

# **The role of S100A4 protein as a regulator of inflammation and bone metabolism in experimental arthritis**

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Gothenburg 2011



To my family



# ABSTRACT

S100A4 belongs to the family of calcium-binding S100 proteins and modulates cell proliferation, cytoskeletal rearrangement, cell motility, and angiogenesis. Increased levels of S100A4 expression correlate with high incidence of metastasis of cancers. Up-regulation of S100A4 protein is demonstrated in synovial tissue and in plasma of rheumatoid arthritis (RA) patients compared with osteoarthritis, and the elevated expression of S100A4 is associated with increased disease activity in patients with RA. Dichloroacetate (DCA) was shown to have a potent anti-tumour effect by facilitating apoptosis and inhibiting proliferation. The aims of this thesis are to investigate contributions of S100A4 in experimental models of septic arthritis and antigen-induced arthritis, and in bone formation using S100A4KO mice. In addition, we also aim to find out the impact of DCA on collagen type II-induced arthritis.

Our studies showed that S100A4 deficiency resulted in reduced joint inflammation and cartilage/bone destruction in both septic and antigen-induced arthritis in mice. Additionally, in septic arthritis, S100A4KO mice had less bone loss and showed a lower bacterial load in the kidneys. S100A4 deficiency resulted in changed pattern of adhesion molecules. In antigen-induced arthritis, S100A4 deficiency resulted in reduced intensity of arthritis and significantly lower frequency of bone destruction, supported by fewer numbers of CD4+ T cells and CD19+CD5+ B cells accumulated in synovia and spleen compared with WT mice. Smaller populations of CD4+ and CD8+ T cells in spleen of S100A4 deficient mice were accompanied by reduced productions of INF- $\gamma$  and IL-17A, and lower expression of Th17 transcription factor ROR $\gamma$ t. Difference in the severity of arthritis was observed in female mice in septic arthritis and in male mice in antigen-induced arthritis. To assess the role of sex hormone on bone, we analysed BMD in S100A4KO and WT mice. S100A4KO mice had higher total BMD and female mice displayed more cortical bone content compared with WT mice. Following ovariectomy (OVX), both S100A4KO and WT mice lost BMD. However, cortical bone loss was more pronounced in S100A4KO mice than in WT supported by high CTX-I level. The loss of trabecular bone was similar in S100A4KO and WT mice. DHEA treatment resulted in a significant increase in the trabecular and cortical BMD both in WT and S100A4KO mice. This increase of BMD was lower in S100A4KO mice. The collagen-type II arthritis model was employed to study the potential effect of dichloroacetate (DCA) treatment on experimental arthritis. Our results showed that mice treated with DCA had a slower onset of CIA, and significantly lower severity and frequency of joint inflammation and cartilage/bone destruction compared with water-treated controls. Moreover, DCA prevented arthritis-induced loss of cortical mineral density. The beneficial effect of DCA was present only in female DBA/1 mice. DCA treatment on the OVX mice did not protect from the development of arthritis, indicating that effect of DCA is potentially estrogen-dependent.

In conclusion, our studies demonstrate that S100A4 plays an important role in inflammation and bone metabolism in experimental arthritis. S100A4 deficiency protects against inflammation and cartilage/bone destruction in staphylococcal and antigen-induced arthritis by changing the expression of adhesion molecules, affecting lymphocyte maturation and functions. S100A4 is a regulator of bone formation in both estrogen sufficient and deficient mice. Our studies indicate that S100A4 protein can be a therapeutic target in arthritis and osteoporosis. We also demonstrate that DCA can be a potential anti-arthritis drug for female patients with RA.

**Key words:** S100A4, arthritis, animal model, bone, inflammation

**ISBN** 978-91-633-9593-2

# ORIGINAL PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals (I-IV)

- I. Li Bian, Paulina Strzyz, Ing-Marie Jonsson, Malin Erlandsson, Annelie Hellvard, Mikael Brisslert, Claes Ohlsson, Noona Ambartsumian, Mariam Grigorian, and Maria Bokarewa.  
**S100A4 deficiency is associated with efficient bacterial clearance and protects against joint destruction during staphylococcal infection**  
*Journal of Infectious Diseases* 2011 Sep;204(5):722-30.
- II. Li Bian, Mattias Svensson, Ing-Marie Jonsson, Malin Erlandsson, Karin Andersson, Mikael Brisslert and Maria Bokarewa.  
**S100A4 deficiency alleviates antigen-induced arthritis by regulating B cell dependent activity of T cells**  
*Submitted for publication*
- III. Malin Erlandsson, Li Bian, Claes Ohlsson, Maria Bokarewa  
**Metastasin S100A4 is a modulator of estrogens and DHEA effects on bone formation**  
*Manuscript*
- IV. Li Bian, Elisabet Josefsson, Ing-Marie Jonsson, Margareta Verdrengh, Claes Ohlsson, Maria Bokarewa, Andrej Tarkowski and Mattias Magnusson.  
**Dichloroacetate alleviates development of collagen II induced arthritis in female DBA/1 mice**  
*Arthritis Research & Therapy* 2009; 11(5): R132.

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## ABBREVIATIONS

APC	Antigen-presenting cell
Bcl-6	Transcriptional repressor B cell lymphoma 6
Blimp 1	B-lymphocyte-induced maturation protein 1
BMD	Bone mineral density
CFU	Colony-forming units
CIA	Collagen-type II induced arthritis
CTX-I	C-terminal telopeptide of type I collagen
CTX-II	C-terminal telopeptide of type II collagen
DCA	Dichloroacetate
DHEA	dehydroepiandrosterone
DTH	Delayed-type hypersensitivity reaction
FDC	Follicular dendritic cell
Foxp3	Forkhead box P3
GATA-3	GATA-binding protein 3
ICOS	Inducible T cell co-stimulator
IFN- $\gamma$	Interferon-gamma
IGF-1	Insulin-like growth factor 1
LPS	Lipopolysaccharide
mBSA	Methylated bovine serum albumin
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
OPG	Osteoprotegerin
OVX	Ovariectomy
pQCT	Peripheral quantitative computed tomography
RAGE	Receptor for advanced glycation end products
RANKL	Receptor activator of nuclear factor kappa-B ligand
RF	Rheumatoid factor
ROR $\gamma$ t	Retinoic acid receptor-related orphan nuclear receptor gamma t
T-bet	T-box expressed in T cells
T <sub>FH</sub>	Follicular B helper T cell
TSST	Toxic shock syndrome toxin

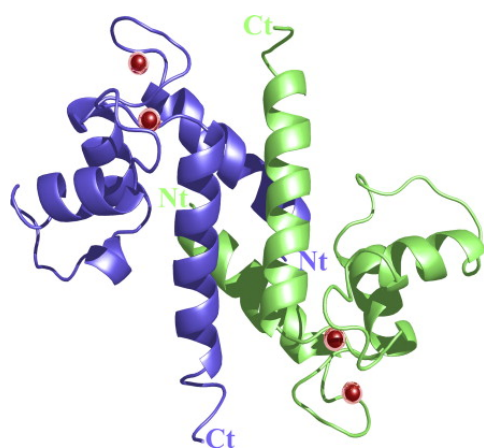


# INTRODUCTION

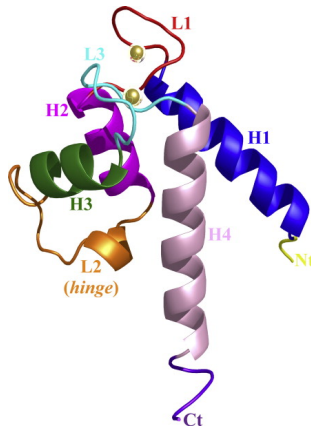
## S100A4 PROTEIN

### Molecule structure

S100A4 (also known as metastasin, FSP1, calvasculin, pEL98, 18A2, p9Ka and 42A) is a 101 amino acid protein with a molecular mass of 12 kDa (Garrett et al., 2006). It belongs to the S100 family of EF-hand calcium binding proteins. The family of S100 proteins is the largest subgroup of EF-hand calcium-binding proteins with 21 members currently. The name of “S100” is based on the observation that they are soluble in 100% saturated ammonium sulfate (Pathuri et al., 2008). S100 proteins normally exist as symmetric homodimers stabilized by noncovalent interactions between two helices from each subunit that form an X-type four-helix bundle (helices 1, 4, 1', 4') (Garrett et al., 2006, Moore, 1965) (Figure 1A, B). Each S100 monomer has two EF-hand  $\text{Ca}^{2+}$ -binding domains. The N-terminal EF-hand (also known as the S100 hand, pseudo EF hand and half EF-hand) is considered as a unique feature of S100 proteins, which harbors 14 amino acids and coordinates calcium weakly (helices 1, 2), while the C-terminal EF hand (also known as typical EF-hand and canonical EF hand) is composed of 12 amino acid residues and coordinates calcium with a higher affinity (helices 3, 4) (Figure 1B). The difference in structure between S100A4 and other S100 family members is that (1) the orientation of the S100A4 typical EF-hand is less like other S100 proteins and (2) the C-terminal loop following helix 4 is quite long and very basic in S100A4, which makes it particularly unique (Garrett et al., 2006, Pathuri et al., 2008) (Figure 1B).



**Figure 1A. Ribbon diagram of the  $\text{Ca}^{2+}$ -bound S100A4 homodimer.** Monomer A is shown in blue, monomer B is shown in green and the calcium ions are represented as red spheres. The N termini and C termini are labeled as Nt and Ct, respectively. Figure adopted with the permission from Elsevier: J Mol Biol (Pathuri P, 2008)

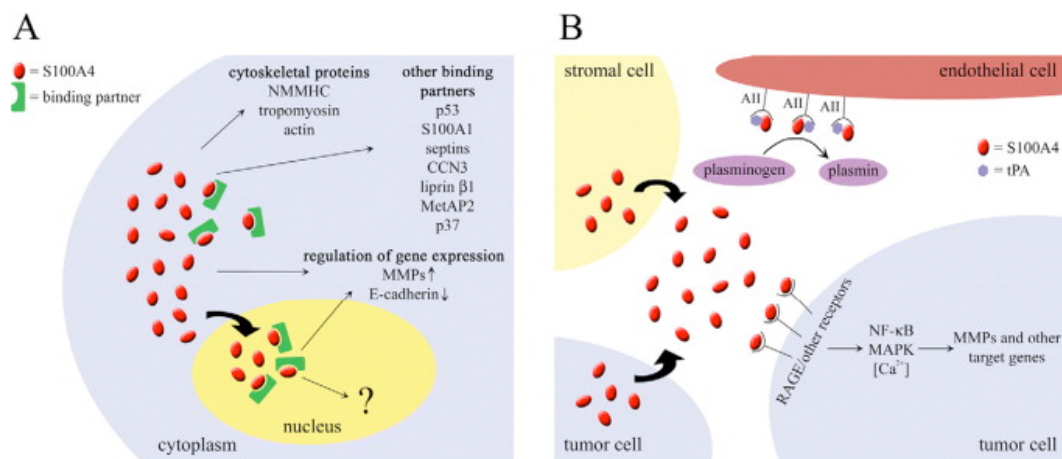


**Figure 1B. Ribbon representation of the Ca<sup>2+</sup>-bound S100A4 monomer.** Helix H1 is shown in blue, helix H2 is shown in dark pink, helix H3 is shown in green, helix H4 is shown in light pink, loop L2 is shown in red, loop L2 (hinge) is shown in orange and loop L3 is shown in cyan. The calcium ions are represented as gold spheres, the N terminus (Nt) is shown in yellow and the C terminus (Ct) is shown in purple. Figure adopted with the permission from Elsevier: J Mol Biol (Pathuri P, 2008)

Most S100 proteins are capable of binding calcium, including S100A4. The apo state is known as the inactive closed conformation, and the calcium-bound state is known as the active open conformation. Upon calcium binding, S100A4 and other dimeric S100 proteins undergo a conformational change in the typical EF-hand. The helix 3 in each S100A4 monomer rotates by  $\sim 60^\circ$  relative to helix 4 and results in the exposure of a hydrophobic binding pocket, which is capable of binding intracellular and extracellular proteins. This calcium-dependent conformational change is necessary for S100A4 to interact with its protein targets and generate a biological effect. The target binding enhances the calcium binding affinity of S100A4 (Garrett et al., 2006, Pathuri et al., 2008).

### **Biological functions**

Expression of S100A4 has a tissue-specific pattern and controls a variety of intra- and extracellular processes (Klingelhofer et al., 2009). S100A4 is expressed in normal tissue of rat, mouse and humans such as smooth muscle, brown adipose tissue, liver tissue, and epithelial cells. A relative high expression was found in the spleen, thymus, bone marrow, lymph nodes and blood, as well as T-lymphocytes, neutrophils, monocytes/macrophages and fibroblasts (Gibbs et al., 1995, Grigorian et al., 1994, Takenaga et al., 1994). Expression of S100A4 in normal cells is mostly in cytoplasm (Takenaga et al., 1994), while in tumor cells its expression is predominant in nuclei (Kikuchi et al., 2006).



**Figure 2. Intracellular function and extracellular functions of S100A4.** (A) Intracellular functions of S100A4. S100A4 is able to interact with cytoskeletal proteins, resulting in increased cell migration. Intracellular expression of S100A4 is also associated with transcriptional regulation of MMPs and E-cadherin. Apart from the functions mentioned above, the biological role of nuclear S100A4 still remains unknown (?). (B) Extracellular functions of S100A4. S100A4 is released from both tumor cells and stromal cells, through the interaction with annexin (AII) and tissue plasminogen activator (tPA) on the surface of endothelial cells, stimulating the conversion of plasminogen to plasmin, thus promoting angiogenesis. S100A4 also interacts with other cell surface receptors, such as RAGE to activate intracellular signal transduction cascades, such as mitogen-activated protein kinases (MAPK), NF- $\kappa$ B and intracellular  $[Ca^{2+}]$ , results in regulation of several target genes. Figure adopted with permission from Elsevier: Am J Pathol (Boye K, Maelandsmo GM, 2010)

S100A4 has no enzymatic activity and exerts its functions mainly through interaction with other proteins (Figure 2). Like other S100 proteins, *intracellular* S100A4 regulates mechanisms associated with calcium transport and cell homeostasis such as protein phosphorylation, transcriptional activity, and cytoskeletal rearrangement (Donato, 2001). Interaction of S100A4 with its intracellular cytoskeleton-associated targets, including non-muscle myosin (Kriajevska et al., 1994), tropomyosin (Takenaga et al., 1994) and liprin- $\beta$  (Kriajevska et al., 2002), facilitates the remodeling of acto-myosin filaments and focal adhesions and enhance **cell motility and invasion**. The interaction with liprin-  $\beta$  and E-cadherin also modulates **cytoskeletal dynamics, cell adhesion and detachment** (Keirsebilck et al., 1998, Kimura et al., 2000, Kriajevska et al., 2002). In addition, S100A4 has also been reported to regulate cell **proliferation** (Endo et al., 2002, Li et al., 2002) **and differentiation** (Li et al., 2002, Sherbet, 2009). Moreover, S100A4 interacts with tumor suppressor protein P53 and may provide a link between S100A4 and **apoptosis** (Grigorian et al., 2001) (Naaman et al., 2004).

When secreted into *extracellular* space, S100A4 exerts its cytokine-like effect. Several line of evidence show that S100A4 as an active extracellular factor influences gene expression by modulation of MAP kinases, ERK, p38, and JNK, and activation of transcription factors NF- $\kappa$ B, and p53. Activation of these pathways mediates the S100A4-driven stimulation of MMPs'

proteolytic activity, angiogenesis and cell survival (Hofmann et al., 1999, Klingelhofer et al., 2007, Novitskaya et al., 2000, Schneider et al., 2007, Yammani et al., 2006). By means of up-regulation of MMPs, S100A4 regulates **remodeling of extracellular matrix**. Down-regulation of S100A4 in osteosarcoma cells led to reduced expression of MMP2 and membrane-type 1 MMP, thus resulting in a reduced ability to migrate through matrigel-coated filters (Bjornland et al., 1999). The invasive ability of human prostate cancer cells is also stimulated by S100A4, at least partly through S100A4 mediating transcriptional activation of MMP 9 (Saleem et al., 2006). Moreover, correlation of S100A4 with epithelial-mesenchymal transition (EMT), a biologic process that allows a polarized epithelial cell changes to a mesenchymal cell with enhanced capacity of migration, invasiveness and resistance to apoptosis, indicates its role in kidney and liver fibrosis, as well as corneal wound healing (Schneider et al., 2008).

S100A4 has also been identified as a potent stimulator of **angiogenesis**. Thrombospondin is an angiogenesis inhibitor and treatment of tumor with S100A4 oligomer induced reduction of thrombospondin 1 gene expression (Schmidt-Hansen et al., 2004). S100A4 is associated with angiogenesis in neoplastic lesion because S100A4 transgenic mice display higher vessel density compared with nontransgenic animals (Ambartsumian et al., 2001). Through interaction with annexin II, extracellular S100A4 promotes the plasmin formation and contribute to angiogenesis (Semov et al., 2005). Receptor for advanced glycation end products (RAGE) has been implied as a cell surface receptor for S100A4 in human articular chondrocytes and pulmonary artery smooth muscle cells (Lawrie et al., 2005, Yammani et al., 2006), while some S100A4-mediated responses appear to be RAGE-independent (Belot et al., 2002, Schmidt-Hansen et al., 2004).

### **S100A4 in cancer metastasis and other diseases**

S100A4 was first described 20 years ago as a metastasis-specific gene product (Ebralidze et al., 1989). The biological function of S100A4 has been investigated most intensively with respect to its role in promoting tumor metastasis, such as cell motility, invasion, angiogenesis and remodeling of extracellular matrix. Overexpression of S100A4 has been observed in several metastatic cancers, including breast (Rudland et al., 2000), pancreatic (Rosty et al., 2002), prostate (Saleem et al., 2005), bladder (Davies et al., 2002), lung cancers (Kimura et al., 2000), colorectal (Gongoll et al., 2002), gastric (Cho et al., 2003), and thyroid (Zou et al., 2005) and

has significant predictive value for early mortality. *In vitro* studies using cell lines also show that overexpression of S100A4 in a benign rat mammary epithelial cell line promotes subcutaneous tumor growth and metastasis to the lungs and lymph nodes (Davies et al., 1993) and the nonmetastatic human breast cancer cell line MCF-7 acquired a metastatic phenotype on S100A4 transfection (Grigorian et al., 1996). Consistent with these observations, inhibition of S100A4 expression in tumor cells suppresses metastatic potential (Maelandsmo et al., 1996, Takenaga et al., 1997, Xue et al., 2003).

Further evidence from genetically engineered mice emphasizes the central role of S100A4 in tumor growth and metastasis. Animals overexpressing S100A4 were phenotypically normal and exhibited no increased frequency of neoplastic transformation in any organ. But when these transgenic mice were crossed into a tumorigenic background, the offspring displayed a markedly increased frequency of lung metastasis, even though they develop primary tumors with incidence and tumor size comparable to those in their nontransgenic littermates (Ambartsumian et al., 1996, Davies et al., 1996). These studies provide compelling evidence that S100A4 is directly involved in the formation of metastasis from different tumors and S100A4 probably regulates the steps in the metastatic cascade without affecting the initiated growth of the primary tumors.

Research on S100A4 during last years revealed new important facets of its functions and involvements, such as disorders in cardio-vascular, nervous, and pulmonary systems, and inflammation. S100A4 was considered as a potent cardiomyocyte differentiation factor in the early stage of cardiomyogenesis (Stary et al., 2006) and overexpression of S100A4 found in animal models of cardiac hypertrophy indicates implication of S100A4 in cytoskeleton remodeling and extracellular matrix reorganization (Ambartsumian et al., 2005, Helfman et al., 2005). As for the nervous system, that S100A4 was found in white matter astrocytes and it was markedly up-regulated after nerve injury indicates that S100A4 possibly involves in tissue reparation since (Kozlova et al., 1999). Pulmonary artery hypertension (PAH) is a fatal vascular human disease associated with abnormal vascular proliferation and overexpression of S100A4 directly correlates to the severity of PAH suggesting possible involvement of S100A4 in the pathogenesis of the disease (Greenway et al., 2004).

## IMMUNITY

**Immunity** is the host resistance to different diseases, specifically infectious diseases. When the immune system encounters a pathogen, within minutes to hours, the **innate immunity** already begins to work. That includes epithelial barriers, phagocytosis by neutrophils and macrophages, opsonization of pathogens by complement system and direct killing by natural killer (NK) cells. During the innate immune response, leukocytes are recruited to the sites of infection. This includes selectin-mediated rolling, integrin-mediated adhesion and transmigration of leukocytes through the endothelium. Then a procedure called phagocytosis can kill microbes.

After 12 hours, the **adaptive immunity** joins the battle against the microbes and noninfectious molecules. The adaptive immunity was divided into two parts: **humoral** and **cell-mediated immunity**. The humoral immunity is mediated by **antibodies** produced by B lymphocytes (**B cells**). Antibodies opsonize and eliminate microbes. Cell-mediated immunity is mediated by T lymphocytes (**T cells**). T cells are activated to become effector cells and wall off the pathogens. The **memory** that is generated by the adaptive immune system makes the combat quicker and more effective when the host reencounters the pathogen. During inflammation, **cytokines** are produced for the communication of the cells in both innate and adaptive immunity.

The importance of the host immune system in limiting infection is underlined by the severity and poor prognosis of diseases in immunocompromised patients. On the other hand, tissue injury and disability as a consequence of inflammation may be also caused by the host immune response to the microbe or self-antigen. Thus, inflammation and immunity are necessary for the protection of host, but they may contribute to the injury.

**Adhesion molecules** play important roles in leukocyte recruitment. They allow the interaction of free-flowing leukocytes with the vessel wall and all subsequent adhesive interactions that are required for emigration into tissue. Adhesion molecule family includes selectins, integrins, immunoglobulins, and other adhesion molecules. The selectin family consists of three different molecules: L-selectin, P-selectin, and E-selectin, which play an important role in leukocyte capturing and rolling on endothelial cells. **L-selectin (CD62L)** is constitutively expressed on almost all leukocytes, whereas the other two members are expressed on endothelial cells. All three selectins mediate rapid low-affinity attachment of leukocytes to endothelium, an early and important step in leukocyte homing. L-selectin also



serves as a homing receptor for naïve T lymphocytes and dendritic cells to lymph nodes. On neutrophils, L-selectin serves the binding to endothelial cells that are activated by cytokines found at the sites of inflammation. It has been shown that soluble L-selectin (sL-selectin) levels in the plasma are elevated in infectious diseases and inflammation (Walzog et al., 2000). E- and P-selectins are also important in migration of leukocytes, including neutrophils and effector and memory T cells to peripheral sites of inflammation. **Integrins** mediate the firm adhesion of leukocytes by binding members of the immunoglobulin family of adhesion molecules expressed on endothelial cells. An important feature of integrins is their ability to respond to intracellular signals by rapidly increasing their avidity to their ligands, thus mediate migration of leukocytes to the sites of inflammation, naïve T cells to lymph nodes, and effector T cells to the site of infection. Integrins is also required for osteoclastic bone resorption by controlling the recognition of bone by osteoclasts (Teitelbaum, 2000). All integrins are heterodimeric molecules consisting of an  $\alpha$ -subunit and a  $\beta$ -subunit and are classified into several subfamilies based on the  $\beta$  chains in the heterodimers. One of the important subfamilies is  $\beta$ 2- (CD18) integrin. This subfamily also called CD11a-cCD18. CD11 refers to different  $\alpha$  chains and CD18 to the common  $\beta$  subunit. CD11bCD18 and CD11cCD18 both mediate leukocyte attachment to endothelial cells and transmigration. CD11bCD18 also functions as a complement receptor on phagocytic cells, binding particles opsonized with the inactivated C3b (iC3b) fragment, thereby mediates phagocytosis of microorganisms.

**Phagocytosis** is the cellular process of engulfing solid particles, for example bacteria. Opsonins such as C3b and antibodies can act as attachment sites and aid phagocytosis of pathogens. Engulfment of material is facilitated by the actin-myosin contractile system. The phagosome of ingested material is then fused with the lysosome, resulting in the formation of phagolysosomes, where most of the microbicidal mechanisms are concentrated. This includes several proteolytic enzymes, reactive oxygen species (ROS) produced by respiratory burst, and nitric oxide (NO) produced by action of inducible nitric oxide synthase (iNOS). Thus, phagocytosed microbes are destroyed in phagolysosomes. At the same time, peptides are generated and presented to T lymphocytes to initiate adaptive immune response. However, when neutrophils and macrophages are strongly activated, lysosomal enzymes, ROS, and NO can be released to the extracellular environment, causing tissue injury.

Hematopoietic stem cells in bone marrow are the precursors of immune cells. The principal effector cells of innate immunity are neutrophils, mononuclear phagocytes, and NK cells.

**Neutrophils.** Neutrophils are one of the first-responders of inflammatory cells to migrate towards the site of inflammation, following the signals from chemoattractants such as IL-8, IFN- $\gamma$  and C5a. Upon activation, neutrophils marginate, undergo selectin-dependent capture followed by integrin-dependent adhesion and move into site of inflammation (Witko-Sarsat et al., 2000). The major role of neutrophils is to phagocytose and destroy infectious agents.

**Monocyte/Macrophages** After a short time in circulation, the monocytes migrate to the tissue and differentiate into macrophage. Macrophages, like neutrophils, are professional phagocytes, but macrophages survive much longer. They play an important role in clearance of microbes and self-tissue that are damaged. In addition to phagocytosis, macrophages also exert antigen-presenting properties and produce cytokines that stimulate T cell proliferation and differentiation

**NK cells** are capable of killing cells that are infected with virus and malignantly transformed.

The main cell types in adaptive immune system are T and B cells. B cells do not develop without the help from T cells, and T cells also need B cells for activation and differentiations.

### **T lymphocyte**

T cells are key regulators of cell-mediated immunity. T cell progenitors migrate from bone marrow into thymus. After positive and negative selection in thymus, two types of mature T cells are produced. CD8<sup>+</sup> T cells, also called cytolytic T cells (CTLs), only recognize antigens presented by major histocompatibility complex I (MHC I), and CD4<sup>+</sup> T cells, also called helper T cells, only recognize antigens presented by major histocompatibility complex II. CD8<sup>+</sup> T cells are cytotoxic T cells that function in killing cells infected with virus and tumors. Effector CD4<sup>+</sup> T cells produce cytokines to activate phagocytes and B lymphocytes.

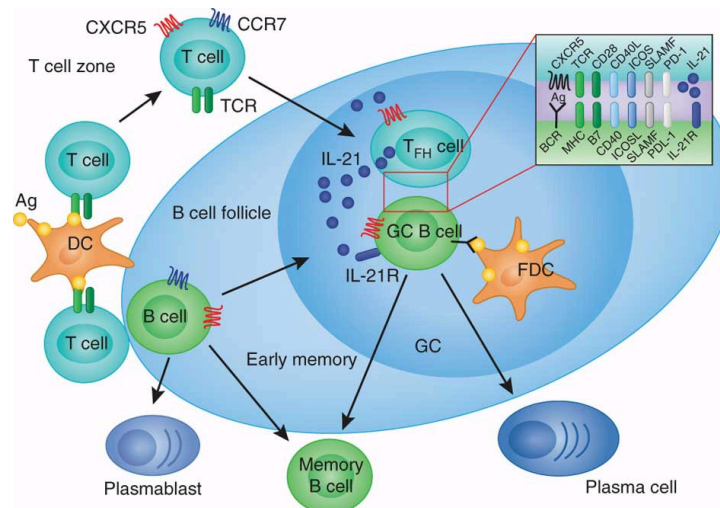
Following activation, CD4<sup>+</sup> T cells differentiate into subsets that are recognized by production of distinct sets of cytokines and perform different functions. These subsets include Th1, Th2, Th17 and Treg (Table 1). The differentiation of Th1, Th2, Th17 and Treg requires lineage-specific transcription factors: T-bet for Th1 cells, GATA3 for Th2 cells, ROR- $\gamma$ t for Th17 cells and Foxp3 for Treg cells.

Table 1. T cell subsets, transcription factors, cytokines secreted and their functions (King et al., 2008)

T cell subsets	Transcription factors	Secreted cytokines	Functions
Th 1	T-bet	IFN- $\gamma$ , TNF	Antiviral, bacterial immunity
Th 2	GATA-3	IL-4, IL-5, IL-13	Immunity to extracellular parasites
Treg	Foxp3	TGF- $\beta$	Regulation/tolerance
Th 17	ROR $\gamma$ t	IL-17	Inflammation, fungal immunity
T <sub>FH</sub>	Bcl-6?	IL-21	T cell help to B cells

**Th1 T cells** secrete IFN- $\gamma$ , IL-2 and mediate delayed-type hypersensitivity reactions (DTH). IFN- $\gamma$  plays a key role in macrophage activation, inflammation, and host defense against intracellular pathogens (Hu et al., 2009). IL-12 produced by macrophages and dendritic cells stimulates the production of IFN- $\gamma$ . IL-2 stimulates survival, proliferation and differentiation of antigen-activated T cells and is required for the survival of regulatory T cells. **Th2 T cells** produce IL-4, IL-5, IL-10 and IL-13, down-regulate inflammation and protect host from helminth infection. IL-10 inhibits activated macrophages and dendritic cells and is thus involved in the control of innate and cell-mediated immunity. Th1 and Th2 are conventional helper CD4<sup>+</sup> T cells. Tregs are supposed to repress immune response in inflammation, but this function is impaired in RA. **Th17 T cells** require IL-6, IL-1 and TGF- $\beta$  for development and maintenance. Th17 cells produce IL-17 and may promote the recruitment of neutrophils and monocytes to the site of infection, and play critical roles in murine arthritis models (Hirota et al., 2007) and in human inflammatory arthritis (Shahrara et al., 2008, Shen et al., 2009).

Recently, a subset of T cells, which are different from Th1 and Th2 cells in their chemokine receptor expression (CXCR5<sup>+</sup>), location (B cell follicular) and function (B cell help) emerged, and have been denoted **follicular B helper T cell (T<sub>FH</sub>)**. T<sub>FH</sub> cells play an important role in the generation of antibody producing plasma cells and formation of germinal centers, as well as the interaction between T and B cells (Haynes, 2008, King et al., 2008) (Figure 3). CXCR5 is responsible for the positioning of B and T cells in the follicular areas of lymphoid tissue and also a marker for T<sub>FH</sub> (Forster et al., 1996, King et al., 2008). Inducible costimulator (ICOS) is another essential molecule for development and maintenance of T<sub>FH</sub>. IL-21 is a functional T<sub>FH</sub>-secreted cytokine that potently stimulates the differentiation of B cells into Ab-forming cells and Bcl-6 has been shown to be a particular transcription factor directing the naïve CD4<sup>+</sup> T cells to the T<sub>FH</sub> cell lineage (King et al., 2008).



**Figure 3. Interaction of T and B cells in secondary lymphoid organs.** Localization to the B cell zone and T cell zone depends on the chemokine receptors CXCR5 and CCR7, respectively. Antigen-specific T cells primed on dendritic cells in the T cell zone, up-regulate ICOS, PD-1 and CXCR5 and migrate towards the B cell follicles. After interacting with their cognate B cells, these T cells mature into T<sub>FH</sub> cells. When follicular B cells encounter antigen, they move to the border of the T cell zone and can further differentiate into extrafollicular plasmablasts, early memory B cells or return to the follicular and undergo rapid proliferation to form a GC. In the GC, T<sub>FH</sub> cells interact with GC B cells through an array of molecular pairing. These interactions culminate in the T-cell-secreting cytokines, particularly IL-4 and IL-21, which are received by the B cells to influence the output of the GC in the form of affinity-matured memory B cells and long-lived plasma cells. Figure adopted with the permission from Nature Publishing Group: Nature Immunology (Stephen LN, 2011).

Following activation, a fraction of antigen-activated T lymphocytes differentiates into long-lived memory cells. **Memory T** cells survive even after infection is eradicated and antigens no longer exist. They do not produce cytokines or kill infected cells, but they may do so rapidly on encountering the same antigen again. CCR7 is a molecule that mediates T cells homing to lymphoid organs for the memory T cells. CCR7<sup>+</sup> T cells present in lymph node, spleen and blood, while CCR7<sup>-</sup> T cells are in blood, spleen and nonlymphoid tissue (Seder et al., 2003). In RA, nearly all of the synovial tissue CD4<sup>+</sup> T cells are of the memory phenotype.

T cell is considered as the main orchestrator of cell-mediated immune responses in RA (Choy et al., 2001) and CIA (Cho et al., 2007), as well as in antigen-induced arthritis (AIA) (Petrow et al., 1996, Pohlers et al., 2004, van den Berg et al., 2007). The prominent T-cell infiltrate into the synovial membrane also suggested these cells are key participants (Firestein, 2003). CD4<sup>+</sup> and CD8<sup>+</sup> T cells have been found in synovial infiltrates in RA patients. CD4<sup>+</sup> T cells directly or through the release of interferon- $\gamma$  and IL-17, stimulate monocytes, macrophages, and synovial fibroblasts to produce interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) and to secrete matrix metalloproteinases (MMPs) (Choy et al., 2001). Adoptive transfer of CD4<sup>+</sup> T cells from donor mice immunized with mBSA or from mice with collagen type II arthritis can induce arthritis in SCID mice (Kadowaki et al., 1994, Petrow

et al., 1996). Anti-CD4 monoclonal antibody reduced the joint inflammation and destruction in antigen-induced arthritis (Pohlers et al., 2004). T cells also contribute to the development of septic arthritis. Depletion of CD4+ T cells resulted in a milder course of *S.aureus* induced arthritis in mice (Abdelnour et al., 1994), while pretreatment of *S.aureus* infected rats with an antibody against  $\alpha\beta$  T cells receptor significantly decreased the severity of arthritis (Bremell et al., 1994).

## **B lymphocytes**

B cells are the central mediators of humoral immunity. Plasma cells are the terminal effector cells originating from B cell lineage. They can neutralize pathogens by secreting pathogen-specific antibodies. B cells play a dual role in RA, presenting yet unknown antigens to T cells, modulating T cell activation and producing auto-antibodies such as rheumatoid factor (RF), antibodies to collagen II and cyclic citrullinated peptides. The **auto-antibodies** bind to auto-antigen, which adhere in the cartilage surfaces. Immobilized antigen-antibody complexes on cartilage surfaces then fix complement and release chemotactic factors such as C5a. Inflammatory cells are subsequently recruited to rheumatoid joint where they are activated and then contribute to local destruction (Firestein, 2003). **Rheumatoid factor (RF)** is the classic IgM autoantibody against the Fc part of the IgG antibody. About 80% of the RA patients are RF positive and its presence predicts a more aggressive, destructive course (Firestein, 2003). However, RF is also detected in healthy individuals and in other inflammatory conditions. **Anti-type II collagen antibodies** are present in 3-27% of all the RA patients (Beard et al., 1980). A study of murine collagen-induced arthritis shows that anti-collagen-type II antibody binds to collagen displayed on the surface of articular cartilage (Firestein, 2003). Another type of autoantibody that was found recently is **anti-citrullinated peptides antibodies (ACPA)**, which is shown to be an early factor predicting development of RA. ACPA, as RF, is associated with increased joint damage and low remission rate (Scott et al., 2010, van der Helm-van Mil et al., 2005).

During the last decade it has become apparent that B lymphocytes exert important regulatory roles independent of their function as antibody-producing cells. This has been demonstrated by the efficacy of B cell depletion therapy using the anti-CD20 monoclonal Ab (rituximab) in RA and other autoimmune diseases. B cell depletion therapy is beneficial not only in autoantibody-mediated diseases, but also in other diseases that are not considered to be mediated by Abs, such as type I diabetes and multiple sclerosis (Fujimoto, 2010). Thus, B cells

have additional functions outside of Ab production, and are likely to play significant roles in autoimmunity. These functions include, *firstly*, antigen presentation and T cell activation. B cells process antigen peptides and present them via MHC II to activate T cells and stimulate the formation of follicular CD4<sup>+</sup> helper T cells (Takemura et al., 2001). B cells as antigen-presenting cells provide important co-stimulatory signals required for CD4<sup>+</sup> T cell clonal expansion and their functions (Nakken et al., 2011). The role of antigen-presentation of B cells is mainly taken by B1 cells (Martin et al., 2001). *Secondly*, production of cytokines. Stimulated B cells can secrete cytokines, such as TNF- $\alpha$ , lymphotoxin and IL-6, which can amplify immune responses (Dorner et al., 2003). They are also able to produce IL-10, which activates follicular dendritic cells (FDCs) and stimulates B cell function (Martinez-Gamboa et al., 2006). The main source for B cell derived IL-10 are B1 cells (O'Garra et al., 1992). Stimulation of B cells with autoantigens, TLR4 and TLR9 ligands leads to IL-10 production (Saraiva et al., 2010). *Thirdly*, interaction with chemokines and their receptors. CXCR13 has been shown involvement in recruitment of B cells to ectopic lymphoid tissue (Martinez-Gamboa et al., 2006). *Fourthly*, Ectopic lymphoneogenesis formation. Ectopic germinal centers in rheumatoid synovial lesion are aggregates of B and T cells and a network of FDC. Ectopic germinal centers are also the sites for B cell development, since they represent the anatomical structure of affinity maturation, differentiation and proliferation of B cells (Martinez-Gamboa et al., 2006). Nevertheless, all facets of B cells in RA pathogenesis have not been completely delineated.

Subdivision of mouse B cells into three cell types is proposed (Engel et al., 2011). Follicular B cells, also known as conventional B cells or B2 cells, represent the vast majority of B cells. The other two B cell subsets are marginal zone B cells (MZ B) and B1 (B1a and B1b) cells. **Follicular B cells** are involved in response to T-dependent Ag, while B1a, B1b and MZ B are more special to T-independent Ag. MZ B cells generate short-lived antibody responses to invading virus and encapsulated bacteria (Engel et al., 2011). **B1 cells** are derived from fetal-liver hematopoietic stem cells. They are particularly in the peritoneal cavity and gut-associated lymphoid tissues. B1a cells express CD5<sup>+</sup>, while B1b cells do not (Engel et al., 2011). B1b cells and MZ B cells may share overlapping functional capabilities, although these two cells occupy different anatomic areas. B1a cells produce natural antibodies and provide innate protection against bacterial infection (Engel et al., 2011), and require the spleen for its generation and /or survival during the adult life (Kruetzmann et al., 2003, Wen et al., 2005). CD5<sup>+</sup> B1 cells was also shown a close association with FDC and involved in induction of FDC (Wen et al.,

2005). Moreover, CD5+ B cells (B1 cells) are considered associated with production of autoantibody, and were found in elevated percentages in RA patients (Becker et al., 1990, Plater-Zyberk et al., 1985).

## **Cytokines**

There is a number of cytokines involved in inflammation during arthritis, such as TNF- $\alpha$ , IL-1, IL-6, IFN- $\gamma$ , IL-17. Here we only discuss the main cytokines that are mentioned in this thesis.

**IL-6** is produced by a variety of cell types including T cells, B cells, fibroblasts, endothelial cells and monocytes. IL-6 is efficient in T cell differentiation and activation and it induces the generation of Th17 cells together with TGF- $\beta$  (Miossec et al., 2009, Neurath et al., 2011). It can also promote B cell differentiation (Kopf et al., 1998, Suematsu et al., 1989). Serum levels of IL-6 are highly elevated during *S.aureus* arthritis (Bremell et al., 1992, Bremell et al., 1994). Elevated IL-6 levels have been observed in both serum and synovial fluid in patients with RA (Madhok et al., 1993, Nishimoto, 2006, Sack et al., 1993). IL-6 has been found to potently affect cartilage and bone destruction in a murine arthritis model (Ohshima et al., 1998). Treatment with anti IL-6 antibodies significantly reduced arthritis activity in collagen-type II induced arthritis (Liang et al., 2009).

**IFN- $\gamma$**  is secreted by CD8+ and Th1 CD4+ T cells, as well as by NK T cells. IFN- $\gamma$  is one of the most important endogenous mediators of immunity and inflammation. It is a major product of Th1 cells and further skews the immune response towards a Th1 phenotype (Schroder et al., 2004). It promotes phagocytosis of microbes by stimulating the antibody production and complement system. IFN- $\gamma$  also stimulates the expression of class II MHC molecules and B7 costimulators on macrophages and dendritic cells, therefore serves as to amplify T cell response. Activations of macrophage and other cell types by IFN- $\gamma$  at sites of inflammation, results in increase of the effector inflammatory components of autoimmune diseases (Hu et al., 2009). In *S.aureus* infection, IFN- $\gamma$  protects the host from sepsis but aggravates arthritis (Zhao et al., 1998). In RA, IFN- $\gamma$  can stimulate monocyte/macrophage and synovial fibroblasts to produce pro-inflammatory cytokines and matrix metalloproteinases (Choy et al., 2001), thus contribute to the joint damage. It has been shown that IFN- $\gamma$  orchestrates the trafficking of specific immune cells to sites of inflammation through up-regulating expression of adhesion molecules and chemokines (Schroder et al., 2004). On the other hand, IFN- $\gamma$  can

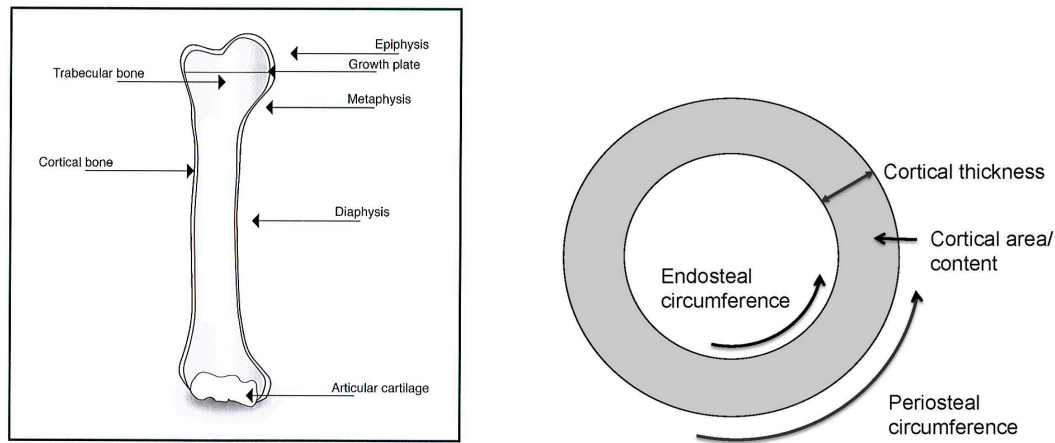
also turn on Treg cells and have a negative role on Th17 cell differentiation and expansion (Chen et al., 2009).

**IL-17** is a pro-inflammatory cytokine mainly produced by Th17 cells. Concordant results using mouse and human models of RA have shown that IL-17 is involved in joint inflammation (Hot et al., 2011, Lubberts, 2008). The arthritis can be induced by a single injection of IL-17 into a normal mouse knee and continuous administration of IL-17 induces massive damage with extensive inflammatory cell migration, bone erosion and cartilage degradation (Lubberts et al., 2005, Waldburger et al., 2009). IL-17 is detectable in synovial fluid from RA patients and enhances osteoclastogenesis by inducing RANKL on mesenchymal cells (Kotake et al., 1999). In RA, the production of TNF, IL-1 and IL-17 by synovial cells is predictive of joint destruction (Kirkham et al., 2006). Most parenchymal cells express interleukin-17 receptors and signaling through these receptors induces target cells to produce pro-inflammatory factors such as IL-6, IL-1, TNF, and MMPs. IL-17 mediates destruction of extracellular matrix by the production of MMPs and cause bone resorption by stimulating osteoblasts to express RANKL (Kirkham et al., 2006). These processes are mostly attenuated in mice with collagen-induced arthritis (CIA) following anti-IL17 antibodies (Lubberts et al., 2004), and induction of IL-17 is effectively suppressed in IL-17 deficient mice (Nakae et al., 2003).

## BONE

**Bone** functions as support for the body and as protection for inner organs. It is also involved in the metabolism of mineral and the bone marrow produces blood cells, which includes all the cells involved in immune system. Bones are made up of two macroscopically different types. **Cortical bone** (compact) is the outer shell of the lamellae and contributes to 80% of the total bone mass. It is mainly in the diaphysis of long bone. **Trabecular bone** (porous) is the internal network of beams and contributes to 20% of the total bone mass. It is found in the metaphysis of long bones and has a faster turnover due to higher metabolic activity (Figure 4).



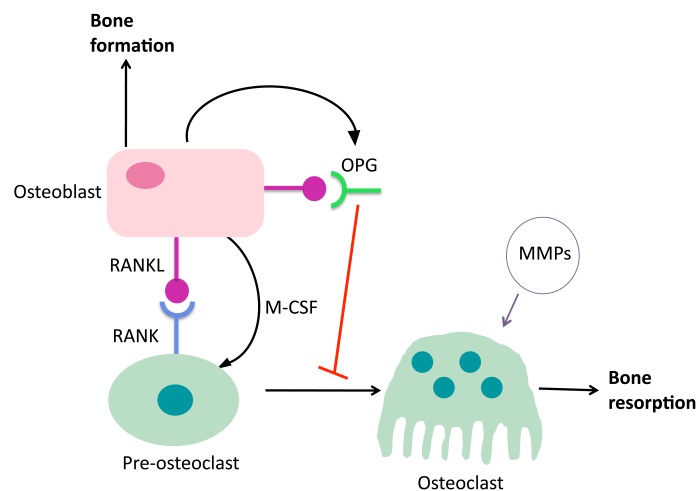


**Figure 4. Bone structure.** The mid-diaphyseal region of femora contains only cortical bone. Trabecular bone is in the metaphysis of long bone (left figure). Trabecular volumetric BMD is measure by metaphyseal pQCT scans of left femora. The scan was positioned in the metaphysis at a distance from the distal growth plate corresponding to 3% of the total length of the femur (an area containing cortical and trabecular bone) (left figure). The trabecular bone region was defined by setting an inner area to 45% of the total cross-sectional area. The mid-diaphyseal region of femur and tibiae in mice contains only cortical bone. Mid-diaphyseal pQCT scans of femora were performed to determine the cortical bone parameters such as cortical volumetric BMD, the cortical cross-sectional area, the periosteal circumference, the endosteal circumference, the moment of resistance, and the cross-sectional moment of inertia (right figure) (Windahl et al., 1999).

About 2/3 parts of the bone are inorganic materials (mineral), which are made up of hydroxyapatite. It provides the stability of the bone. Another 1/3 part of bone is organic material, which can be divided into the matrix and cells and is responsible for the flexibility of the bone. 90% of the organic matrix is collagen type I. Organic section is composed also of cells. **Osteoblasts** (4-6%) originate from mesenchymal stem cells. They are responsible for bone formation by secreting bone proteins of matrix, which includes collagen type I, and other proteins such as osteocalcin and osteonectin. They are also responsible for the mineralization of the matrix. **Osteoclasts** (1-2%) develop from hematopoietic stem cells, which is the cell lineage that can also become monocytes and macrophages. Osteoclasts are multinuclear cells and are responsible for bone resorption. Formation of osteoclasts needs macrophage colony-stimulating factor (M-CSF) and receptor activator of NF- $\kappa$ B ligand (RANKL). The binding of RANKL to RANKL is crucial for osteoclast development. This binding is inhibited by the decoy receptor osteoprotegerin (OPG). Osteoclasts are characterized by expression of tartrate-resistant acid phosphatase (TRAP) and cathepsin K. **Osteocytes** (90-95%) develop from osteoblasts and are mechanosensors, which sense loading of the bone. They are important for regulation of bone remodeling.

**Bone remodeling** occurs throughout lifetime. It is a dynamic process that includes bone resorption and bone formation and controls the reshaping of bone during growth and

repairing following fracture (Figure 5). The process requires close cooperation of two types of cells, osteoblasts and osteoclasts. The resorption by osteoclasts occurs on the surface of trabeculae in trabecular bone, while a tunnel is formed in the cortical bone. In a healthy individual, the rate of bone resorption is balanced to the rate of bone formation. The coupling of bone resorption and formation determines the bone mineral density and hence the bone strength. A net increase in bone formation results in osteopetrosis and a net decrease in bone formation results in osteoporosis. During bone resorption, type I collagen degrades and some fragments (C-terminal telopeptides) are released into the circulation and levels of C-terminal telopeptides (CTX-I) are a useful marker of bone resorption.



**Figure 5. Bone remodeling.** Bone is dynamically remodeled where osteoblasts are responsible for bone formation and osteoclasts are responsible for bone resorption. The osteoblast expresses receptor activator of NF- $\kappa$ B ligand (RANKL) on its surface. When it binds to RANK on the pre-osteoclast, with the help of macrophage colony-stimulating factor (M-CSF), it promotes the osteoclast differentiation and formation, leading to bone resorption. The osteoblast also secretes osteoprotegerin (OPG), a decoy receptor for RANKL. OPG blocks the interaction of RANKL/RANK by binding to RANKL and prevents osteoclast formation. The balance between RANKL and OPG determines the activity of osteoclasts, bone formation and resorption.

Bone remodeling is controlled by several factors, including loading of bone, parathyroid hormone, estrogen, growth hormone and cytokines. Both estrogens and androgens are important in the development of bone and maintenance of bone mineral density throughout life (Callewaert et al., Krum, 2011). Estrogen is an important regulator of bone metabolism (Riggs et al., 2002). During bone growth, estrogen is considered as an inhibitor, while androgen is a stimulator (Frank, 2003). Men generally have higher bone mass than women. Women experience a rapid bone loss during menopause due to estrogen deficiency. Estrogen is protective in bone loss. A reduction of estrogen levels during menopause leads to a decrease in both cortical and trabecular bone mineral density. Mice following ovariectomy display the same results. Androgen affects the skeleton in both men and women and androgen deficiency is associated with low bone mineral density (Krum, 2011, MacLean et al., 2010). Regarding

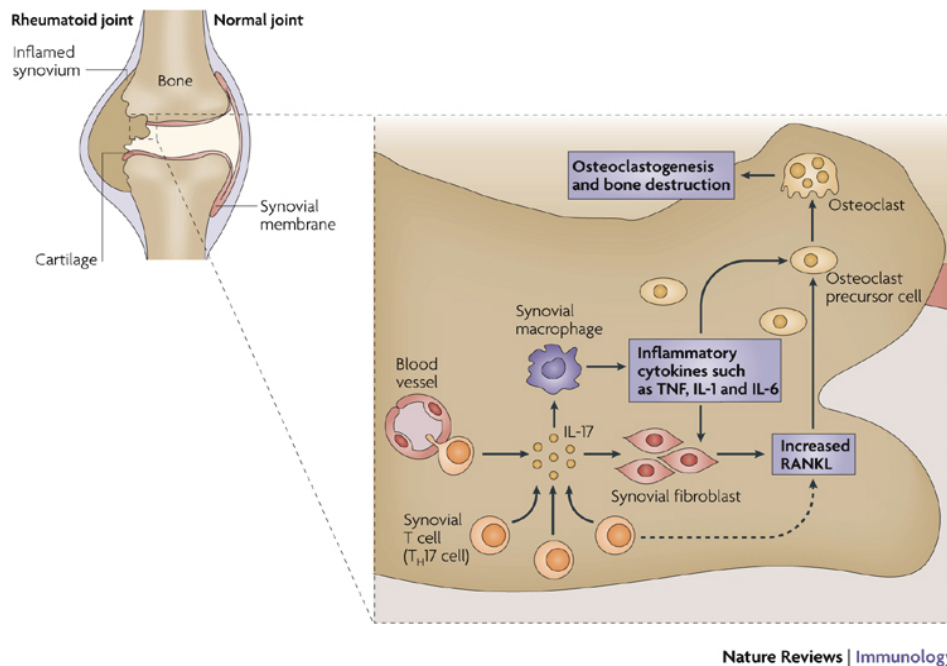
growth hormone and IGF-1, it shows that mice with low IGF-1 have reduced total BMD and cortical thickness, whereas mice with higher IGF-1 levels show increased total BMD and femoral cortical thickness (Yakar et al., 2010). Cytokines such as IL-6, INF- $\alpha$  and IL-17 are important for the bone metabolism.

## IMMUNE SYSTEM AND BONE REMODELING IN ARTHRITIS

The balance between bone formation and resorption is important for normal physiological functions. But this balance is broken during inflammation and arthritis. Inflammatory cells and secreted cytokines promote the development of osteoclasts and bias the balance to bone resorption, leading to bone loss. RANKL, RANK and OPG are central regulators in osteoclast function. **RANKL** promotes osteoclast differentiation and activation by binding to RANK, its receptor on pre-osteoclast (Leibbrandt et al., 2009, Li et al., 2000). It also stimulates mature osteoclast to “eat” bone (Burgess et al., 1999) and inhibits osteoclast apoptosis (Lacey et al., 2000).

Inflammatory cells including T cells (Takayanagi, 2007), B cells (Takayanagi, 2010), macrophages (Takayanagi, 2010), synovial fibroblasts (Shigeyama et al., 2000), dendritic cells (Takayanagi, 2010) and neutrophils (Haynes, 2007, Poubelle et al., 2007) have been shown to be involved in osteoclast development in inflammation. Recently, Dickkopf 1, a negative regulator of Wnt family, was shown to be associated with increased bone erosions in inflammatory and degenerative joint diseases (Diarra et al., 2007). T cells are the cell type studied most in bone metabolism in arthritis (Figure 4). Among different types of T cell, Th17 cells, which are characterized by secreting IL-17, are very potent in inducing RANKL expression. During inflammation, such as RA, activated T cells, usually Th17, secrete IL-17, which leads to increased expression of pro-inflammatory cytokines from macrophages, such as TNF, IL-1 and IL-6. These inflammatory cytokines in turn leads to increased RANKL expression in preosteoclasts/fibroblasts in inflammatory joints. IL-17 also stimulates fibroblasts to produce chemokines for neutrophils that contribute to cartilage destruction (Ruddy et al., 2004). Moreover, Th17 cells express RANKL themselves, which also increases the osteoclast development (Takayanagi, 2007). Under the stimulation of IFN- $\gamma$  and IL-17, monocytes/macrophages and synovial fibroblast produce MMPs (Choy et al., 2001, Li et al., 2010), leading to bone and cartilage destruction. MMPs can contribute to osteoclastic bone resorption by removing unmineralized osteoid from the bone surface for osteoclasts binding (Uchida et al., 2001). However, some studies showed that MMPs have little effect on

cartilage/bone destruction in animal models of arthritis (Calander et al., 2006, Cox et al., 2010, Mudgett et al., 1998) and treatment with MMPs inhibitor did not protect against joint damage for patients with rheumatoid arthritis (Milner et al., 2005).



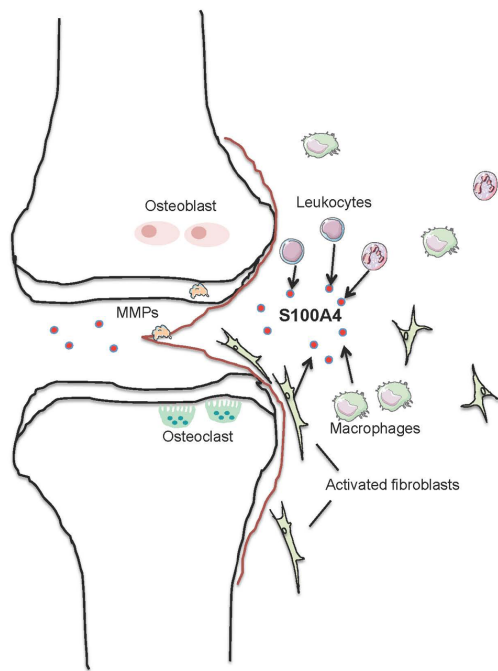
**Figure 6. The role of T cells in osteoclastogenesis in autoimmune arthritis.** In RA, inflammatory synovium invades and destroys bone and this is mediated by osteoclasts that are induced by RANKL. The link between T cells and activation of osteoclast-mediated bone resorption has been shown here: IL-17 producing T help cells (Th17 cells) are the only osteoclastogenic Th cell subset. Th17 cells produce IL-17 that induces RANKL on synovial fibroblasts. IL-17 also stimulates the local inflammation and activates synovial macrophages to secrete pro-inflammatory cytokines such as TNF- $\alpha$ , IL-1 and IL-6. These cytokines activate osteoclastogenesis by either directly acting on osteoclast precursor cells or inducing RANKL on synovial fibroblasts. Th17 also express RANKL on their membrane, which partly contributes to the enhanced osteoclastogenesis. Figure adopted with the permission from Nature Publishing Group: Nature Review Immunology (Takayanagi H, 2007)

In addition to T cells, B cells and dendritic cells are also demonstrated new players that are associated with osteoclasts (Takayanagi, 2010). B lymphocyte lineage cells can serve as osteoclast precursors (Manabe et al., 2001). Neutrophils also express RANKL that regulates the bone erosion (Haynes, 2007). In septic arthritis, granulocytes and macrophages are the dominating cell types and responsible for the cartilage and bone destruction in the early phase by secreting inflammatory cytokines (Verdrengh et al., 2006). RANKL expressed by activated neutrophils is likely to induce bone loss in septic arthritis (Chakravarti et al., 2009, Sakurai et al., 2003). Inhibition of RANKL signaling significantly prevents bone loss in a murine staphylococcal arthritis model (Verdrengh et al.).

## S100A4 IN INFLAMMATION AND BONE METABOLISM

Several S100 proteins have been found to accumulate in inflammation sites and this may indicate their role in inflammation. Expression of S100 proteins has been shown in synovial tissue and fluid of rheumatoid arthritis (S100A8/S100A9) (Foell et al., 2004), ulcerative colitis (S100A8/S100A9)(Foell et al., 2004) and psoriasis (S100A7) (Anderson et al., 2009). In addition, certain members of S100 family have bactericidal properties. S100A8/S100A9 heterodimer inhibits microbial growth in *Staphylococcus aureus* abscesses by metal chelation (Corbin et al., 2008). S100A7 exhibits membrane - permeabilizing properties against Gram-positive bacteria at low pH (Michalek et al., 2009). S100A4 contributes not only to the regulation of tumor progression and metastasis, but participates also in the process of inflammation and cartilage destruction (Oslejskova et al., 2008, Yammani et al., 2006). Recently, strong up-regulation of S100A4 was found in the dermis of patients with psoriasis and it is an essential contributor to the pathogenesis of psoriasis (Zibert et al., 2009). Increased expression of S100A4 in inflamed muscle tissue indicates its role in pathogenesis of inflammatory myopathies (Andres Cerezo et al., 2011). The most intensive study of S100A4 on inflammation is about its role on rheumatoid arthritis. An association of S100A4 and inflammation was first observed by the detection of up-regulated S100A4 mRNA in proliferating synovial fibroblasts of RA patients. Moreover, S100A4 has been detected in the lining and sublining layer of RA synovial tissues, while its expression was not observed in healthy synovial tissue (Masuda et al., 2002). Further more, studies by Grigorian M and coworkers demonstrated a strong up-regulation of S100A4 protein in synovial tissue and fluid in patients with RA and most cell types (fibroblasts, immune and vascular cells) populating the synovial tissue contributed to the production of S100A4. The local up-regulation of S100A4 was accompanied by high plasma concentration of S100A4 protein, existing in the bioactive oligomeric form (Klingelhofer et al., 2007, Senolt et al., 2006). Most importantly, they also showed expression of S100A4 at sites of cartilage and bone destruction and that S100A4 influences the production of matrix metalloproteinases (MMP 3, 1, 9, 13) as well as p53 functions (Senolt et al., 2006) (Schmidt-Hansen et al., 2004). Consistent with these findings, clinical data showed that the plasma level of S100A4 protein are correlated with disease activity of RA and the multimer (bioactive) S100A4 declined after successful TNF- $\alpha$  blocking therapy (Oslejskova et al., 2009). In addition, high levels of S100A4 are associated with a poor clinical response to anti TNF- $\alpha$  treatment infliximab. (Erlandsson M et al, Rheumatology (Oxford), 2011 in press).

Not so many studies focus on the role of S100A4 in bone metabolism. There are two *in vitro* studies that showed that low levels of S100A4 were associated with markedly increased mineralization nodules accompanied with increased expression of type I collagen. Further analysis showed that the expression of osteoblastic markers such as osteopontin (OPN), bone sialoprotein (BSP), osteocalcin (OCN), as well as osteoblast-specific transcription factors Runx2/Cbfa1 and Osterix were increased. This may imply that the inhibition of S100A4 increase osteoblast differentiation (Duarte et al., 2003, Kato et al., 2005).



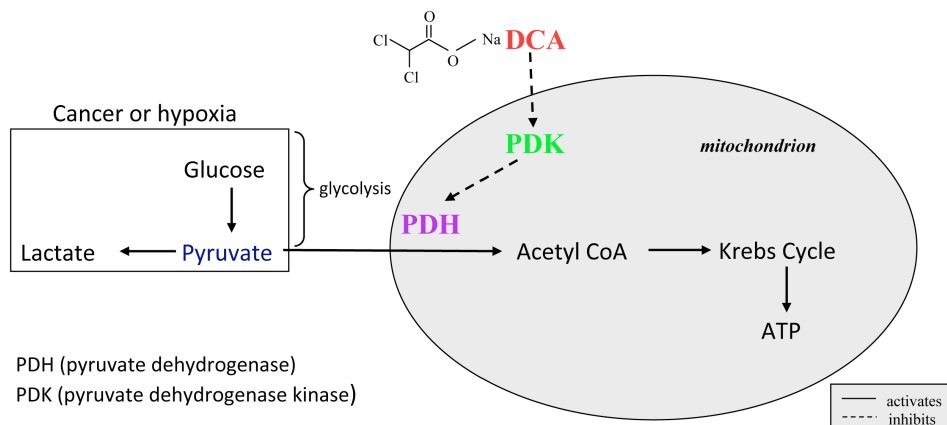
**Figure 7. Assumed effects of S100A4 in rheumatoid arthritis.** S100A4 proteins are secreted by inflammatory cells populating the synovia and synovial fluid. S100A4 proteins may interact with these cells and modulate the immune response, causing inflammation and joint destruction in RA.

Based on these findings, one may speculate that S100A4 plays an important role in the pathogenesis of inflammation and arthritis. However, it is still rather far from a comprehensive understanding of the mechanisms that implicate S100A4 in different disorders. It remains unclear what the role is for S100A4 in host defense during bacterial infections. S100A4 has been shown to have effects on functions of macrophages, fibroblasts and T cells (Cunningham et al., 2010, Grum-Schwensen et al., 2010, Li et al., 2010), but we do not know whether S100A4 have influence on cell formation, such as B cell formation. Moreover, studies about S100A4 on bone metabolism are limited. In this thesis, we tried to explore some mechanisms through which S100A4 modulates inflammation and bone metabolism in arthritis by using different murine arthritis models.

## DICHLOROACETATE

Dichloroacetate (DCA) is a well-established drug that has been used in clinic to treat lactic acidosis and inherited mitochondrial disorder (Figure 6). Recently, Bonnet and coworkers

found that DCA has a potent anti-cancer effect both *in vivo* and *in vitro* because of its pro-apoptotic and anti-proliferative properties. DCA can induce the efflux of pro-apoptotic mediators, such as cytochrome C and apoptosis-inducing factor (AIF), and inhibit nuclear factor of activated T lymphocytes (NFAT) and decrease the expression of survivin, a marker of resistance to apoptosis and tumor aggressiveness (Bonnet et al., 2007). Because of the common feature of cancer and inflammation in arthritis, we studied the impact of DCA treatment in collagen-induced arthritis in paper IV.



**Figure 8. Mechanism of dichloroacetate on treatment of lactic acidosis.** In normal situation, most of glucose metabolism enter into Krebs cycle and produce ATP. In lactic acidosis, increased glucose metabolism will produce more lactate. The mechanism of DCA treatment is through inhibition of pyruvate dehydrogenase kinase (PDK), increasing pyruvate dehydrogenase (PDH) activity, therefore shifting pyruvate metabolism from glycolysis and lactate production to glucose oxidation. Figure was modified with the permission from Elsevier: Cancer cell (Bonnet S, 2007)

## AIMS

- I. Study the role of S100A4 in the development of staphylococcal arthritis in mice
  
- II. Study the role of S100A4 in the development of antigen- induced arthritis in mice
  
- III. Evaluate the role of S100A4 in bone formation regulated by estrogen
  
- IV. Investigate the effects of dichloroacetate in the development of collagen-type II induced arthritis in mice

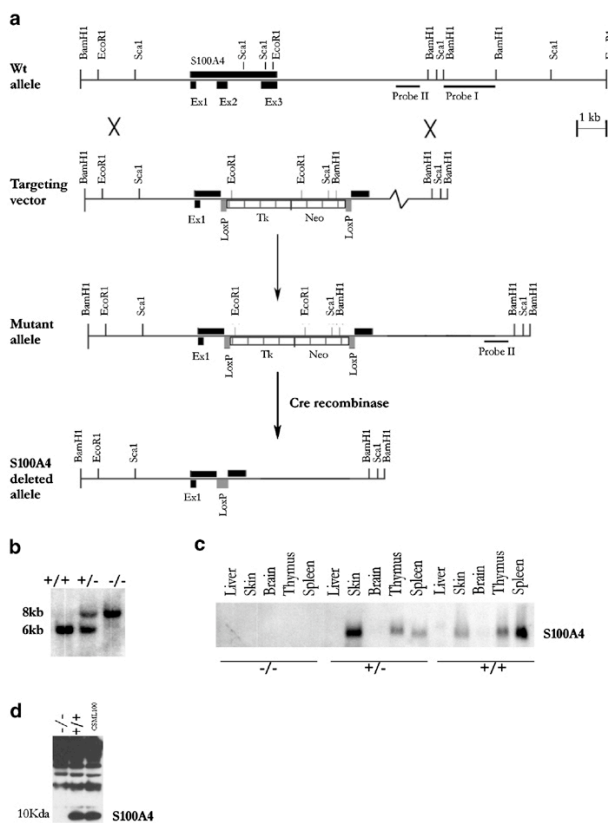


## METHODS

Characteristics of major methods employed in this thesis are described below. Details of each method are provided in corresponding papers.

### Mice (paper I, II and III)

The murine S100A4 gene was inactivated by homologous recombination in embryonic stem cells (ES cells), using a targeting strategy depicted in figure 1a (Naaman et al., 2004). Mice bearing the deletion of S100A4 were identified by Southern blot analysis (Figure 1b). The S100A4<sup>-/-</sup> mice were born viable. Histological analysis of the tissues obtained from 12- and 24- weeks old animals did not reveal any abnormalities as compared to the control mice. S100A4 RNA and protein were not detected in the tissues of these mice (Figure 1c and d)



**Figure 1. Generation of S100A4 knockout mice.**

(a) Targeting strategy at S100A4 locus. Exons 2 and 3 were replaced by the neomycin- and thymidin kinase-containing cassette in a recombinant allele generated by homologous recombination. Selection cassette was excised by transfection of selected clone with Cre-recombinase bearing plasmid. (b) Southern blot analysis of genomic DNA. 8kb represents the knockout allele whereas 6kb represents the wild type. (c) Northern blot analysis of total RNA isolated from different organs of +/+, +/- and -/- animals. Hybridization with S100A4 probe. (d) Immunoprecipitation of S100A4 from primary embryonic fibroblasts isolated from +/+ and -/- animals

### Animal model of septic arthritis (paper I)

**Staphylococcus aureus arthritis model** (paper I). *S.aureus* is the most common bacterium causing septic arthritis. The majority of human infectious arthritis has a haematogenic spread,

therefore the i.v. route of bacterial administration is particularly relevant and permits the study of interaction between bacteria and host defense. An animal model of *S.aureus* haematogenous arthritis was established in our laboratory (Bremell et al., 1992{Bremell, 1994 #649). The advantage of this model is the ease of handling of animals and the availability inbred and genetically defined mouse strains. Histopathological examinations of joints, following i.v. infection with *S.aureus*, reveal an early influx of polymorphonuclear cells (PMNCs) and a later appearance of mononuclear cells (MNCs). The initial joint destruction is localized to the cartilage-synovium junction, with pannus formation and subsequent cartilage and bone destruction (Bremell et al., 1992). Rapid joint destruction during the first days is mediated by PMNCs, followed by a secondary phase where T cells triggered by superantigens are instrumental in joint destruction (Abdelnour et al., 1994, Bremell et al., 1995, Verdrengh et al., 1997).

#### Animal models of rheumatoid arthritis (paper II, IV)

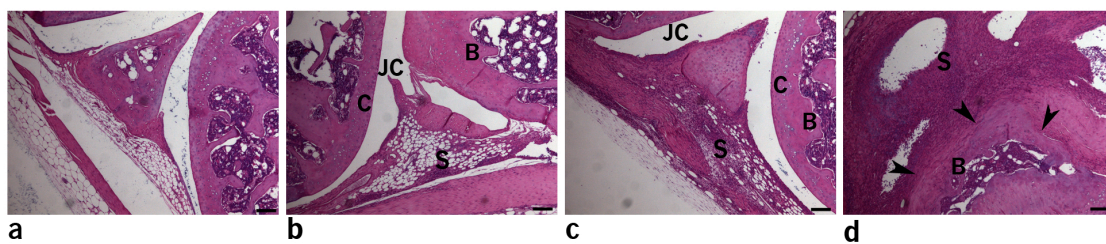
Several different animal models of RA have been established. They become useful tool for studying the pathogenesis of RA and developing anti-arthritic therapy, although human disease is more complex than either of these animal models. In this thesis, two animal models for RA have been used.

***Collagen-type II induced arthritis model*** (paper IV). Collagen-type II induced arthritis is a well-established murine arthritis model for human RA and is widely used in the study of the pathogenesis of RA as well as new anti-arthritic treatment. Like in humans where susceptibility to RA is highly associated with the HLA-DR1 and HLA-DR4 MHC II molecules, collagen type II arthritis can be induced in mouse strains of DBA/1 and B10.Q that have I-A<sub>q</sub> and I-A<sub>r</sub> haplotypes and are highly susceptible to CIA (Cho et al., 2007). The incidence of CIA is around 80%. CIA has symmetrical swelling of joints and engagement of small joints, followed by cartilage and bone erosion and finally loss of function. T- and B- lymphocyte response is the main driven force and the patterns of synovial infiltration and histological joint destruction are similar to RA.

***Antigen-induced arthritis model*** (paper II). We used methylated bovine serum albumin (mBSA) as antigen in our arthritis model. This arthritis model is not haplotype dependent and can be induced in a variety of mouse strains. Arthritis was induced by intra-articular injection of 30µg mBSA at day 21 in the left knee of pre-immunised animals (Forsman et al., 2011). Mice were pre-immunized on day 0 and day 7 with an antigen emulsion containing 200µg and 100µg mBSA, respectively. The antigen was prepared by diluting mBSA in PBS, which

was then emulsified 1:1 in Freund's adjuvant. At the termination of each experiment on day 28, serum samples were collected for serological analysis. Spleens can be processed for proliferation assays, cytokine secretion measurements and flow cytometry, and mRNA analysis. The injected knee joints were taken for morphological evaluation and synovial tissue for flow cytometry.

The histopathological features of antigen-induced arthritis resemble those of human RA (Figure 2). Beyond the 100% incidence of arthritis, another major advantage of antigen-induced arthritis model is that the time point of induction of arthritis is known (Frey et al., 2005). The morphologic differences between antigen-induced arthritis in animals and human rheumatoid arthritis may be due to the greater chronicity of human disease.



**Figure 2. Representative images of joint destruction and inflammation in mBSA-induced arthritis.** A scoring system is used to measure the inflammation in joints, a) represents a healthy joint scored 0, b) mild synovitis = +, c) moderate synovitis = ++, and d) severe synovitis = +++, this micrograph also shows erosion of bone and cartilage as indicated by arrow heads. S = synovitis, C = cartilage, B = Bone, JC = joint cavity. Hematoxylin and eosin staining.

### DTH and olive oil induced inflammation (paper IV)

Delayed type II hypersensitivity reaction (DTH) to oxazolone is a T cell/macrophage mediated, antigen-specific inflammatory response with a subsequent formation of edema on the ear. Olive oil induced inflammation is triggered by a single intradermal injection of olive oil in the mouse paw, and it induces a strong swelling reaction due to infiltration of PMNCs, reaching maximal intensity in 24 hours. So it is granulocyte dependent but T cell/macrophage independent (Josefsson et al., 1993). These two models were used in the study of paper IV to investigate the impact of dichloroacetate on *in vivo* cell-mediated inflammatory response.

### Ovariectomy (paper III, IV)

Ovariectomy (OVX) was used for studying the effects of sex steroid deficiency. OVX was performed by removal of the ovaries after a flank incision. The control mice had sham-operation, having ovaries exposed but not removed. The operation procedure was carried out

under Ketalar/Domitor anaesthesia. The mice were left to rest for 1-2 weeks before the starts of experiments (Jochems et al., 2005).

### Phagocytosis test (paper I)

Phagocytosis test is to investigate the phagocytic function of granulocytes and monocytes. It allows the quantitative determination of leukocyte phagocytosis in heparinized whole blood. Phagocytosis test measures the overall percentage of monocytes and granulocytes that have ingested bacteria. The evaluation was done by flow cytometry. Heparinized peripheral blood (100ul) was incubated with fluorescein isothiocyanate (FITC)-labeled *S.sureus* at 37°C in a water bath. Control blood samples were incubated with FITC-labeled bacteria on ice. Erythrocytes were lysed and leukocyte surface-attached bacteria were quenched and washed. Cells containing FITC-labeled bacteria were assessed by flow cytometry {Strandberg, 2009 #470}

### Bacteria distribution *in vivo* (paper I)

This experiment was to investigate the bacterial accumulation by injecting intravenously FITC-labeled bacteria to the mice. FITC-labeled *S.aureus* was inoculated intravenously to S100A4KO and WT mice. One hour and three hours after inoculation, mice were killed. Spleens, lymph nodes, kidneys, and lungs were removed, processed to cell suspension, and subjected to flow cytometry to examine the leukocytes loaded with bacterial in each organ.

### Proliferation assay (paper II)

Proliferative activity of leukocytes was examined by *in vitro* cultures of spleen cells with different stimuli {Ying, 2011 #758}. Briefly, spleen cells were seeded in 96-well cell culture plates and stimulated with the stimuli. After 48h, 50µl of the supernatants were collected from each well, and [<sup>3</sup>H]-thymidine was added to the cultures. The cells were harvested through Glass fibre filter on FilterMate Harvester after a further 10-12h, and the amount of incorporated [<sup>3</sup>H]-thymidine was counted in a beta-counter.

### Flow cytometry (paper I, II)

Flow cytometry was employed to distinguish phenotypes of cells. Leukocytes retrieved from spleen and synovial tissue were processed to get signal cell suspension and then were lysed with NH<sub>4</sub>Cl to get rid of erythrocytes (Amu et al.). After incubating with Fc block for elimination of non-specific binding of antibodies to Fc receptor, cells were incubated with

primary antibodies against different cell surface markers to identify cell phenotypes. Next, cells were collected and analysed using FACSCantoII equipped with FACSDiva software. Analysis was performed using FlowJo 887 software, and fluochrome minus one (FMO) was used for determination of positive populations and gating when needed.

### Gene expression analysis (paper II)

The mRNA expressions of different transcription factors were measured by real time PCR. Total RNA was extracted from splenocytes, and the concentration and quality of RNA were evaluated spectrophotometrically and by gel electrophoresis. cDNA was synthesized from the total RNA, and real-time RCR using SYBR Green was performed to measure mRNA levels. The reactions were performed using 500nM of forward and reverse primers, and 4ng total RNA in a final volume of 25ul. The results were expressed as the fold change compared with the expression levels in the WT control cells with the ddCq-method (2 reference genes were used).

### Evaluation of bone mineral density (paper I, III, IV)

Bone mineral density (BMD) was analysed in the left femora of mice using peripheral quantitative computed tomography (pQCT) scan (Windahl et al., 1999). The mid-diaphyseal region of femora and tibiae in mice contains only cortical bone. Mid-diaphyseal pQCT scans of femora were performed to determine the cortical bone parameters such as cortical volumetric BMD, the cortical cross-sectional area, the periosteal circumference, the endosteal circumference, the moment of resistance, and the cross-sectional moment of inertia. Metaphyseal pQCT scans of left femora were performed to measure trabecular volumetric BMD. The scan was positioned in the metaphysis at a distance from the distal growth plate corresponding to 3% of the total length of the femur (an area containing cortical and trabecular bone). The trabecular bone region was defined by setting an inner area to 45% of the total cross-sectional area.

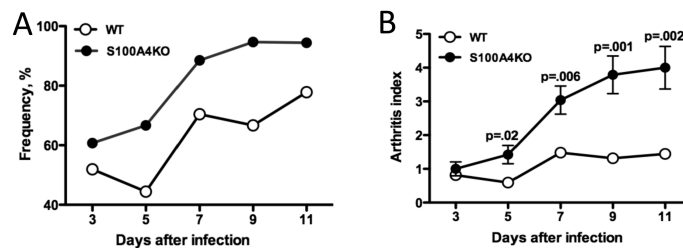
### Statistic analysis (paper I, II, III, IV)

The statistic evaluations in this thesis were performed by Mann-Whitney *U* test between different groups. Frequency comparison used Fisher exact test and chi-square test. Values were reported as median [interquartile range] or mean  $\pm$  standard error of the mean (SEM). A *P* value  $< 0.05$  was considered significant.

## RESULTS

### **S100A4KO mice displayed more severe clinical arthritis following intravenous inoculation of *Staphylococcus aureus* (paper I)**

In paper I, S100A4KO mice showed more severe clinical arthritis including redness and swelling compared with WT mice following intravenously inoculation of *S.aureus*. Both the frequency and severity of arthritis were higher in S100A4KO mice compared with their WT controls throughout the course of infection (Figure 1A, B). This more severe clinical arthritis was supported by increased serum level of IL-6 ( $p = 0.002$ ) and sL-selectin ( $p = 0.015$ ) in S100A4KO mice.



**Figure 1. Frequency (A) and severity (B) of clinical arthritis are higher in S100A4KO (n=33) compared with WT mice (n=34) following intravenous inoculation of *S.aureus*.** The frequency of arthritis is presented as a percentage of the number of infected mice. The severity of arthritis is represented by the arthritis index constructed by adding the scores from all 4 limbs from each mouse.

However, Morphological evaluation of joint on day 11 showed the severity of synovitis and erosion were similar between S100A4KO and WT mice (% synovitis:  $2.4 \pm 0.1$  vs.  $2.3 \pm 0.1$ ; erosion:  $2.3 \pm 0.2$  vs.  $2.3 \pm 0.2$ , respectively). To the same extent, frequency of synovitis and erosion in histological sections were also similar between two groups (Table 1).

Table 1. Morphological evaluation of joints following hematogenous staphylococcal infection

	Synovitis		Bone destruction/erosions	
	Frequency (%) <sup>a</sup>	Severity	Frequency (%)	Severity
Day 7				
S100A4KO, n = 11	43 ± 7	2.0 ± 0.2	23 ± 4	1.4 ± 0.2
WT, n = 12	35 ± 4	1.7 ± 0.2	18 ± 4	1.1 ± 0.1
Day 11				
S100A4KO, n = 18	53 ± 4	2.4 ± 0.1	39 ± 5	2.3 ± 0.2
WT, n = 18	54 ± 3	2.3 ± 0.1	41 ± 4	2.3 ± 0.2

Note: Histological score was constructed as described in (Bremell et al., 1991), where 0 points = no damage, and 1 point = mild, 2 points = moderate, and 3 points = severe arthritis.

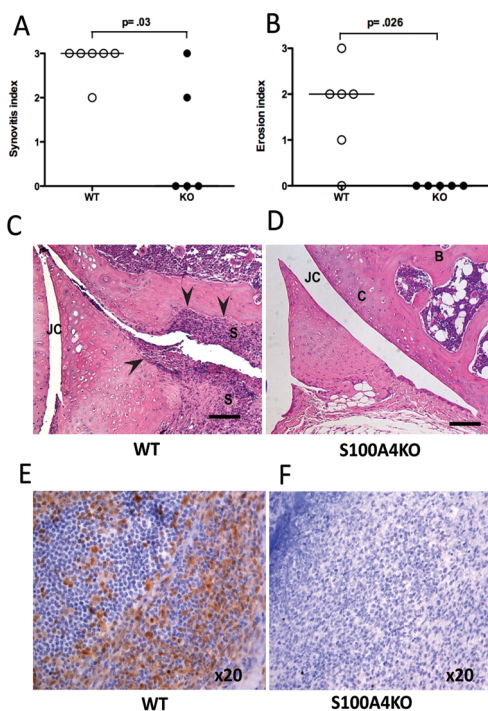
<sup>a</sup> Frequency represents the proportion of affected joints to all inspected joints (%).

<sup>b</sup> Severity represents the score (mean ± SEM).

**In septic arthritis, S100A4 deficiency resulted in significantly reduced synovitis and cartilage/bone destruction compared with WT counterparts following intra-articular injection of *S.aureus* (paper I)**

After a single injection of *S.aureus* into the knee joints, morphological evaluation showed that signs of synovitis were significantly less pronounced in S100A4KO mice than that in WT controls ( $p = 0.03$ , Figure 2A). Joint erosions were seen in 83% of WT mice, but in none of S100A4KO mice ( $p = 0.026$ , Figure 2B-D). Staining of WT mice synovia showed accumulation of S100A4 and none were detected in S100A4KO mice (Figure 2E, F)

The synovia and spleens of infected WT mice showed increased levels of granulocyte compared with non-infected mice (median, synovial: 33% vs. 12%,  $p = 0.001$  and spleen: 12% vs. 6%,  $p = 0.003$  respectively). Influx of granulocytes into the synovial and spleens of infected S100A4KO mice was less pronounced and did not differ significantly from that seen in non-infected S100A4KO mice (data not shown).

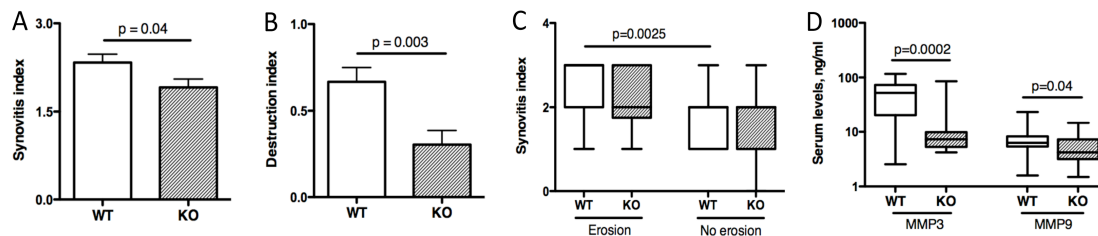


**Figure 2. Morphological finding of joints 4 days after intra-articular injection of *S.aureus* ( $1 \times 10^4$  cfu/mouse).** (A) Leukocyte influx into the joint cavity (synovitis). (B) Cartilage and bone erosion. (C) A representative image of a histological section from a knee joint of a WT mouse. (D) A representative image of a histological section from a knee joint of a S100A4KO mouse. Synovial tissue of WT mice (E) and S100A4KO mice (F) were staining with rabbit anti-mouse S100A4 antibodies (10ug/ml). Scale bar = 100 $\mu$ m. Arrowheads denote bone erosions. B, bone; C, cartilage; S, synovitis.; JC, joint cavity.

**S100A4 deficiency resulted in decreased joint inflammation and cartilage destruction during antigen-induced arthritis (paper II)**

In paper II, we studied the impact of S100A4 deficiency on another murine arthritis model, antigen-induced arthritis. Our results showed that after intra-articular challenge with methylated mBSA, preimmunised S100A4KO mice ( $n = 33$ ) had less synovitis ( $p = 0.04$ , Figure 3A) and milder cartilage/bone destruction ( $p = 0.003$ , Figure 3B) compared with WT controls

(n=33). The frequency of cartilage destruction was significantly lower in S100A4KO mice than in WT controls (30% vs. 67%,  $p=0.006$ ), while the frequency of synovitis was similar (97% vs. 100%, respectively). Moreover, S100A4KO mice displayed significantly lower levels of MMP3 and MMP9 compared to WT counterparts (MMP3,  $p=0.0002$ , MMP9,  $p=0.04$ , Figure 3D). Furthermore, mice with joint erosion was analysed separately. The comparison of mice with and without erosions showed that a high synovitis index in WT mice was associated with erosions ( $p=0.0025$ ), while no such association was seen in S100A4KO mice (Figure 3C). Additionally, S100A4KO mice with erosion had higher levels of soluble RANKL ( $p=0.04$ ) and lower OPG/RANKL ratios compared with WT mice. This difference was not seen in mice without cartilage destruction (data not shown).

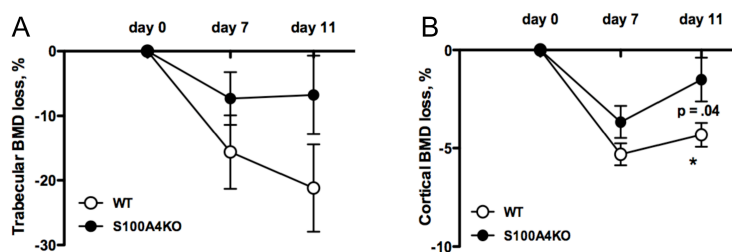


**Figure 3. Joint inflammation and cartilage destruction were decreased in S100A4KO mice compared with WT mice during mBSA arthritis.** (A) Morphological evaluation of injected knee joints showed that S100A4KO mice had less inflammation. (B) S100A4KO mice showed less cartilage/bone destruction. (C) Synovitis in mice with/without erosion showed that synovitis in WT mice was associated with the presence of erosion, while no such association was seen in S100A4KO mice. (D) Levels of matrix metalloproteinases were lower in S100A4KO mice compared with WT.

### **S100A4KO mice had less bone loss compared with WT controls after intravenous inoculation of *S. aureus* (paper I).**

The left femur of each mouse was subjected to pQCT scan to measure the bone mineral density (BMD) when the experiment was terminated. Bone loss was evaluated following staphylococcal infection. Reduction of BMD in arthritis was showed here because bone mineral density is different before infection. Both S100A4KO mice and WT counterparts had bone loss during staphylococcal infection. However, S100A4KO mice had less bone loss compared with WT controls (Figure 4). Loss of BMD was predominantly in trabecular bone (on day 11, S100A4KO -7.5% vs. WT -21.2%, Figure 4A). Cortical bone loss was also smaller in S100A4KO mice (on day 11, S100A4KO, -1.5% vs. WT -4.3%,  $p=0.04$ , Figure 4B). The less bone loss in S100A4KO mice was supported by significantly lower serum levels of RANKL (on day 7, S100A4KO  $9.1 \pm 1.0$  vs. WT  $11.1 \pm 0.8$  ng/ml,  $p = 0.04$ ; on day 11, S100A4KO  $2.6 \pm 0.3$  vs. WT  $3.3 \pm 0.2$  ng/ml,  $p = 0.03$ ).

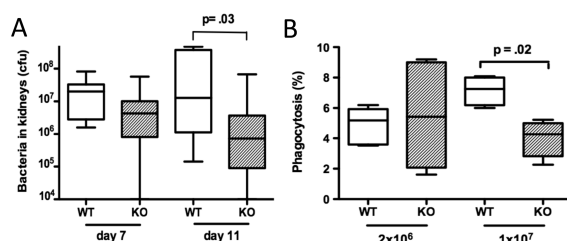




**Figure 4. Bone loss in S100A4KO and WT mice during the course of septic arthritis.** Reduction in trabecular (A) and cortical (B) bone mineral density (BMD) on day 7 and day 11 were more pronounced in WT mice (n=12) than in S100A4KO mice (n=14).

### Bacterial load in the kidneys were lower in S100A4KO after intravenous inoculation of *S. aureus*, while the phagocytostic properties of S100A4KO mice was impaired (paper I)

The comparison of *S.aureus* load in the kidneys showed that S100A4KO mice had consistently lower bacterial counts than WT mice (Figure 5A). To find out why S100A4 deficiency causes efficient bacterial clearance in the kidneys, bacterial distribution *in vivo*, phagocytosis *in vitro* and bactericidal effect of S100A4 were evaluated. Our results showed that S100A4KO mice had higher bacterial accumulation in the kidneys one hour after intravenous inoculation of FATC-labeled bacteria compared with WT mice (2.17% vs. 0.37%, p=0.015, respectively). However, phagocytic capacity of S100A4KO mice was significantly reduced (Figure 5B) at higher bacterial concentration and S100A4 had no bactericidal effect.

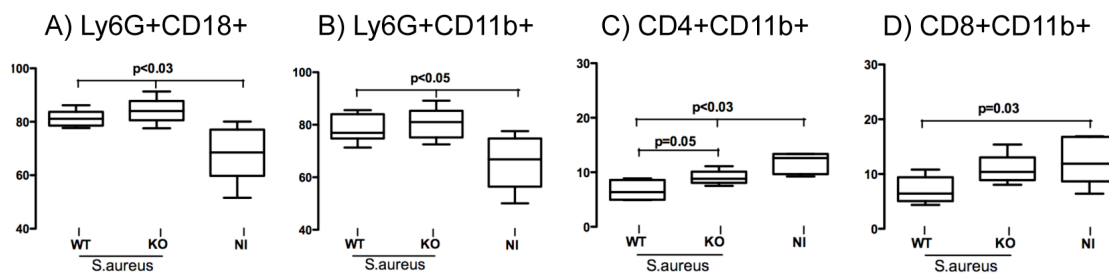


**Figure 5. Bacterial load in kidneys and phagocytosis capacity of S100A4KO and WT mice.** (A) Bacterial load in the kidneys of S100A4KO (n=22) and WT mice (n=23) 7 and 11 days after intravenous inoculation of *S.aureus* ( $1.4 \times 10^7$  cfu/mouse). (B) Phagocytosis of fluorescein isothiocyanate (FITC)-labeled *S.aureus* were assessed in whole blood of S100A4KO (n=10) and WT mice (n=9). Two concentrations of bacteria were used ( $2 \times 10^6$  or  $1 \times 10^7$  cfu/sample).

### Adhesion molecules changed during staphylococcal infection (paper I)

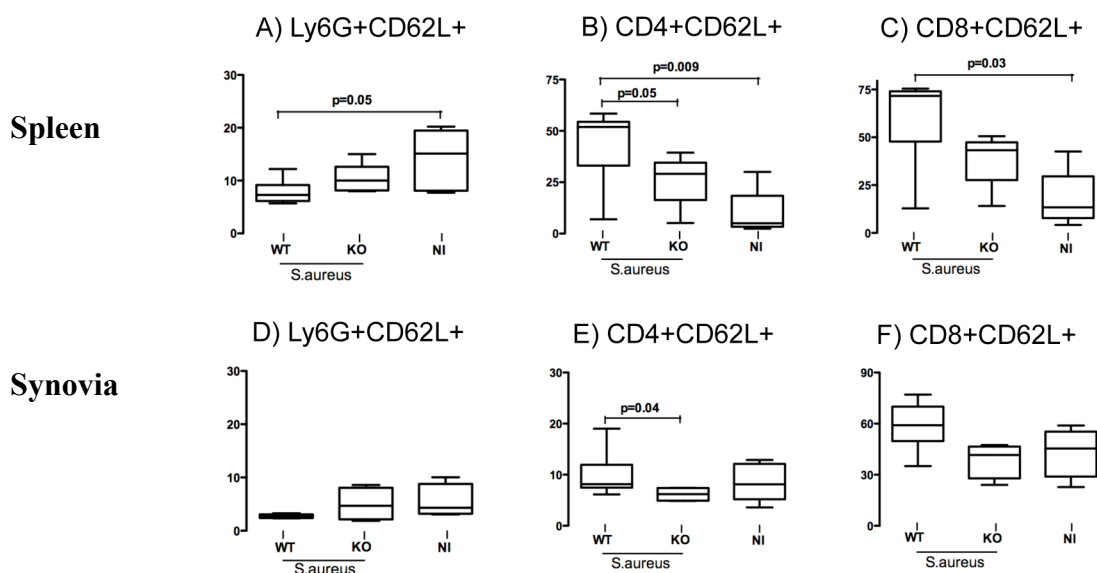
Adhesion molecules play an important role in lymphocyte recruitment and phagocytosis (May et al., 2001). The expression of adhesion molecules on synovial and splenic leukocytes was assessed in both non-infected and those given intra-articular injection of *S.aureus* on T lymphocytes (CD4+ and CD8+ cells) and granulocytes (Ly6G+ cells) of S100A4KO and WT mice.

In infected mice, CD18 and CD11b expression were increased on granulocytes (Figure 6A, B), and CD11b expression was decreased on CD4+ and CD8+ lymphocytes compared with non-infected mice in spleen (Figure 6C, D). Further analysis showed that S100A4KO mice had higher expression of CD11b on CD4+ and CD8+ T cells when compared with WT mice (Figure 6C, D). In synovia, infected mice had an increased expression of CD11b and CD18 on leukocytes and it is more pronounced on Ly6G+ granulocytes and CD4+ lymphocyte populations, while no difference was found between S100A4KO and WT mice (data not shown).



**Figure 6.** The expression of leukocyte adhesion molecules was assessed in the spleens from noninfected mice and after intra-articular challenge with *S.aureus*. Single cell cultures from spleens of S100A4KO (n = 6) and WT (n = 10) mice were stained for integrins (CD18+ and CD11b+) and their expression on granulocyte (Ly6G+) and T lymphocyte (CD4+ and CD8+) were analysed by flow cytometry. Results are presented in percent to granulocyte, CD4+ T cells and CD8+ T cells respectively.

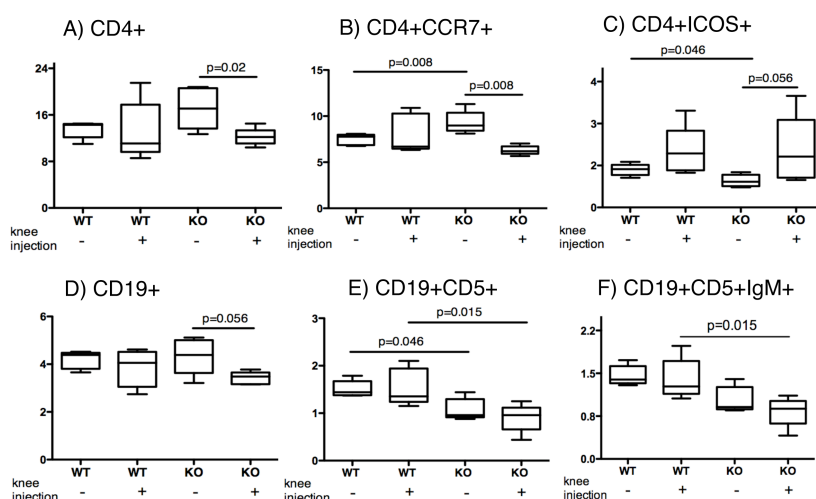
As for L-selectin (CD62L), spleen granulocytes from infected mice showed reduced CD62L expression (Figure 7A), while T lymphocytes (CD4+ and CD8+) had increased levels of CD62L expression (Figure 7B, C). Further analysis showed that S100A4KO mice had slightly higher expression of CD62L on granulocytes (Figure 7A) and significantly lower expression of CD62L on CD4+ and CD8+ cells (Figure 7B, C). In synovia, CD62L expression was also reduced on CD4+ and CD8+ lymphocytes in S100A4KO mice compared with WT mice (Figure 7E-F).



**Figure 7. L-selectin (CD62L) expression on granulocytes and CD4+ and CD8+ lymphocytes was assessed both in spleen and synovia from noninfected mice and after intra-articular challenge with *S.aureus*.** Single cell cultures from spleens and synovia of S100A4KO (n = 6) and WT (n = 10) were stained for L-selectin (CD62L+) and its expression on granulocyte (Ly6G+) and T lymphocyte (CD4+ and CD8+) was analysed by flow cytometry. Results are presented in percent to granulocyte, CD4+ T cells and CD8+ T cells respectively.

### **Influx of T and B cells in synovial tissue of S100A4KO and WT mice during mBSA-arthritis (paper II)**

Lymphocytes play a leading role in antigen-induced arthritis. The cells retrieved from synovial tissue were analysed by flow cytometry in mBSA immunized S100A4KO and WT mice prior to and 7 days after intra-articular injection with mBSA. Our results showed that S100A4KO mice had loss of total CD4+ T cells following intra-articular challenge of mBSA (Figure 8A). This was mainly pronounced in CD4+CCR7+ T cells (Figure 8B). Loss of CCR7 in CD4+ T cells may indicate a transformation from memory into effector cells. Analysis of B cell subpopulations showed that CD19+ cells decreased after challenging of mBSA (Figure 8D) and CD19+CD5+ B1 cells was significantly reduced in both prior to and after intra-articular injection of mBSA (Figure 8E-F).



**Figure 8. Influx of T and B cells in synovial tissue of S100A4KO and WT mice** Synovial cells were retrieved from knee joints of pre-immunised mice prior to intra-articular injection (n=5+5) or 7 days after the injection (n=5+5). Reduced influx of CD4+CCR7+ and CD19+CD5+ cells populations is obvious in the synovia of S100A4KO mice. CD4+ T cell subpopulations (A-C) are presented in percent to the total leukocyte number. CD19+ B cell subpopulations are presented in percent to the total leukocyte number.

### Dysregulation of T and B cell populations in spleens of S100A4KO and WT mice in mBSA-induced arthritis (paper II)

The splenocyte subpopulations were assessed when the experiments was terminated and shown in Table 2. S100A4KO mice had a significantly higher proportion of CD19+ B cells, as well as a smaller CD4+ and CD8+ T lymphocytes compared to WT controls, while the macrophage and dendritic cell populations were similar.

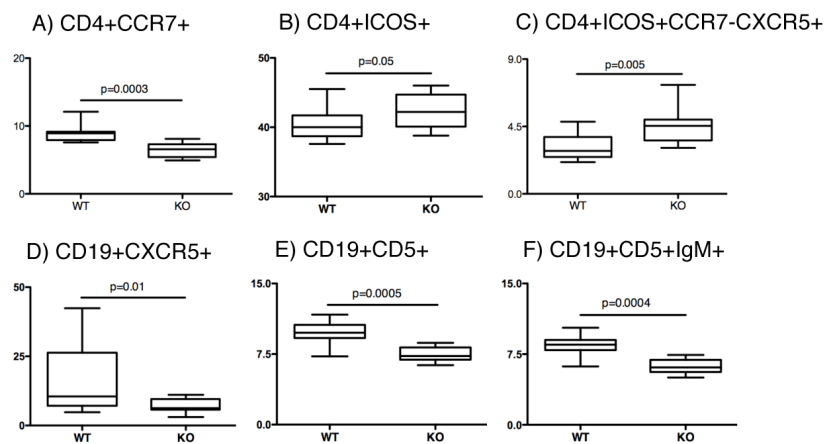
Table 2. Main leukocyte populations in spleens of S100A4KO and WT mice

	Wild-type (n=11)	S100A4KO (n=11)	P-values
CD4+ T cells	15 [14-16]	13 [12-15]	0.02
CD8+ T cells	4.6 [4.4-4.8]	3.9 [3.2-4.6]	0.035
CD19+ B cells	45 [40-47]	51 [49-52]	0.001
CD11c+B220- cells	2.1 [2.0-2.3]	2.3 [2.0-2.5]	N.S.
F4/80+ cells	1.6 [1.5-1.8]	1.55 [1.3-1.6]	N.S.

\*Note. The values were showed as % of total splenocytes: median [interquartile range]

Further analysis of T cell subpopulations showed that S100A4KO mice had a smaller population of CD4+ memory cells, due to reduction of the CD4+CCR7+ subpopulation (p=0.003, Figure 9A), as compared to WT mice. Additionally, S100A4KO mice showed increases in the CD4+ICOS+ population (p=0.05, Figure 9B) and T follicular helper cells (p=0.005, Figure 9C), which is defined as CD4+ICOS+CCR7-CXCR5+.

Analysis of B cell subpopulations showed a reduction of CD19+CXCR5+ B cells ( $p=0.01$ , Figure 9D), a smaller CD19+CD5+ population ( $p=0.0005$ , Figure 9E), and consequent reduction of CD19+CD5+IgM+ subpopulations ( $p=0.0004$ , Figure 9F) in S100A4KO mice.



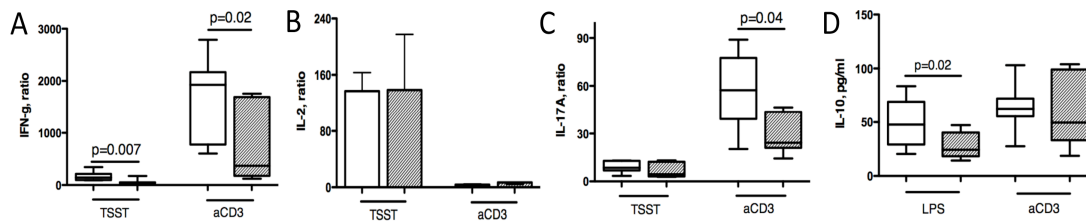
**Figure 9. T and B lymphocytes in spleens of S100A4KO and WT mice during mBSA-induced arthritis.** Memory T cell population (A) is significantly smaller in S100A4KO mice compared with WT mice. Follicular T cell populations (B,C) are higher in S100A4KO mice, while CD19+CXCR5+ cell population (E) and CD19+CD5+ cell populations (F,G) are smaller in S100A4KO mice. Subpopulations of CD4+ T cells are presented as a percent to total CD4+ T cells. Subpopulations of CD19+ B cells are presented as a percent to total CD19+ cells. S100A4,  $n = 11$ ; WT mice,  $n = 11$ .

### Impaired T cell function in S100A4KO mice during mBSA-arthritis (paper II)

T cell response was assessed in S100A4KO and WT mice following stimulation with anti-CD3 (aCD3), TSST-1, and LPS. The proliferative response, cytokine production and expression of transcription factors distinctive for subpopulations of T cells were analysed.

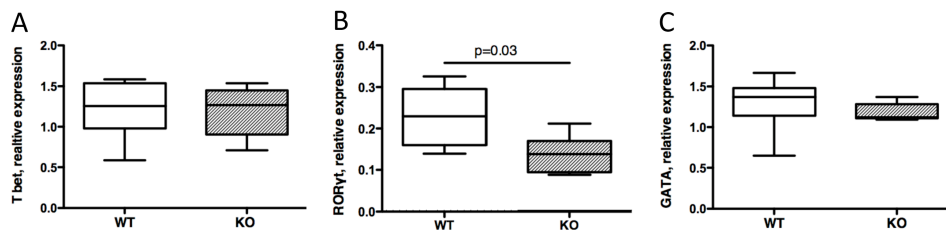
Splenocyte proliferation in S100A4KO and WT mice was similar in response to any stimuli applied. The production of IL-2 was similar between S100A4KO and WT mice (Figure 10B).

The productions of cytokine IFN- $\gamma$  and IL-17A were significantly lower in aCD3 stimulated splenocyte cultures of S100A4KO mice compared with WT mice (Figure 10A, C). S100A4KO mice had a reduced production of IL-10 following LPS stimulation, while after aCD3 stimulation, IL-10 production was similar in S100A4KO and WT mice (Figure 10D).



**Figure 10. Reduced Th cytokines in S100A4KO mice during the course of mBSA arthritis.** Lymphocyte cultures (n=7+7,  $2 \times 10^6$ /ml) from the spleens of S100A4KO and WT mice were stimulated with anti-CD3 antibodies, TSST or LPS when the experiments were terminated on day 28. Cytokines released in the supernatant were analysed. (A), (B), (C), (D) present the levels of cytokine IFN- $\gamma$ , IL-2, IL-17A and IL-10, respectively. Results showed here are relative changes compared to the baseline level of each cytokine, except IL-10 (D), which showed the absolute value because of the extreme low baseline production. S100A4KO mice: dark bar; WT mice: empty bar.

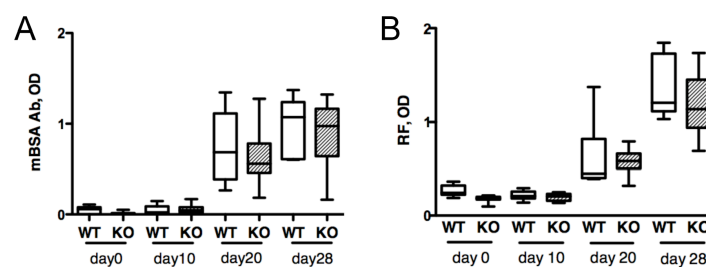
The expression of the Th17 specific transcription factor ROR $\gamma$ t was reduced in S100A4KO mice (Figure 11B), while the expression of Th1 transcription factor T-bet was similar in S100A4KO and WT mice (Figure 11A). No difference in mRNA expression of Th2 transcription factor GATA 3 can be detected between S100A4KO and WT mice (Figure 11C).



**Figure 11. mRNA expression of Th cell transcription factors in S100A4KO and WT mice.** Total mRNA was prepared from splenocyte pellets and assessed for transcription factors of T-bet (A), ROR $\gamma$ t (B) and GATA-3 (C). S100A4KO mice, n=7; WT mice, n=7.

### Non-changed antibody production in S100A4KO and WT controls (paper II)

The specific mBSA-antibody productions in S100A4KO and WT mice were assessed on days 10, 20 and 28 after immunized with mBSA. A gradual increase in the titres of anti-mBSA antibodies was found on both S100A4KO and WT mice. The increase was identical between two groups of mice (Figure 12A). Levels of rheumatoid factor, antibodies against the Fc-fragment of IgG, did not differ between S100A4KO mice and WT counterparts (Figure 12B).



**Figure 12. The serum levels of mBSA-specific IgG antibodies and rheumatoid factor were similar in S100A4KO and WT mice.** Antibodies levels were assessed in blood in different time point (day 0, 10, 20 and 28). (A) mBSA-specific IgG antibodies. (B) Rheumatoid factor (RF).

### **S100A4 deficiency led to increased bone formation (paper III)**

During the septic arthritis, we found that female S100A4KO mice had higher BMD compared with WT mice. Further analysis showed that this difference exists before infection. In paper III, we first studied bone formation in S100A4KO and WT mice. Total BMD in female S100A4KO mice was higher than in WT mice (mg/mm<sup>3</sup>: 529 [475-588] vs. 493 [379-536], p=0.01). Further analysis showed that trabecular density was higher in S100A4KO mice (Table 3). S100A4KO mice had a significantly larger cortical area (p =0.003) and higher cortical content (p = 0.03) (Table 3) than WT controls. This resulted in the increase on bone strength. S100A4KO mice had 21% higher resistance to twisting (moment of inertia, p = 0.004) and 12.5% higher tolerance to bending (moment of resistance, p = 0.01) (Table 3)

Table 3. Bone parameters in naïve female WT and S100A4KO mice

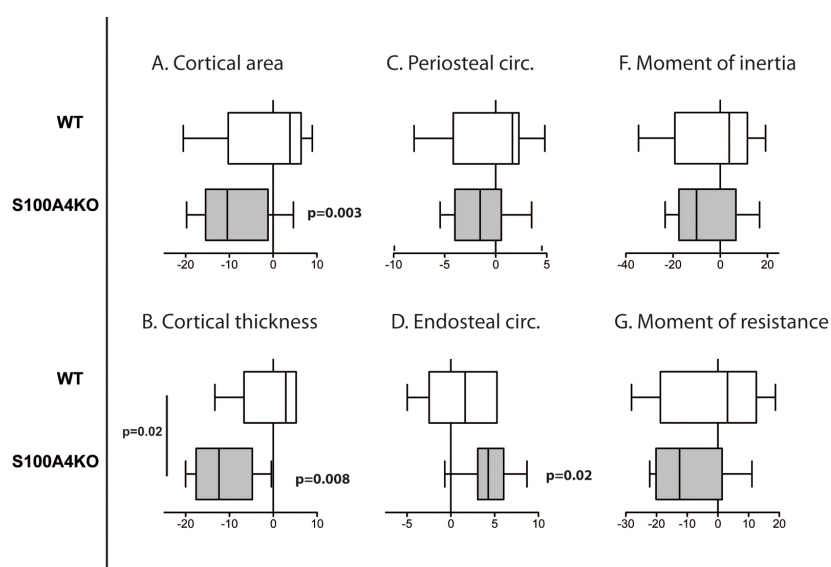
Mouse strain	WT N=13	S100A4KO N=11	P value
Trabecular BMD.mg/cm <sup>3</sup>	286 [164-430]	355 [209-508]	N.S.
Cortical density. mg/cm <sup>3</sup>	1260 [1203-1290]	1237 [1172-1319]	N.S.
Cortical content. mg/mm	0.99 [0.84-1.099]	1.07 [0.87-1.26]	0.03
Cortical thickness. mm	0.21 [0.20-0.23]	0.23 [0.20-0.25]	0.05
Cortical area. mm <sup>2</sup>	0.78 [0.70-0.86]	0.86 [0.74-0.97]	0.003
Endosteal circ.. mm	3.00 [2.68-3.19]	3.08 [2.88-3.23]	0.03
Periosteal circ.. mm	4.32 [4.09-4.54]	4.55 [4.33-4.65]	0.001
Moment of inertia	0.25 [0.20-0.31]	0.30 [0.23-0.37]	0.004
Moment of resistance	0.32 [0.28-0.37]	0.36 [0.29-0.41]	0.01

\*Note. Bone parameters were presented as median [interquartile range].

### **Loss of cortical bone in S100A4KO mice following estrogen deprivation (paper III)**

As we described previously, bone BMD was increased in female S100A4KO mice. So ovariectomy (OVX) was performed to find out if estrogen plays a role. OVX resulted in significant loss of total BMD (WT: -20% vs. S100A4KO: -19%) and trabecular BMD (WT: 39.5% vs. S100A4KO: 34.5%). The degree of these bone loss is similar for both S100A4KO and WT mice.

However, evaluation of cortical bone loss following OVX gave different results. WT mice showed no change on either cortical bone or bone strength. In contrast, OVX S100A4KO mice had a significant more bone loss in cortical compartment. Following OVX, the reduction of cortical bone thickness was more pronounced in S100A4KO mice than WT mice (% S100A4KO: -12.4 [-17.6, - 4.8] vs. WT: +2.9 [-6.7, 5.2],  $p = 0.02$ , Figure 13B). To the same extent, the changes of other cortical parameters were more obvious in OVX S100A4KO mice. The increased endosteal circumference ( $p = 0.02$ , Figure 13D) led to the reduction of cortical thickness ( $p=0.008$ , Figure 13B) and cortical area ( $p = 0.003$ , Figure 13A) compared with sham-operated S100A4KO mice. This resulted in a decrease of cortical bone strength in resistance to both twisting and binding (Figure 13 F, G).



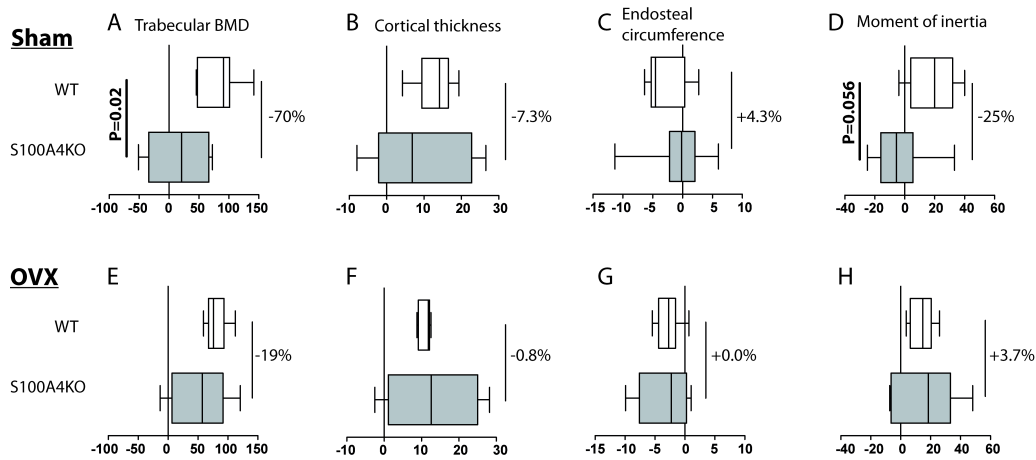
**Figure 13. S100A4KO mice lose cortical bone after ovariectomy.** Sex and age matched S100A4KO mice and WT mice were ovariectomised (OVX) or sham operated and left for 5 weeks. At termination, the left femora were subjected to pQCT. This figure compares the cortical compartments of S100A4KO mice and WT mice. The data are shown as relative change (%) comparing OVX values to the median value in the sham operated mice of the same genotype. P values shown represent statistical evaluation (Mann-Whitney U test) between the OVX and sham groups. A. Cortical area, B. Cortical thickness, C. Periosteal circumference, D. Endosteal circumference, F. Moment of inertia, G. Moment of resistance.

### BMD in WT and S100A4KO mice after DHEA treatment (paper III)

DHEA treatment resulted in the increase of BMD both in sham-operated and OVX mice (Figure 14). In sham-operated mice (Figure 14, upper row), this increase is more pronounced in WT mice than in S100A4KO mice. Changes in trabecular bone BMD is significantly higher in WT mice compared to S100A4KO mice (91% vs. 21%,  $p=0.02$ , Figure 14A). WT mice showed 14% increase in cortical thickness ( $p=0.0012$ ), while the increase of S100A4KO mice was not significant (4%, Figure 14B), which led to the increased strength to twisting in WT mice (Figure 14D). DHEA treatment on OVX mice also increased trabecular and cortical



BMD in WT and S100A4KO (Figure 14, down row). The degree of the increase of BMD was similar in estrogen-deprived WT and S100A4KO mice (Figure 14E-H).



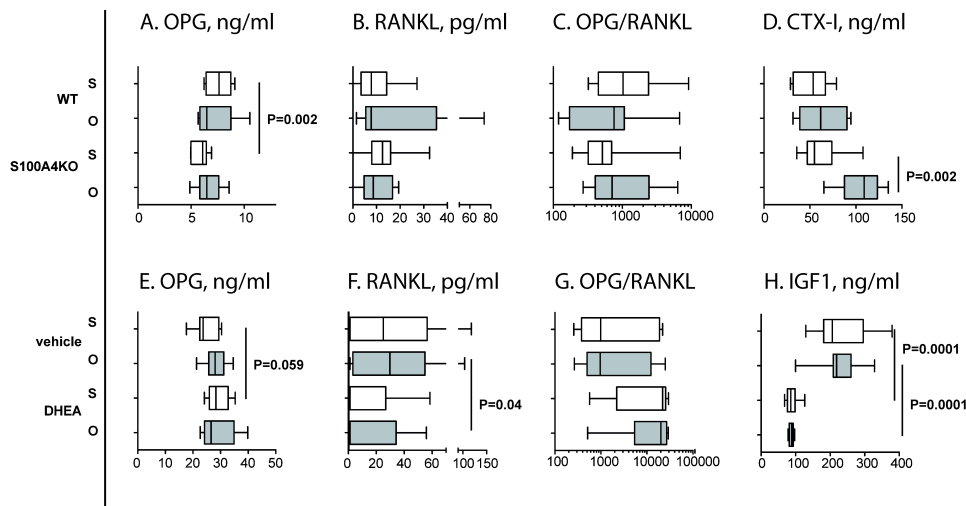
**Figure 14. DHEA treatment resulted in less bone formation in sham operated S100A4KO mice.**

S100A4KO mice and WT mice were sham operated or ovariectomised (OVX) and left for 10 days to rest followed by 5 weeks treatment with dehydroepiandrosterone (DHEA). Data are shown as relative changes (%) comparing the values from DHEA-treated mice to the median value in the vehicle-treated mice. P values represent a comparison of the treatment changes in WT and S100A4KO mice using Mann-Whitney U test. A-D, sham-operated mice. E-H, OVX mice. A and E, Trabecular BMD. B and F, Cortical thickness. C and G, Endosteal circumference. D and H, Moment of inertia.

### Serum levels of IGF-1 and markers for bone turnover (paper III)

Serum levels of IGF-1 were similar in sham operated S100A4KO and WT mice, while it showed a slight increase in OVX S100A4 mice than WT mice (ng/ml: 362[282-447] vs. 311[232-420], respectively). IGF-1 was significantly decreased after DHEA treatment (Figure 15H).

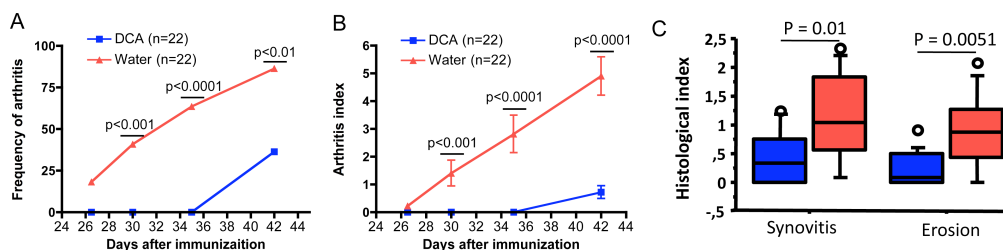
The marker of bone degradation CTX-I was similar in sham-operated mice, while levels of CTX-I was 200% increased ( $p=0.002$ , Figure 15D) in OVX S100A4KO mice than OVX WT mice. This is consistent with the excessive loss of cortical bone in S100A4KO mice under estrogen deprivation. Sham operated S100A4KO mice had a lower level of OPG compared with WT mice (Figure 15A). After OVX, WT mice had a slight rise of RANKL level followed by a decrease OPG/RANKL ratio (1023[321-9130] vs. 758[119-6762]; Figure 15B, C), while OVX S100A4 had no increase in RANKL following estrogen deprivation. DHEA treatment improved OPG levels (Figure 15E) and decreased levels RANKL (Figure 15F), leading to an increase in OPG/RANKL ratio (Figure 15G).



**Figure 15. Bone turnover markers.** OPG, RANKL and the collagen type-I fragment (CTX-I) were measured in serum by ELISA. Figures 3A-D are from the first experiment with WT and S100A KO mice subjected to OVX or sham operation, Figures 3E-F are from the experiment with DHEA treatment of S100A4KO mice. Statistical comparisons were made with the Mann-Whitney U test.

### Dichloroacetate (DCA) treatment significantly reduced frequency, severity of arthritis and bone destruction in collagen-type II induced arthritis mice (paper IV)

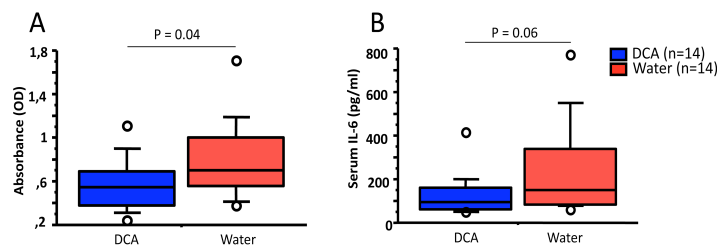
In paper IV, we use another murine arthritis model, collagen-type II induced arthritis to evaluate the effect of dichloroacetate (DCA) on arthritis. We found that DCA treatment alleviated the development of collagen type II arthritis in female DBA/1 mice, but not in male mice. DCA treated female mice had a significantly slow disease onset (on day 35, none of DCA drinking mice had arthritis vs. 73% of water treated mice had arthritis), lower frequency and much less severe severity compared with water treated DBA/1 mice (Figure 16 A, B). Morphological evaluation confirmed that DCA group had a reduced arthritis. Importantly, the destruction of bone and cartilage was significantly diminished in DCA drinking group compared with water controls (Figure 16C). For male mice, there is no difference between DCA-drinking group and water treated group (data not shown).



**Figure 16. Arthritis induced by collagen type II was reduced in DCA treated female mice.** Frequency (A) and severity (B) of arthritis were significantly decreased in DCA treated mice compared with water treated mice. (C) Morphological finding showed that DCA treated mice had significantly reduced synovitis and bone erosion.

## Circulating anti-collagen II IgG antibody levels and IL-6 level were lower in DCA-treated female DBA/1 mice (paper IV)

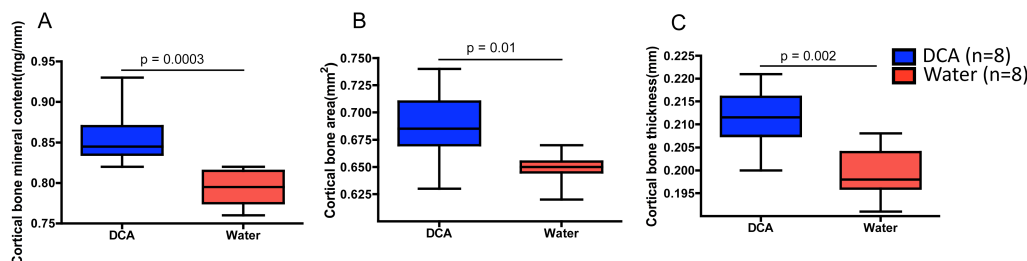
The ameliorated effect of DCA on female DBA/1 mice may be because of the suppression of specific anti-collagen II IgG antibodies production and pro-inflammatory cytokine IL-6 (Fig 17A, B). Our results showed that circulating anti-collagen II IgG antibodies and IL-6 were decreased ( $p = 0.04$  and  $p = 0.06$ , respectively) in DCA drinking group compared with water treated mice.



**Figure 17. DCA impact on *in vivo* inflammatory immune response.** (A) Serum level of anti-collagen II IgG antibodies and (B) IL-6 in DCA drinking group and water treated group. OD: optical density.

## Cortical bone loss were prevented in DCA-treated female DBA/1 mice (paper IV)

Analysis of bone mineral density showed that the female mice immunized with collagen II and treated with DCA displayed higher cortical bone content ( $p = 0.0003$ , Figure 18A), increased cortical bone area ( $p = 0.01$ , Figure 18B) and increased cortical bone thickness ( $p = 0.002$ , Figure 18C) when compared with water treated mice. No difference was observed with respect to total BMD and trabecular BMD between the DCA drinking group and water treated group (data not shown).



**Figure 18. Dichloroacetate (DCA) effect on cortical bone mineral density (female mice only).** Cortical bone mineral content (A), cortical bone area (B) and the thickness of cortical bone (C) are significantly higher in DCA treated mice compared with water drinking mice.

## Impact of DCA treatment had no effect on *in vivo* cell-mediated inflammatory response (paper IV)

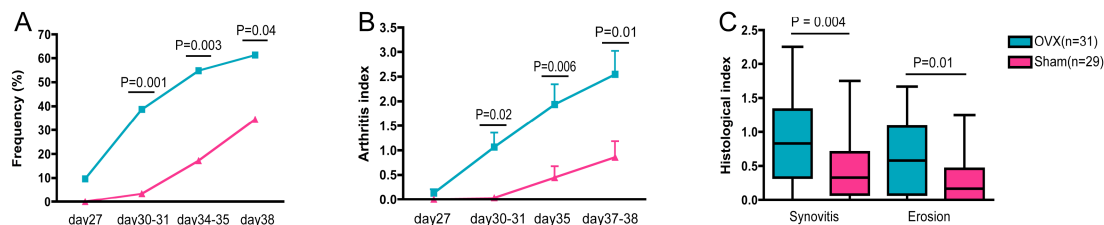
DTH is a T cell/macrophage mediated immune reaction and olive oil-induced inflammation is a granulocyte-dependent and T cell- and macrophage-independent immune response. The mice pretreated with DCA and water were immunised with OXA. DTH reactivity was

evaluated by measuring the increase of thickness on ears 24 hours after the challenge. Olive oil response was assessed by the increase of paw thickness after 24 hours. Our results showed that both the DTH response and olive oil response were similar in the DCA and water drinking groups.

#### OVX mice treated with DCA had more severe arthritis compared with sham-mice (paper IV)

We speculated that the beneficial effect of DCA on collagen type II-induced arthritis is estrogen dependent because only female DBA/1 mice response to DCA. To test our hypothesis, one group of mice was ovariectomized and another group had sham surgery. Then both groups were induced collagen type II arthritis and treated with DCA. Our results showed that the OVX group treated with DCA had significantly higher frequency and more severe arthritis all the way during the course of arthritis (Figure 19A, B). Morphological evaluation confirmed that the OVX group had more synovitis ( $p = 0.004$ ) and bone erosion ( $p = 0.01$ ) compared with sham surgery group treated with DCA (Figure 19C). However, no significant difference was detected with respect to serum levels of collagen type II antibodies and IL-6 (data not shown).

The weights of uteri, an indirect indicator of estrogen levels, were measured in intact mice. No difference was found between from DCA treated and water treated mice.



**Figure 19. Impact of ovariectomy (OVX) on dichloroacetate (DCA) treatment of collagen type II arthritis.** Frequency (A) and severity (B) of arthritis were more severe in OVX mice treated with DCA than sham-operated mice treated with DCA. (C) Morphological evaluation confirmed that OVX mice treated with DCA had more synovitis and erosion than sham-operated mice treated with DCA.

# CONCLUSIONS

The major conclusions from this thesis are:

## ***PAPER I***

- S100A4 deficiency results in reduced synovitis and cartilage/bone destruction in staphylococcal arthritis following both systemic (intravenous) and intra-articular instillation of bacteria
- S100A4 deficiency results in less bone loss accompanied by lower level of RANKL and MMPs
- S100A4 deficiency results in a lower bacterial load in kidneys
- S100A4 protein has no bactericidal capacity

## ***PAPER II***

- S100A4 deficiency results in reduced intensity of synovitis and joint destruction during the course of antigen-induced arthritis. This protective effect was observed in male S100A4KO mice
- S100A4 deficient mice display lower CD5+ B1 cell population in synovia and in spleen
- S100A4 deficient mice have smaller CD4+ and CD8+ T cells populations and dysfunctional Th1 and Th17 cells

## ***PAPER III***

- S100A4 deficiency in female mice gives enhanced cortical bone formation compared to WT counterparts
- S100A4 deficient mice have an excessive cortical bone loss following estrogen deprivation
- S100A4 deficiency is associated with an insufficient bone formation in response to DHEA treatment

## ***PAPER IV***

- DCA treatment delays the onset and alleviates the severity of collagen-type II induced arthritis, and prevents cortical bone loss during the course of arthritis in female mice
- The protective effect of DCA is significantly diminished in estrogen deprived (ovariectomised) mice

## DISCUSSION

In this thesis, the roles of S100A4 in arthritis were studied by using S100A4 KO mice (S100A4KO) and their wild-type counterparts (WT) in septic arthritis (paper I) and in mBSA-induced arthritis (paper II). The results showed that S100A4 deficiency resulted in reduced inflammation and cartilage/bone destruction in joints in both septic and mBSA-induced arthritis, as well as less bone loss in septic arthritis. S100A4 deficiency is also associated with efficient bacterial clearance. The impact of S100A4 deficiency on arthritis is more pronounced in male mice during mBSA-induced arthritis. Moreover, lack of S100A4 resulted in high BMD in estrogen sufficient mice and excessive loss of cortical bone in estrogen deficient mice (paper III). In addition, we demonstrated that dichloroacetate (DCA) alleviated collagen-type II induced arthritis in female DBA/1 mice (paper IV).

This thesis studied several mechanisms of potential importance for the arthritis protection in S100A4 deficiency mice. Expression of adhesion molecules is one of the mechanisms. Our studies showed that S100A4 deficiency resulted in changes of adhesion molecule expressions. In septic arthritis (paper I), staphylococcal infection caused the increase of CD18 and CD11b expression on the granulocytes and the decrease of CD18 and CD11b on CD4<sup>+</sup> and CD8<sup>+</sup> T cells. The expression of L-selectin on granulocytes was reduced, while expression of L-selectin on T lymphocytes was increased in spleen. The most striking difference between WT and S100A4KO mice in the expression of adhesion molecules was seen in the CD4<sup>+</sup> T cell population. In staphylococcal infection, the expression of integrin CD11b, CD18, and L-selectin on CD4<sup>+</sup> T cells in S100A4KO mice was changed less compared to WT mice. This suggests that S100A4KO mice were less reactive and less adjustable in the expression of adhesion molecules as compared to WT mice during septic arthritis.

We also found that influx of granulocytes into the synovia and spleens of infected WT mice were significantly increased compared with non-infected mice, while this increase in cell infiltration was not seen in infected S100A4KO mice. This suggests a limited cell migration to the sites of inflammation in S100A4KO mice and this may be dependent on less reactive adhesion molecules. Adhesion molecules play an important role in cell adhesion and detachment, as well as cell migration. S100A4 has been shown to modulate cell adhesion by regulating E-cadherin mediated cell-to-cell adhesion system (Kimura et al., 2000). Changed patterns of adhesion molecules in S100A4KO mice may reduce leukocytes adhesion and

migration to the inflamed joints. The decreased infiltration of leukocytes led to less inflammation and bone destruction.

During the course of septic arthritis, alteration in adhesion molecules might be a reason of the impaired endothelial adhesion and phagocytosis, followed by lower bacterial load in kidneys in S100A4KO mice (paper I). We found an early accumulation of staphylococci in kidneys of S100A4KO mice followed by rapid bacterial clearance and lower bacterial load in kidneys in the late phase of infection. In addition, the phagocytic capacity in S100A4KO was impaired at a high dose of bacterial inoculation. Both phagocytosis and leukocyte migration require recognition of bacteria by adhesion molecules (May et al., 2001). Macrophages lacking the expression of  $\beta$ -integrins show defective phagocytosis (Wang et al., 2008). Impaired bacterial attachment to the surface of cells and cartilage facilitate bacterial killing by bactericidal peptides in circulation. S100A4KO mice showed high levels of soluble L-selectin (sL-selectin). Soluble L-selectin is released following shedding from the cell surface by Adam-17 (Tang et al., 2011). High levels of sL-selectin have been previously identified as a marker of inflammation severity (Maekawa et al., 1998).

Our results also showed that S100A4KO mice had lower levels of MMP3 and MMP9 compared with WT mice both in infected and in naïve mice (paper I, II). It has been shown that S100A4 stimulates the production of MMPs in different pathways. Extracellular stimulation of endothelial cells using recombinant S100A4 resulted in the increase of MMP13 expression and was followed by subsequent release of the proteins from the endothelial cells (Schmidt-Hansen et al., 2004). Receptor for advanced glycation end products (RAGE) is one of the potential receptors that mediate extracellular effects of S100A4. Binding of S100A4 to RAGE stimulates MMP13 production by chondrocytes (Yammani et al., 2006). Intracellular effects of S100A4 on MMPs have been studied on S100A4 overexpressing cells. Overexpression of S100A4 significantly increases MMP9 expression by transcriptional activation and subsequently increases its proteolytic activity (Saleem et al., 2006). Inhibition of S100A4 transcript on osteosarcoma cell line reduced the mRNA levels of MMP2 (Bjornland et al., 1999). S100A4 deficiency mice displayed lower levels of MMP3 and MMP9. However, in our experimental arthritis models, we could not differ between extracellular and intracellular effects of S100A4 deficiency on MMPs expression.

Are lower levels of MMPs in S100A4KO mice one of the mechanisms that underline the protective effect on arthritis? Studies about the effects of MMPs on arthritis are controversial. Table 2 presents a number of studies on MMPs in animal models of arthritis. Different MMPs seem to have different impacts on joint destruction and inflammation. In clinical studies, it has been shown that MMP inhibitors have little effect on rheumatoid arthritis (Milner et al., 2005). Thus, it remains unclear if the lower levels of MMPs in S100A4KO mice contribute to the protective effect of arthritis.

Table 1. Effects of some MMPs in joint inflammation and destruction in animal models of arthritis

MMPs	Joint destruction	Joint inflammation	Arthritis *	References
MMP1	↑	↑	OA	(Wu et al., 2008)
MMP2	↑		MOA	(Mosig et al., 2007)
MMP3	↑		OA	(Blom et al., 2007)
	↑	↑	Streptococcal arthritis	(Koenders et al., 2005)
	↓	↓	CII	(Mudgett et al., 1998)
MMP8	↓	↓	Serum transfer arthritis	(Garcia et al., 2010)
	↓	↓	Adjuvant arthritis	(Cox et al., 2010)
MMP9	↑	↑	Streptococcal arthritis	(Koenders et al., 2005)
	↓	↑	Staphylococcal arthritis	(Calander et al., 2006)
MMP13	↑	↑	Streptococcal arthritis	(Koenders et al., 2005)
	↑↓		OA	(Little et al., 2009)
MMP14	↑		hTNF arthritis	(Schurigt et al., 2008)

\* Note. OA, osteoarthritis. MOA, multicentric osteolysis with arthritis. CII, collagen-type II induced arthritis. hTNF, human tumor necrosis factor-transgenic mouse model.

The impact of S100A4 on lymphocyte development is unknown. In the antigen-induced model of arthritis, we found that S100A4 deficiency may impair the formation of T and B lymphocytes. S100A4KO mice had reduced CD19+CD5 and CD19+CXCR5+ B cell subpopulations. The reduction of these B cell populations is of potential importance for the development of arthritis due to the abundance of CD5+B cells at inflammation sites (Wen et al., 2005) and enrichment of CD5+B cells in peripheral blood in RA (Becker et al., 1990, Plater-Zyberk et al., 1985). CXCR5+ B cells have been shown important regulators of immunological memory and auto-reactivity (Lee et al., 2009). We used LPS-stimulation of splenocytes to study a specific B cell response in S100A4KO mice. The analysis of B cell specific production of IL-10 showed reduced levels of IL-10 produced in S100A4KO



splenocytes compared to WT mice. This may suggest a dysfunctional regulatory B cells in S100A4KO mice (Fujimoto, 2010).

We also found that CD4<sup>+</sup> and CD8<sup>+</sup> T cell populations were smaller in the spleens of S100A4KO mice compared with WT mice. Additionally, production of IFN- $\gamma$  and IL17 in S100A4KO mice was significantly decreased, indicating insufficient formation of Th1 and Th17 cell populations.

The interaction of B and T cells in the follicular of secondary lymphoid organs is important for the formation of antibody producing plasma cells, and it also affects T cells activities. B cells can regulate T cell activation and expansion, and modulate T cell functions such as cytokine productions and macrophage activation (Engel et al., 2011, Martinez-Gamboa et al., 2006). When CD4<sup>+</sup> T cells were transferred into SCID mice, the animal developed arthritis, only in the presence of B cells. When B cells were depleted, no disease occurred, suggesting a critical dependence of CD4<sup>+</sup> T cell and arthritis pathophysiology on the presence of B cells (Takemura et al., 2001). Our results showed that S100A4KO mice had a big CD4<sup>+</sup>ICOS<sup>+</sup> population in follicular T cells, but had small CD19<sup>+</sup> CXCR5<sup>+</sup> and CD19<sup>+</sup>CXCR5<sup>+</sup>IgM<sup>+</sup> cell populations compared to WT mice. This indicates a potential dysfunctional interaction between T and B cell populations in the follicular of S100A4KO mice. However, the levels of mBSA specific antibodies and rheumatoid factors are similar between S100A4KO and WT mice. This suggests that the antibody-producing function of B cells was not impaired in S100A4KO mice.

During our studies, we found that S100A4KO mice were protected from both bone loss in both septic arthritis (paper I) and formation of bone erosion in mBSA-induced arthritis (paper II). Moreover, S100A4KO mice had increased bone formation compared to WT mice (paper III). These findings indicate the importance of S100A4 in bone metabolism. RANKL/OPG system is important in bone homeostasis. RANKL is a product of osteoblasts and its binding to RANK on the preosteoclasts induces maturation of osteoclasts (Rehman et al., 2001). In septic arthritis, we found that S100A4KO mice had lower levels of RANKL compared with WT mice suggesting an impaired osteoblast function and /or less osteoclast formation (paper I). In mBSA-induced arthritis, levels of RANKL were similar between S100A4KO and WT mice. But when we analysed the mice with bone erosions, RANKL was higher in S100A4KO mice compared with WT mice (paper II). This indicates that the production of RANKL in mBSA-arthritis did not elicit their bone resorption functions. Moreover, RANKL levels in S100A4KO

mice were not increased with estrogen deprivation as they were in WT mice. This indicates that regulation of osteoclast formation by estrogen is limited in S100A4KO mice.

As for the production of OPG, a decoy receptor for RANKL, its levels did not differ in septic arthritis between S100A4KO and WT mice. This implies that OPG did not elicit its competitive function for the RANKL in S100A4KO mice in septic arthritis. And it tipped the OPG/RANKL ratio to the osteoblastic side. In mBSA-induced arthritis, levels of OPG were similar between S100A4KO and WT mice. However, OPG levels were lower in naïve S100A4KO mice compared with WT mice that correlates to the increased BMD in S100A4KO mice.

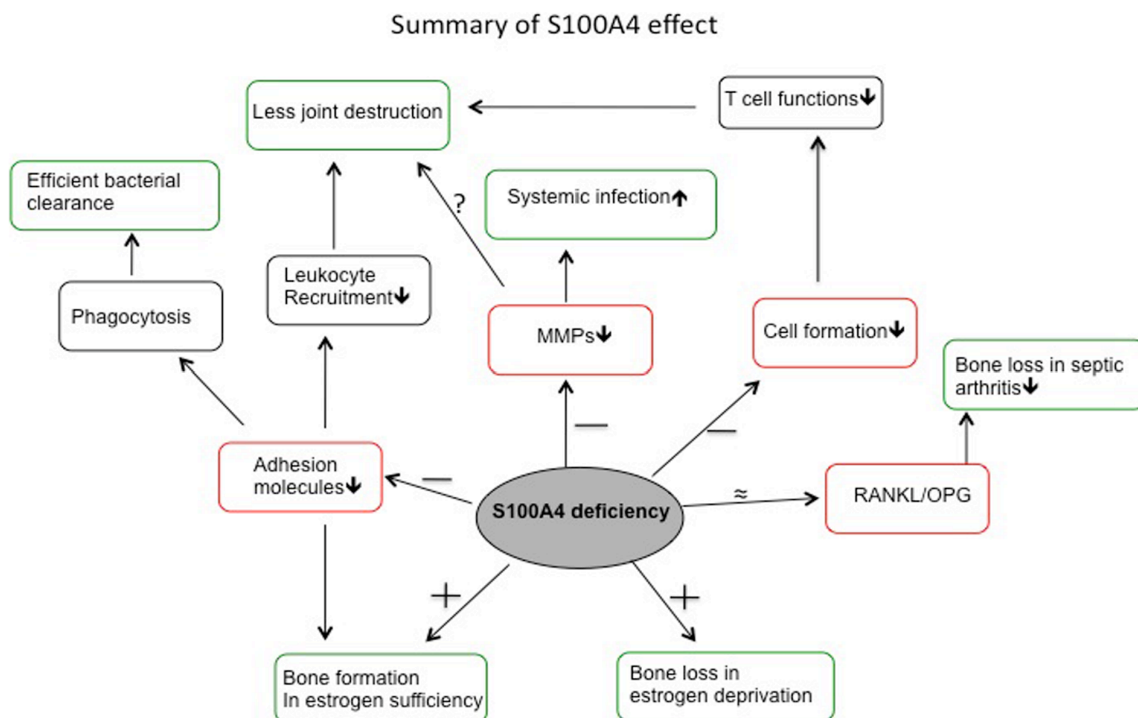
We studied the effect of estrogen on bone formation in S100A4KO mice. Our results showed that S100A4 deficient female mice had an increase of BMD in cortical bone. Excessive bone loss was seen in estrogen deficient (OVX) S100A4KO mice compared with OVX WT. OVX S100A4KO mice had a reduced cortical bone content, and decreased cortical thickness and area compared to estrogen sufficient (sham) mice. The decrease of cortical bone led to a weaker bone strength. In contrast, OVX WT mice had no change of cortical bone parameters and no change of bone strength. This suggests that the effect of S100A4 on bone is associated with estrogen.

Increased bone parameters in cortical bone of female S100A4KO mice indicate these mice have a “male like” cortical bone. In arthritis, male mice were more influenced by S100A4 deficiency in antigen-induced arthritis, while female mice showed no difference between S100A4KO and WT mice with respect to joint inflammation and destruction. These finding suggests that the role of S100A4 on bone and inflammation is sex dependent and may be mediated by estrogen.

Treatment with dichloroacetate (DCA) on collagen-type II induced arthritis showed that the DCA effect was also sex dependent (paper III). In this study, DBA/1 mice treated with DCA had a significantly slower onset of disease and less severe arthritis compared with water-treated mice. Moreover, the destruction of cartilage/bone was almost totally abolished and cortical bone BMD was significantly higher in mice treated with DCA. Our results showed that only female, not male DBA/1 mice were beneficial from DCA treatment. This indicates that estrogen may play a role in the DCA effect. An ameliorating effect of estrogens on arthritis has been demonstrated in both mice and human (D'Elia et al., 2003, Holmdahl et al., 1986, Jochems et al., 2005). Indeed, protective effect of DCA was absent in OVX group treated with

DCA compared with sham-operated group also treated with DCA. This suggests that the beneficial effect of DCA is mediated by estrogen. However, a control OVX group without DCA treatment may provide more evidence for our finding of DCA protective effect on arthritis, although the confounding factor that OVX treatment in itself can worsen CIA (Jochems et al., 2005) may be excluded because the effect of DCA treatment is of several magnitudes greater than OVX impact on arthritis.

Taken together, S100A4 deficiency on experimental arthritis caused altered expression of adhesion molecules, impaired lymphocyte maturation and formation, and resulted in reduced leukocyte infiltration and less cartilage/bone destruction in joints, efficient bacterial clearance and less bone loss. The effect of S100A4 deficiency on arthritis may be sex dependent. The impact of S100A4 deficiency on bone is also sex dependent and is mediated by estrogen. Interestingly, DCA treatment on collagen type II induced arthritis was also sex dependent and had impact on cortical bone BMD. Our studies indicate that S100A4 protein can be a therapeutic target in arthritis and osteoporosis, and dichloroacetate can be a potential drug for female patients with RA.



## POPULÄRVETENSKAPLIG SAMMANFATTNING

Under evolutionen har vår kropp utvecklat ett vapen som kallas immunitet, för att bekämpa invasion av främmande mikroorganismer, som bakterier, virus och svampar. Immunsvar mot mikroorganismer leder till inflammation. Immunitet hindrar oss från skador orsakade av främmande inkräktare. Men när immunsvaret mot mikroorganismer är för starkt, kan vår kroppsvävnad skadas. Immunsystemet kan angripa våra egna celler av någon okänd anledning, vilket leder till autoimmuna sjukdomar, exempelvis reumatoid artrit (RA), som är en ledsjukdom som leder till funktionshinder. En vanlig bakterieorsakad infektion är septisk artrit, en annan gemensam sjukdom som kan leda till nedsatt funktion i leder, döden utan behandling. Även om septisk artrit och reumatoid artrit har olika orsaker, innebär de båda inflammation i leder och brosk-/ benförstöring, och immunsystemet spelar en viktig roll i båda sjukdomar. För att ta reda på mekanismer för dessa sjukdomar och utveckla behandling, används experimentella djurmodeller i stor utsträckning. I denna avhandling använde vi en septisk artrit modell, antigen-inducerad artrit modell och kollagen typ II inducerad artrit modell i mus för att studera eventuella mekanismer för septisk och reumatoid artrit.

S100A4 är ett protein som endast finns i ryggradsdjur. Det upptäcktes först som ett cancer metastas-specifikt protein för 20 år sedan. Nivån på S100A4 ökar om cancer är metastaserande. Så S100A4 protein kan användas som en markör för att prognostisera metastasering av tumör. Höga halter av S100A4 hittades också i ledvävnad och blod från patienter med RA. Detta är inte förvånande eftersom cancer och inflammation under artrit har vissa gemensamma drag. De har båda okontrollerad celltillväxt som invaderar vävnad med alltför mycket blodkärlsgeneration. Dichloroacetat (DCA) är ett läkemedel som traditionellt används för att behandla lactacidosis, en sjukdom med överskott av mjölksyra i kroppen. DCA har visat sig ha en potent anti-tumör effekt eftersom det kan döda tumörceller och hämmar tumörcellsproliferation.

Syftet med denna avhandling är 1) att studera vilken roll S100A4 har i septisk artrit hos mus. 2) att studera effekten av S100A4 i antigen-inducerad artrit modell. 3) att studera vilken roll S100A4 har för benmetabolism. 3) att studera DCA-behandling på kollagen inducerad artrit (CIA).

Det huvudsakliga arbetet vi gjorde var att jämföra av reaktionerna från möss utan S100A4 (S100A4KO) och möss med S100A4 (WT) i olika experimentella artritmodeller. Vår studie visade att S100A4KO-möss hade mindre ledinflammation och ledförstörelse i både septisk artrit (artikel I) och antigen-inducerad artrit (artikel II). Dessutom hade S100A4KO-möss mindre benförlust vid septisk artrit och färre bakterier i njurarna. Minskad ledinflammation och förstörelse i S100A4KO-möss kan bero på att mindre cell-migration till ledhålan. I septisk artrit-modellen fann vi en förändring hos adhesionsmolekyler, en grupp molekyler som hjälper celler vidhäfta och migrera. Adhesionsmolekyler är också viktiga vid bakterieclearance. I antigen-inducerad artrit fann vi att nivån av CD5 + B-celler, en subpopulation av B-lymfocyterna, var signifikant sänkt hos S100A4KO hanmöss. Nivån av CD5 + B-celler är vanligtvis hög i blodet hos patienter med reumatoid artrit. Så förändrat uttryck av adhesionsmolekyler kan vara orsaken till mildare artrit och färre bakterier i njurarna i septisk artrit och färre CD5 + B-celler kan vara orsaken till mindre artrit i antigen-inducerad artrit.

Vi har också studerat effekten av S100A4 på ben. Möss som sakar S100A4 har en tjockare benstomme än WT-möss. Ökad bentäthet var mer uttalad i kortikalt ben än i trabekulärt ben. I artikel III visade vi också att efter ooforektomi, en operation som tar bort äggstockarna för att ta bort effekten av östrogen (kvinnligt köns hormon), förlorade S100A4KO möss mycket mer kortikalt ben jämfört med icke-opererade möss, medan WT möss inte gjorde it. Så S100A4 är en hämmare av benbildning i möss med ästrogen och kan förhindra kortikal benförlust i möss som sakar östrogen.

I artikel IV använde vi DCA för att behandla kollagen typ II-inducerad artrit i möss. Vi fann att möss som behandlats med DCA hade en senare sjukdomsdebut och betydligt mildare ledinflammation och ledförstörelse än vattenbehandlade kontrollmöss, men bara honmöss, inte hanmöss som fått DCA behandling. Genom att göra ooforektomi, hittade vi att DCA-effekten medieras av östrogen.

Sammanfattningsvis visar studierna i denna avhandling visar att S100A4-proteinet spelar en viktig roll för inflammation och benmetabolism i experimentell artrit. Brist på S100A4 skyddar mot ledinflammation och ledförstörelse i både septisk artrit och antigen-inducerad artrit hos mus. Mekanismerna för den skyddande effekten av S100A4 på artrit kan vara det ändrade mönstret av adhesionsmolekyler, minskad cell-bildning och nedsatta cellfunktioner. Brist på S100A4 leder också till ökad bentäthet. Våra resultat tyder på att S100A4 kan vara en

viktig måltavla för behandling av septisk och reumatoid artrit, liksom benskörhet, en sjukdom med låg bentäthet. Vi visar också att dichloroacetat kan vara ett potentiellt läkemedel för behandling av kvinnliga patienter med RA.

## ACKNOWLEDGEMENTS

First of all, my sincere gratitude is given to my original supervisor **Andrej Tarkowski**, for giving me opportunity to start rheumatology research in the department, for his never ending encouragement and always letting me feel “Excellent!”. His great personality and enthusiasm to life and science make him an unforgettable idol.

**Maria**, I am really grateful that you took over the supervision after Andrej. You guided the research throughout the work of this thesis. Thank you for sharing your broad knowledge and experience in both clinic and research, for inspiring me with your sharp ideas, for your patience and effort in checking countless revisions of papers and presentations. You are a model of hard-working and never giving up.

**Mattias M**, you are always so patient and helpful whenever I have questions. Thank you for spending time on reviewing every single word of the DCA manuscript. It is a good experience of writing together with you.

**Ing-Marie and Margareta**, both of you are the first people who taught me lab techniques. Ing-Marie, thank you for teaching me lab skills, for sharing your experience and knowledge, for all the histological sections you have done for me, for the thoughtfulness when I was writing this thesis. You are the best! Margareta, thank you for all the techniques you taught me, I enjoy working with you.

**Malin**, thank you for taking care of everything we need in the lab, for all the help to me and for good discussions. You are a good teammate.

**Mikael**, you let me know what flow cytometry is. Thank you for sharing your enthusiasm and knowledge of B cells and for inspiring discussions.

**Tao**, thank you for introducing me to the group, for your optimistic attitude to research and life, for all the help and advice. I am glad I've gotten known you.

**Elisabet**, I enjoy our interesting chat during lunchtime and late evening. You always help me to figure out the puzzles – life and science. Thank you for correcting my Swedish and reading my thesis. I wish we keep in touch no matter where we are.

**Sofia L**, My roommate from start, thank you for encouragement and support, for sharing all the happiness and sadness, for sharing every step of the disputation. I will always remember the time we've been together!

**Lois**, I miss the time when you sat in our room. Thank you for sharing your laugh and tears. Nothing is cuter than you!

**Annelie**, thank you for all the encouragement and support. You can always cheer me up when I am a bit down. I will miss you and good luck!

**Mattias S**, thank you for all the help, especially with FACS, for nice suggestions and discussions. Good luck with your research!

**Mia**, you are such a lovely girl. Thank you for all the joy and chocolate you bring.

**Sofia A**, I appreciate the small discussion with you!

**Inger G**, I am really grateful for all the advice from you both in science and life, for the patience and support when I have any difficulty.

**Esbjörn**, thank you for being so helpful when I have questions about computer and immunology.

**Mats, Rille, Anna-Karin H-E, Kaisa, Catharina L, Janne, Lena**, thank you for all the nice and interesting discussions.

**Other Doctorander: Alex, Annica, Cicci, Andrey, Kuba, Karin C, Lucija, .....** I enjoy the time spent with you in the lab and outside the lab!

**Marie L, Anna K, Urika, Anna Karin L, Karin A, Johan B, Alexandra S, Merja, Pernilla J, Nina, Inger N, .....** thank you for nice suggestions about research and for the “grillning”.

**Maggan**, this thesis would not be finished without your excellent work of taking care our mice. Thank you very much!

**Eva, Harriet and Sofie**, thank you all for the big help when I need some instructions for the complicated paper work!

**Hans and Kristina**, both of you created a relaxing and joyful atmosphere for the people working here. You are good at your job. Kristina, thank you for your kind offer during this summer.

**My Chinese friends and their families, Yuan Wei, Meng Liu, Yihong, Huamei, Fei, Meimei, Mattias, Jianhua and Bingbing**

Thank you for every moment we spent together, for all the help and suggestions when I had any difficulty. I am lucky to have all of you as good friends.

**My mom and sisters**, for your never-ending support, for helping me taking care of Lucas. You are the ones who love me without any condition.

特别感谢我的妈妈和妹妹及其家人帮助我们照顾杭希佳，感谢两个妹妹在妈妈身边照应。你们的理解和毫无保留的爱是我不断向前的动力。

**My husband Darren and our little Lucas**, Lucas, you are such a little angel. Nothing makes me happier than to hear your laugh or see you smile. I love you soooooo.....much! Darren, I do not know if there will be enough words in the dictionary to express how much I appreciate the love and support from you. Your understanding, wisdom, kindness, hard working, and sense of humour, .....

all help me make this thesis possible!



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