid treatment and increased risk for osteoporosis is well studied. Due to its strong anti-inflammatory effects, glucocorticoid treatment is common to use during several inflammatory diseases. However, in a study by Steinbuch et al, patients treated with glucocorticoids had an almost 17-fold increased risk for vertebral fractures and 7-fold increased risk for hip fractures[56]. And observational studies show fractures in 30-50% of the patients on long-term glucocorticoids[57]. Glucocorticoids affect the bone

homeostasis in several ways. For example, they direct mesenchymal stem cells towards adipocytes and reduce the lifespan and function of osteoblasts[58].

1.3.4 TREATMENT OF OSTEOPOROSIS

There are multiple choices of osteoporotic drugs on the market. The risk:benefit ratio needs to be assessed for each individual. Which drug to be used depend on many factors, e.g. age, underlying diseases, and what other medications the patient is on. All osteoporosis treatments are prescribed together with calcium and vitamin D supplementation, and the majority of the available drugs have the indication postmenopausal osteoporosis.

There are two main categories of osteoporotic drugs; anabolic and antiresorptive. Anabolic treatments focus on increasing the bone-building properties, such as parathyroid hormone, which can be administered intermittently to enhance bone formation. Antiresorptive drugs include bisphosphonates, denosumab, calcitonin, estrogens, and selective estrogen receptor modulators (SERMs).

Bisphosphonates have been used for more than 50 years and remain the first-line treatment for osteoporosis. The main function of bisphosphonates is to promote osteoclast apoptosis[59]. There are, however, some concerns regarding bisphosphonates. There are reports indicating risk for osteonecrosis of the jaw as well as atypical femur fractures[60]. However, both of these risk factors are rare and have been debated. Bisphosphonates are not recommended in fertile women[61], since it has been shown in animal studies to induce fetal abnormalities[62].

Denosumab consists of monoclonal antibodies against RANKL, and treatment for 10 years resulted in low fracture incidence, improved BMD, and few adverse events[63]. However, that study lacked a long-term control group but an estimation analysis revealed a long-term efficacy with denosumab to reduce major osteoporotic fractures and hip fractures in osteoporotic postmenopausal women[64]. Also, there is a lack of safety data regarding denosumab for patients on immunosuppressive agents[65]. Calcitonin is a hormone that binds to osteoclasts and inhibits bone resorption, however it was found to be less effective than bisphosphonates in increasing hip and spine BMD[66] and more data are needed to verify its ability to prevent fractures. Estrogens and SERMs are the osteoporotic drugs of interest in this thesis. They will be described in section 1.4.

1.3.5 OSTEOPOROSIS IN SLE

Osteoporosis is a common, but yet relatively neglected secondary complication in women with SLE. The risk for osteoporosis is higher in women with SLE compared with an age-matched healthy population[67]. They also have a nearly 3-fold increased risk for vertebral fractures[68].

There are multiple possible explanations for this increased risk for osteoporosis and associated fractures in SLE patients. These include earlier menopause, systemic inflammation, sun avoidance (leading to vitamin D insufficiency), renal failure and the use of glucocorticoids[69]. The levels of SLE-induced organ damage have also been shown to influence BMD in lupus patients[70].

Osteoporosis is however not only a problem in postmenopausal SLE; in one study pre-menopausal SLE patients were shown to have a lower BMD compared with healthy controls[71] and in another presented with osteopenia[72].

There are currently no anti-osteoporotic drugs with the indication secondary osteoporosis in SLE.

1.4 ESTROGEN

1.4.1 INTRODUCTION TO ESTROGEN

Estrogens are a group of sex steroid hormones. There are three main endogenous estrogens; estrone (E1), estradiol (E2), and estriol (E3). E2 is the most potent estrogen and the most prominent in females between puberty and menopause. At menopause, the levels of E2 decrease and E1 is the major estrogen. E3 is present at high levels during pregnancy. E2 will be the estrogen in focus in this thesis.

The estrogen biosynthesis pathway starts with cholesterol, and after several steps estrogens are formed from androstenedione and testosterone, *Figure 3*.

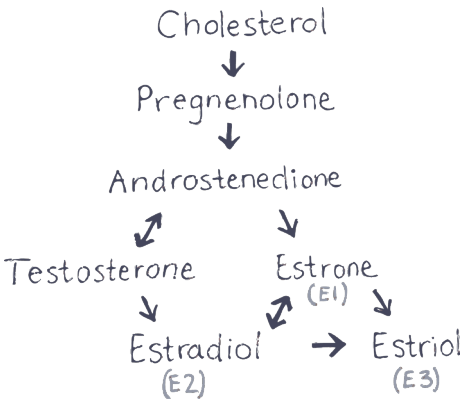

Figure 3. Simplified biosynthesis pathway of estrogens.

1.4.2 ESTROGEN RECEPTORS AND SIGNALING

Estrogens are hydrophobic and can therefore diffuse easily through the cell membrane, where they act through estrogen receptors (ERs) that bind to DNA sequences and activate gene transcription. ERs are nuclear receptors and present on most cells in the immune system[73, 74]. There are two ERs; ERα and ERβ, which are encoded by different genes and expressed to a different extent in different tissues. For example, ERα is the main ER in bone, mammary glands, and uterus, while both ERα and ERβ are expressed in the ovaries[75].

The main pathways for estrogen signaling are the classical and the non-classical. The classical pathway contains ligand binding of estrogen to the ER, and dimerization of the complex. This complex then translocate into the nucleus to function as a transcription factor by binding to estrogen-response elements (ERE) in DNA and thus affecting the gene transcription. In the non-classical pathway the dimerized estrogen-ER complex bind to other transcriptional factors, such as specificity protein 1 and activator protein 1. There are also estrogen pathways resulting in non-genomic effects; signaling through membrane-bound ERs or membrane-bound G protein-coupled estrogen receptor-1[76].

Apart from regulating female reproduction, estrogen has a major impact on bone, as well as the nervous system, the cardiovascular system and the immune system. Estrogens are present in the serum, and the bioavailability of estrogen depends on the levels of sex hormone-binding proteins.

1.4.3 ESTROGEN AND THE IMMUNE SYSTEM

Estrogen has multiple effects on the immune system. It can activate the immune system by for example stimulating B cells to increase their antibody production and decrease macrophage and dendritic cell apoptosis[77]. Estrogens also increase cytokines such as IL-2, IL-10, and IFNγ[77], while decreasing pro-inflammatory cytokines such as IL-1, IL-6, IL-17, and TNFα[78, 79]. Estrogen is further known to inhibit both B- and T-lymphopioesis[80, 81]. Although these are some of the main effects of estrogen, it is important to acknowledge that estrogen can have contradictive effects depending on various conditions, such as which target organ, what immune stimuli are present, and the concentrations of estrogen, reviewed by Straub[82]. Estrogen also have different, and even opposite, effects on the immune system depending on the dose[83, 84]. For example a low dose estrogen can enhance production of pro-inflammatory cytokines, while a high dose estrogen can inhibit the same cytokines. Estrogen is known to have a U-shaped, (non-monotonic) dose response curve in some tissues. The mechanism for this phenomena is related to the interactions between the ligand and the receptors[85].

1.4.4 MENOPAUSE AND HRT

As a part of the natural aging process, women go through menopause. The endogenous production of estradiol decreases and many of these women will suffer from menopausal symptoms. These include for example vasomotor symptoms (VMS; often described as hot flushes), urogenital problems, and sleep disruptions. The estrogen deprivation can after a few years into menopause eventually cause osteoporosis. Hormone replacement therapy (HRT) containing estrogen, is efficient in counteracting VMS as well as the development of osteoporosis. In HRT estrogen is given in combination with progestin to protect against endometrial cancer, but progestin has also had some negative side effects, such as increasing the risk for breast cancer[86].

In the women's health initiative (WHI) study, more than 16000 postmenopausal women were treated with estrogen plus progestin, estrogen alone, or placebo[87]. The results from that study showed an increased risk for breast cancer and no protective effects on coronary heart disease in the estrogen plus progestin group. After this study, general HRT treatments were no longer recommended. Later, the WHI study has been reevaluated and showed that short-term hormone therapy in younger patients, early after menopause onset, could benefit from the treatment[88]. Recently, it has also been suggested, that HRT should be reconsidered as a primary option for treatment of postmenopausal osteoporosis when the individual risk/benefit is favorable, reviewed by Gosset et al[89]. Especially early post-menopausal women, within 10 years of menopause onset, with no contradictions and great lifetime risk for fractures might benefit from this treatment.

1.4.5 ESTROGEN AND BONE

The bone destructive properties of estrogen deficiency speak its clear language, and the bone-protective effects of estrogen are pronounced.

The estrogen deprivation following menopause will increase both osteoclastic and osteoblastic activities. However, the net result is higher osteoclastic activities, which results in a net bone loss that increases the risk for fractures. There are multiple mechanisms in which estrogen exerts its protective effects on bone[90]. For example estrogen reduces bone resorption by lowering the sensitivity to parathyroid hormone or by increasing calcitonin. The bone resorption is also decreased due to an increased calcium resorption from the intestine and a reduced calcium excretion from the kidney[90].

1.4.5.1 DIRECT EFFECTS OF ESTROGEN ON BONE

Estrogen also has direct effects on bone remodeling, reviewed by Khosla et al[91]. Estrogen deficiency increases osteocyte apoptosis, which in turn increases bone remodeling and resorption. Further, estrogen directly targets osteoclasts and induces apoptosis and increases RANKL-induced osteoclast differentiation. Estrogen also has direct effects on osteoblasts by inhibiting apoptosis and increasing their life-span.

1.4.5.2 INDIRECT EFFECTS OF ESTROGEN ON BONE

Some of the indirect effects of estrogen on bone remodeling contain the estrogenic effects on cytokines that in turn affect bone remodeling. For example, estrogen deficiency leads to an increase in IL-1 and TNFα. By using drugs to block each of these cytokines, bone resorption markers were markedly reduced in postmenopausal women[92]. Estrogen deficiency also results in up-regulated macrophage colony-stimulating factor (M-CSF), which in turn enhances osteoclast formation. Treatment of ovariectomized mice with an M-CSF neutralizing antibody resulted in a prevented bone loss[93]. Further, the levels of IL-6 are also increased by estrogen deprivation, and thereby also the number of osteoclasts is increased. This is important since serum IL-6 is a predictor for osteoporosis in young postmenopausal women[94].

Removal of endogenous estrogen by ovariectomy of mice showed how the upregulation of IFNγ caused upregulation of MHC class II and thus increased antigen presentation by macrophages, T cell activation, and the lifetime of activated T cells[95]. Estrogen deprivation in postmenopausal women also upregulated RANKL expression on preosteoblastic stromal cells, T cells, and B cells and it correlated with levels of bone resorption markers[96]. A study by Onal et al investigated the ovariectomy-induced bone loss in conditional knock-out mice lacking RANKL in B or T cells[97]. They found no impact by knock-out in T cells, while mice with B cells lacking RANKL had protection from trabecular bone loss. This protective effect was associated with lower numbers of osteoclasts. However, no protection was found regarding cortical bone loss.

1.4.6 SERM

Selective estrogen receptor modulators (SERMs) are compounds that are high-affinity ligands to ERs and their activity can range from being agonistic to antagonistic. What effect a SERM will have on a specific tissue depends on multiple circumstances, e.g. which co-activators are present, competition by other receptors, and whether the gene promoter accepts the conformation.

The first SERM, Tamoxifen, had already been on the market for use in hormone-dependent breast cancer and was considered a pure anti-estrogen when it was discovered that this drug had estrogen agonistic effects in bone tissue[98]. A new class of drugs was found. The objective for developing more SERMs, and increase their area of use, is to provide drugs with a better benefit/risk ratio compared with HRT. SERMs are for example used in treatment of postmenopausal osteoporosis and some cancers.

There have been over 70 SERMs developed[99] but in this thesis, only a few will be mentioned. The first generation SERM Tamoxifen is, as previously mentioned, used for ER-positive breast cancer, however it increases the risk for malignant endometrial lesions[100]. The second generation of SERMs include Raloxifene, which has a bone protective effect comparable to estrogen, without stimulating the endometrium[101] and is used for treatment and prevention of postmenopausal osteoporosis, as well as prevention of invasive breast cancer[102]. However, Raloxifene treatment increases the risk for venous thromboembolism[103] and hot flushes[104]. The third generation of SERMs includes for example Lasofoxifene and Bazedoxifene (Bza). Lasofoxifene was approved for treatment of osteoporosis and shown to prevent non-vertebral fractures[105]. However, it was never marketed and the approval was withdrawn. Further, Lasofoxifene was associated with an increased risk of venous thromboembolic events[106]. Bza is approved for treatment of postmenopausal osteoporosis. Bza is the first SERM on the market to prevent both vertebral and non-vertebral fractures[107, 108]. The five-year follow-up analysis on Bza treatment revealed an overall favorable safety profile regarding adverse events such as breast and endometrial cancer[108].

1.4.7 TSEC

A tissue-selective estrogen complex (TSEC) is constructed of a SERM combined with estrogen. The first TSEC to be approved and used in the clinic is Bazedoxifene combined with conjugated estrogens (CE). The idea of the TSEC consisting of Bza + CE is to have estrogen's positive effects on bone and VMS while the negative side effects on breast and endometrium are antagonized by Bza. TSEC was approved in the US 2013 and EU 2015 for treatment of VMS and prevention of postmenopausal osteoporosis. These approvals were based on the five phase-three trials called the Selective estrogens, Menopause, And Response to Therapy (SMART) trials. In the SMART trials, the safety and efficacy of the treatment were thoroughly investigated. Additionally, quality of life was assessed, which is of significant impact to postmenopausal women suffering from VMS and osteoporosis. One of the advantages of TSEC is that, unlike estrogen, it does not have to be given with progestin, thereby reducing the risk for nuisance bleeding[86].

The TSEC consisting of estrogen + Raloxifene has also been investigated. However, Raloxifene was associated with endometrial thickening and, compared with Bza, Raloxifene had a more prohibitive effect on the CE cofactor recruitment, thus hindering the desired estrogenic effects[109].

1.4.8 ESTROGEN AND SERM IN SLE

The correlations between hormones and SLE are a complex subject. For example, pregnancy can induce lupus disease flares[110] while contraceptive hormonal treatment does not increase the risk for flares in pre-menopausal SLE patients with stable disease[111]. Estrogen has been known to worsen the SLE disease in MRL/*lpr* mice[112], while the SERM Tamoxifen, ameliorated the disease severity[113]. In a study by Zhang et al, NZB/W F1 mice were treated with the combination of Raloxifene and E2, which showed a lowering of kidney damage and levels of anti-dsDNA antibodies compared with E2 treatment alone[48].

HRT treatment studies in SLE patients have also been found contradictive. For example, in a study by Buyon et al in SLE patients, HRT treatment did not significantly induce any severe flares versus placebo[114], while other studies show it can induce SLE flares and should only be used in SLE patients with inactive disease[115].

Neither Bza nor TSEC has been investigated for treatment of osteoporosis in SLE.

2 AIM

The overall aim of this thesis was to investigate TSEC as a potential anti-osteoporotic drug in a mouse model of postmenopausal lupus. To achieve this goal, the work was divided into more specific aims;

1. To characterize the bone loss in ovariectomized (OVX) MRL/*lpr* mice and evaluate the suitability for the MRL/*lpr* mouse strain to be used for experimental studies on osteoporosis in postmenopausal lupus. (Paper I)

2. To investigate whether osteoporosis in OVX MRL/*lpr* mice can be treated with TSEC. (Paper II)

3. To study the effects of TSEC on B-cell development and function in healthy mice. (Paper III)

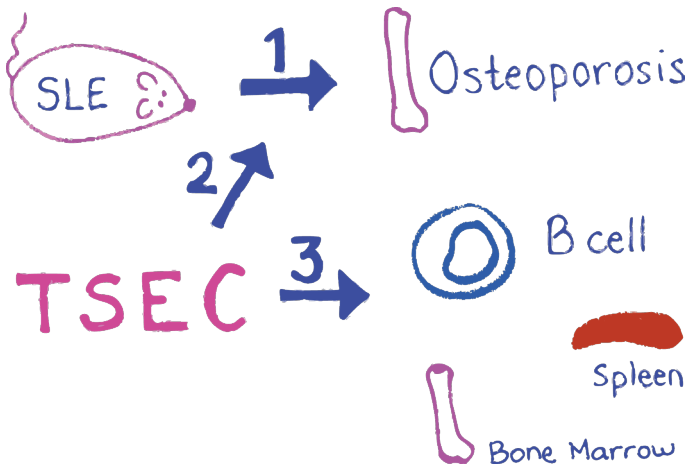

Figure 4. Illustration of the specific aims.

3 METHODOLOGICAL CONSIDERATIONS

In this section the methods used in this thesis will be presented, however a more detailed description of the experiments can be found in Paper I, Paper II, and Paper III.

3.1 MOUSE MODEL

Mouse models are a valuable tool in the search for new medical treatments or new indications for drugs available on the market. SLE is a complex disease with potential involvement of in principle every organ in the body. In vitro studies do not take this into consideration, and by using in vivo studies we lay the groundwork for what could be of interest to study in a setting involving SLE patients.

3.1.1 MRL/*lpr* MICE

The MRL/MpJ-*Fas*^{lpr}/J (MRL/*lpr*) mouse model was found by strain crossing experiments by Murphy and Roths[116]. MRL/*lpr* mice have a homozygous mutation in the gene encoding Fas protein, resulting in a massive lymphoproliferation. The Fas mutation causes MRL/*lpr* mice to spontaneously develop lupus symptoms such as production of autoantibodies, systemic inflammation, and glomerulonephritis. The lupus disease is more severe in female MRL/*lpr* mice compared with males and the development of lupus disease in these mice is rather rapid compared with other mouse models of lupus, thus allowing for a short treatment period at a young age. MRL/*lpr* mice were used in Paper I and Paper II.

3.1.2 MRL/++ MICE

MRL/MpJ (MRL/++) is the parent and congenic control strain for MRL/*lpr* mice. With time MRL/++ mice also develop symptoms of lupus disease to some extent, but the progression is much slower, and their survival time is much longer. When MRL/*lpr* mice die from lupus symptoms, the MRL/++ mice at corresponding age have no indications of lupus disease. MRL/++ mice were used in Paper I as controls to MRL/*lpr* mice.

3.1.3 C57BL/6 MICE

The C57BL/6 mouse strain is one of the most commonly used inbred strains in medical research. It was the also the first mouse strain to have its entire genome sequenced. This strain was used in Paper III to evaluate the effects of TSEC treatment on B cell development in healthy mice.

3.2 OVARIECTOMY AND TREATMENTS

Mice do not undergo menopause as humans do, but we can mimic an estrogen-deficient postmenopausal state in mice by performing ovariectomy (OVX), which removes the endogenous estrogens.

In Paper II and Paper III, mice were injected subcutaneously five times per week for three weeks. The substances were E2, Bza, or TSEC (E2 + Bza) dissolved in miglyol oil. Vehicle (Veh) control treatment consisted of mygliol oil only. The use of inert oil allows the substances to be slowly released into the tissues of the mice.

3.3 URINE ANALYSES

MRL/*lpr* and MRL/++ mice (Paper I, II) were assessed for proteinuria and hematuria using dipsticks. The procedure encompasses restraining of the mouse and collecting a urine droplet on the dipstick. The color on the test surface is compared to a reference color chart provided by the manufacturer. Urine analyses were initiated at an early age in order to start measurements before any of the mice displayed proteinuria and hematuria, The analysis was performed twice weekly from the age of 10 weeks, since the mice were suspected to soon start displaying proteinuria and hematuria.

3.4 BONE ASSAYS

The mice skeleton was analyzed using different bones and different measuring techniques.

3.4.1 DUAL-ENERGY X-RAY ABSORPTIOMETRY

Dual-energy X-ray absorptiometry (DXA) is a technique using 2 laser beams at different wavelength. The soft tissues and bone absorb the laser beams differently and a two-dimensional image is created. Parameters obtained from

DXA include areal BMD (aBMD) and areal bone mineral content. DXA is used both in clinical practice and animal studies. In Paper II a Lunar PIXImus (Wipro GE Healthcare) densitometer was used. An area of interest was created; for whole body analysis this area was between the mouse neck and beginning of the tail. For analysis of the vertebras the area of interest was set to include lumbar vertebras L3-L6. The greatest advantage of this technique is that it can be performed on live mice during the experiments.

3.4.2 PERIPHERAL QUANTITATIVE COMPUTED TOMOGRAPHY

Peripheral quantitative computed tomography (pQCT) is a method that creates a three-dimensional image by rotating the laser around the sample, and thus a volumetric BMD can be obtained. The high resolution allows for a distinction between cortical and trabecular bone, such as measurements of cortical thickness and trabecular BMD. For the pQCT XCT Research M (version 5.4B; Norland), used in Paper I and Paper II, the bones need to be dissected before analysis.

3.4.3 HIGH-RESOLUTION MICRO-COMPUTED TOMOGRAPHY

In high-resolution micro-computed tomography (μCT) a three-dimensional measurement can be constructed by placing the specimen on a rotating plate, creating two-dimensional images that are combined into a three-dimensional image. By using μCT the image resolution is higher compared with pQCT; within the μm range. This technique allows for determination of the bone microstructure and parameters such as trabecular thickness, separation, and number. In Paper I and Paper II the 1172 μCT (Buker MicroCT) was used.

3.4.4 THREE-POINT BENDING

In order to measure the bone strength a three-point bending test was used. The tibias were placed on two supporters and loaded at the mid-tibia until failure. Measured parameters include bone strength (defined as maximum load at failure, Fmax), stiffness (resistance to deformation under load, i.e. the slope in the elastic state), and toughness (the amount of energy needed to cause material failure, i.e. the area under the stress-strain curve). The machine used in Paper I was an Instron 3366 (Instron) with software Bluehill 2, version 2.6.

3.5 IMMUNOLOGICAL ASSAYS

3.5.1 SERUM ANALYSES

Enzyme-linked immunosorbent assay (ELISA) is a commonly used method for serum analysis. In short, the method involves binding of the molecule of interest to coated plates, labeling the molecules with antibodies, and measuring absorption with a monochromatic xenon beam at a predetermined wavelength. We measured serum BAFF (Paper III), IL-6 (Paper I, II), anti-dsDNA antibodies (Paper I, II), IgG (Paper I, II), IgM (Paper II), C-terminal telopeptide of type I collagen (CTX-I, Paper I) and N-terminal propeptide of type I procollagen (P1NP, Paper I) using ELISA kits. Blood Urea Nitrogen was measured using a colorimetric assay kit.

3.5.2 FLOW CYTOMETRY

Fluorescence-activated cell sorting (FACS) was used in Paper III to determine the extent of different cell populations in mouse bone marrow and spleen using a FACSVerse (BD). We stained the cells with fluorochrome-labeled antibodies specific to surface epitopes of interest. In the flow cytometer, the cell suspension is converted into a beam of single cells, which is then exposed to lasers and the emitted light measured by detectors. The cells were analyzed for forward scatter versus side scatter as well as for which labeled antigens they present on their surface. Forward scatter is used to differentiate the cells depending on size, while side scatter differentiate the granularity, or complexity of the cell.

3.5.3 ELISPOT

The enzyme-linked immunospot (ELISPOT) assay was used to quantify the number of antibody-producing primary bone marrow and spleen cells in Paper III. The cells are isolated from fresh bone marrow and spleens respectively, diluted in three different concentrations, and added, in duplicates, to a plate pre-coated with capture antibodies for IgM, IgG, and IgA. The plates were incubated for 3.5 hours at 37 degree Celsius before secondary antibodies towards IgM, IgG, and IgA was added and incubated overnight. A developing substrate was used and there were spots visible to the eye. Each spot corresponded to a single antibody-producing cell. The dilution for each organ corresponding to approximately 20-50 spots per well was chosen. The wells were photographed and the spots were counted.

3.6 QUANTITATIVE POLYMERASE CHAIN REACTION ANALYSIS

In Paper I quantitative polymerase chain reaction (qPCR) was used to examine whether the cortical bone of OVX MRL/*lpr* had up- or down-regulated certain genes, that are associated with bone remodeling, compared with controls. mRNA was extracted from femoral shafts and reverse transcribed into cDNA. The cDNA was amplified and analysed for Runx2, Tnfsf11 (encoding RANKL), Tnfrsf11b (encoding osteoprotegerin, OPG), and Bglap (encoding osteocalcin). 18S ribosomal RNA was used as the internal standard and the delta-delta CT method was used to calculate which genes were up- or downregulated in the different groups.

3.7 STATISTICS

In Paper I and Paper III statistical analysis was performed using Statistical Package for the Social Sciences (SPSS, IBM). We checked for normality and used logarithmic transformations when needed for normal distribution. Analysis of covariance (ANCOVA) was used to adjust for covariates, which in these studies was for day-to-day variation at termination. Two groups were compared using Student's *t*-test, or Welch's t-test if Levene's test showed unequal variances (Paper I). Multiple groups were compared using Tukey's post-hoc test or Dunnett's T3 post-hoc test if Levene's test was significant (Paper III). Pearson's correlation test was used to check for correlations (Paper I).

In Paper II GraphPad Prism (Graph Pad software inc.) was used for statistical analysis. The groups were compared using one-way ANOVA and Sidak's post-hoc analysis. Log-Rank tests were used to compare the Kaplan-Meier curves for proteinuria and hematuria.

4 RESULTS

4.1 PAPER I

Female MRL/*lpr* and MRL/++ mice were OVX. They were monitored for lupus symptoms such as proteinuria and hematuria. After termination the skeleton was analyzed.

Main findings

- MRL/*lpr* mice had decreased total BMD in long bones, and decreased cortical thickness in both tibia and lumbar vertebra L5.
- The tibial cortical thickness was positively correlated with bone strength (measured using three point bending analyses).
- MRL/*lpr* mice had decreased trabecular thickness in both tibia and L5.
- qPCR analysis of cortical bone mRNA implied an elevated osteoclastic activity in MRL/*lpr* mice, as well as a decreased osteoblastogenesis and osteoblastic activity, compared with MRL/++ mice.

Conclusion

At the age of 21 weeks, OVX MRL/*lpr* mice presented with a more osteoporotic bone phenotype compared with OVX MRL/++ mice.

4.2 PAPER II

In this paper we investigated whether TSEC or Bza had a protective effect or the ability to treat osteoporosis in a model of postmenopausal lupus. OVX MRL/*lpr* mice were treated with E2, Bza, TSEC, or Vehicle during early or more active phases of disease progression. Different treatment doses were investigated and the mice were monitored for lupus symptoms and the skeleton analyzed during the experiment and at termination.

Main findings

- Treatment with E2, Bza or TSEC did not influence the lupus symptoms proteinuria or hematuria regardless of treatment doses or time-point for initiation of treatments. Nor did the treatment affect serum levels of anti-dsDNA antibodies, IL-6, or urea.
- Medium dose TSEC treatment, administered early in the lupus disease course, showed protective effects on trabecular bone.
- A high dose treatment of E2, but not Bza or TSEC, administered in early lupus, showed improvement on bone parameters.
- Using a low dose treatment of E2, Bza, or TSEC, administered in established lupus disease, did not protect the osteoporotic bone phenotype in MRL/lpr mice.

Conclusion

Using a medium dose TSEC early in the lupus disease protects the trabecular bone in OVX MRL/*lpr* mice without affecting proteinuria, hematuria or serum parameters.

4.3 PAPER III

Healthy C57BL/6 mice were OVX operated and treated with E2, Bza, or TSEC. Flow cytometry was performed on bone marrow and spleen cells, and the different development stages of the B cell lineage was analyzed.

Main findings

- The uteroproliferation by E2 was completely blocked by adding Bza to the treatment; e.g. using TSEC.
- TSEC treatment resulted in an inhibition of B cell differentiation from the Pre-BI stage in bone marrow, until the development into marginal zone B cells in the spleen.
- The number of antibody-producing B cells in bone marrow was increased in mice treated with TSEC.
- The B cell differentiation and number of antibody producing B cells was comparable between E2 and TSEC treatment.

Conclusion

When healthy OVX mice were treated with TSEC the unwanted uteroproliferation by E2 was antagonized by Bza, while the addition of Bza to E2 treatment did not affect the B lymphopoiesis.

5 DISCUSSION

Patients with SLE have a decreased quality of life compared with the healthy population[117]. But they don't just have to tackle the many complications associated with the disease itself, they are also at high risk for secondary complications, such as cardiovascular disease and osteoporosis. The elevated fracture risk associated with osteoporosis adds to the patient's pain, disability, and even risk of death. Why the risk of osteoporosis is elevated in these patients is yet not completely known, but for example autoimmune inflammation, hormonal factors, and SLE-induced organ damage can be possible influencing factors[72, 118]. Osteoporosis results in a high economical burden on our society due to high numbers of incident fractures and long hospitalizations[52]. With the increasing aging of the population, the burden of the disease is expected to continue to increase. Osteoporosis is a disease where an economical net profit can be reached by prevention.

Women are affected to a higher degree than men of both SLE and osteoporosis. And even though the association between SLE and osteoporosis has been known for about 30 years now, there are still no optimal treatment options available for these patients. The upcoming newer treatments for osteoporosis have not been systematically tested in SLE, and this patient group needs to be included in drug advances that are made.

In order to create possibilities for testing new drugs, or drugs available but not verified in these patients, we established an animal model for postmenopausal lupus-associated osteoporosis. This was the work performed in Paper I. We chose the MRL/*lpr* mouse model due to several reasons; the display of many autoimmune SLE-like symptoms (such as the production of anti-dsDNA antibodies and kidney involvement) and due to its rapid disease course, which could be of advantage for future short term treatment studies. A study by Schapira et al, examined the bone tissue metabolism of MRL/*lpr* and MRL/++ mice and found a decreased bone formation in MRL/*lpr* mice and suggested this strain to be suitable for studies concerning skeletal changes in SLE[119]. In Paper I we used pQCT and μCT analyses to provide further evidence of the osteoporotic bone phenotype in the MRL/*lpr* mouse model. As mentioned, mice do not enter menopause, but by removing the ovaries an estrogen-deficient post-menopausal state can be induced. In the

first paper, we could conclude that the ovariectomized MRL/*lpr* mouse had an osteoporotic bone phenotype caused by the lupus disease itself.

An interesting question is; how do we know that the osteoporotic bone phenotype was caused by the lupus disease and not only due to OVX? Since we compared OVX MRL/*lpr* and OVX MRL/++ mice, the difference between these two groups lies in whether they have lupus disease or not. We also examined the bone properties of sham operated MRL/*lpr* and sham operated MRL/++ mice, and found a decreased bone mass in MRL/*lpr* mice (unpublished data). Thus, we concluded that MRL/*lpr* mice have an osteoporotic bone phenotype regardless of whether ovariectomy is performed or not.

With the model for osteoporosis in postmenopausal lupus established, it was time to evaluate the drug of choice, TSEC (E2 and Bza), in these mice (Paper II). As previously mentioned, TSEC has the ability to have agonistic effects on bone, while other tissues that are usually negatively affected by estrogen, such as endometrium, will be safe due to the antagonistic effects of Bza. Paper II focused on the outcomes regarding the skeleton when OVX MRL/*lpr* mice were treated with TSEC. In this study, TSEC treated mice were compared with Vehicle-treated mice as well as with mice treated with only E2 or Bza. We found bone-deteriorating effects mainly on trabecular bone and it was ameliorated by TSEC treatment. Also, treatment with Bza or E2 alone had a protective effect. This is in concurrence with earlier studies in both healthy mice and mice with collagen-induced arthritis[120, 121]. The trabecular bone loss is more rapidly induced and estrogen deficiency usually has a stronger effect on trabecular compared with cortical bone. The mice in Paper II were treated for three weeks only and directly terminated, but we speculate that cortical effects on the bone would be seen given a longer treatment time.

In comparison, the bone deteriorating effects seen in OVX MRL/*lpr* mice compared with OVX MRL/++ mice were more pronounced in cortical bone, however also the trabecular bone was affected (Paper I). In SLE patients, it is cortical bone parameters that are affected to a larger extent, compared with trabecular bone parameters[122]. In Paper I, qPCR of cortical bone showed that MRL/*lpr* mice had down-regulated mRNA expression of genes associated with osteoblast differentiation and activity. Interestingly, patients with SLE were suggested to have an impaired osteoblast differentiation, based on a

study on bone marrow mesenchymal stem cells from SLE patients[123]. However, the microarchitecture of trabecular bone has become of increasing importance in the clinic for SLE patients, since they present with vertebral fractures without meeting the criteria for osteoporosis[124]. Measuring the trabecular bone score might therefore be of high importance for SLE patients[125].

Considering the fact that osteopenia and osteoporosis are found already in pre-menopausal SLE patients, one could argue about the choice to ovariectomize the MRL/*lpr* mice in this work. However, osteoporosis is exacerbated by estrogen deficiency, which leads to additional fracture risk and thus a condition that definitely requires preventative treatment. Also, the anti-osteoporotic drugs on the market are with the indication postmenopausal osteoporosis and new prospective drugs are investigated in the postmenopausal patient group. Hence it is likely that also a future study including anti-osteoporotic drugs for SLE patients would be conducted in the postmenopausal patient group. Therefore, the OVX MRL/*lpr* mouse serves its purpose for preclinical investigations in the research field of osteoporosis in postmenopausal lupus. Additionally, the OVX mouse model is well established for studies of postmenopausal osteoporosis, and with our study it can now also be used in the setting of lupus disease.

As a part of elucidating the effects of TSEC treatment on the immune system, we investigated the effects on B cells in healthy C57BL/6 mice (Paper III). In the TSEC-treated group, Bza blocked the uteroproliferative effect by estrogen, while the estrogenic effects on B cell differentiation and antibody production remained. Specifically, TSEC treatment resulted in an inhibition of B cell differentiation from the Pre-BI stage in bone marrow, until the development into marginal zone B cells in the spleen, which were increased compared with Vehicle treatment. TSEC treatment also resulted in elevated numbers of antibody producing B cells in bone marrow compared with Vehicle treatment. These were the same effects as treatment with only E2 had on the B cell development and number of antibody producing B cells. Bza treatment alone resulted in a significantly lower number of antibody-producing B cells in the bone marrow compared with E2 treatment, which was in line with a previous study from our lab performed in healthy mice[80]. The administered doses of E2 and Bza in Paper III were low. Whether Bza can block or reduce the E2-induced increase in antibody-producing B cells

could be further studied by repeating this study using a TSEC with higher doses of E2 and Bza.

Since Bza treatment does not increase the number of antibody-producing B cells like E2, it was suggested by Bernardi et al as treatment of osteoporosis in SLE[80]. As previously mentioned, E2 has for long been associated with disease-worsening effects on lupus. However, during the years contradictory findings have been reported. For example, a short course HRT treatment did not increase the risk of severe flares compared with placebo[114], and patients with stable disease had no increased risk of flares when administered oral contraceptives[111]. In a study by Mok et al, SLE patients with glucocorticoid-induced osteoporosis were treated with the SERM Raloxifene, which improved the lumbar spine BMD without increasing the rate of disease flares[126]. Further, lupus-prone NZB/W F1 mice were treated with the TSEC Raloxifene plus E2, which resulted in lower titers of anti-DNA antibodies and reduced kidney damage[48]. In addition, Raloxifene was suggested to have antagonistic effects on auto-reactive B cells.

In Paper III, the number of antibody-producing B cells was comparable between TSEC treatment and E2 treatment, and in Paper II there were no differences between the treatment groups regarding antibody levels in serum (except for IgM). Also, there were no differences in proteinuria or hematuria with any of the treatments. Hence the results from Paper II suggest that the medium dose TSEC is able to exert its bone protective effects without worsening the lupus disease course. However, further studies are needed to provide more detailed data regarding the effects of TSEC and Bza treatment on the lupus disease. Such studies could include histological examination of for example the kidney to assess the level of glomerulonephritis as well as the degree of T cell infiltrates and immune complexes.

To conclude, our studies found that OVX MRL/*lpr* mice have a lupus-induced osteoporotic bone phenotype. By treating the mice with TSEC early in the disease course, we could protect against trabecular bone loss. The TSEC treatment did not exacerbate the lupus disease according to the parameters analyzed in this work. Hopefully, in the future, this work will benefit SLE patients suffering from osteoporosis.

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