Tyrosine Kinase Flt3/Flt3-Ligand Signaling in the Modulation of Immune Responses in Experimental Arthritis

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Cover illustration: Mattias Svensson, inspired by music and science.

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ABSTRACT

Rheumatoid arthritis (RA) is an autoimmune, chronic systemic inflammatory disorder that primarily affects flexible joints resulting in severe joint destruction and disability if left untreated. Today, advances in treatment have significantly improved the outcome for patients, although the pathogenesis of RA remains relatively unknown. Signaling through the tyrosine kinase receptor fms-like tyrosine kinase 3 (Flt3) has been suggested to play a part in the RA pathogenesis. Flt3 is primarily expressed on hematopoietic stem cells and lymphoid progenitors in the bone marrow and has an important role in early B-cell development and formation of dendritic cells (DC). Furthermore, the ligand for Flt3 (Flt3L) serves as a regulator of regulatory T-cell (Treg) homeostasis and has been suggested to support differentiation of bone-resorbing osteoclasts.

This thesis aimed to investigate the effect of Flt3/Flt3L signaling on the immune system during development of arthritis using an experimental animal model of human RA. Our study shows that Flt3 signaling supports formation of DCs and Treg cells during arthritis development. Treg expansion associated with Flt3L treatment resulted in a reduced production of inflammatory cytokines, reduced levels of antigen-specific antibodies and reduced bone destruction. On the contrary, lack of Flt3L was associated with reduced Treg formation resulting in loss of control over T-cell proliferation, and bone destruction during arthritis. Flt3L was found to positively influence the transcription of the osteoclast-regulating factor IRF8, and could by this mechanism influence osteoclast formation. Impaired signaling through Flt3 resulted in low IRF8 expression, accumulation of osteoclasts in the arthritic joint and an increased loss of femoral trabecular bone. Conversely, Flt3L treatment was associated with increased IRF8 expression, reduced osteoclast formation and restoration of trabecular bone formation in mice lacking Flt3L (Flt3LKO). Finally, we could identify a previously unacknowledged role for Flt3 in peripheral B-cell responses. We demonstrated that Flt3 was re-expressed on activated B-cells following LPS stimulation *in vitro* and on a population of germinal center B-cells *in vivo*. By using Flt3LKO mice we could identify an important role for Flt3L in class switch recombination (CSR) to IgG1. B-cells from Flt3LKO mice were found have reduced activation of Stat6 after IL-4 stimulation, resulting in impaired initiation of CSR to IgG1 and highly reduced formation of IgG1+ B-cells and IgG1 production.

In summary this thesis shows that Flt3L has an important function in regulating DC and Treg homeostasis and function during arthritis. Furthermore, Flt3L has a regulatory role on osteoclast development and on trabecular bone formation. Finally, signaling through the Flt3 receptor on activated B-cells has an important role in the CSR process and deficiency of Flt3L leads to a skewed antibody

response towards the more potent IgG subclasses IgG2b and IgG2c. Together, these results suggest that Flt3L might play a protective role during arthritis by reduction of bone destruction, induction of regulatory T-cells and regulation of antibody effector functions. The conclusion of this thesis is that signaling through the tyrosine kinase Flt3 plays an important role in modulating immune responses during experimental arthritis.

Keywords: Flt3, Flt3L, dendritic cells, regulatory T-cells, B-cells, osteoclasts, rheumatoid arthritis

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LIST OF PAPERS

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

I. Mattias N. D. Svensson, Sofia E. M. Andersson, Malin C. Erlandsson, Ing-Marie Jonsson, Anna-Karin H. Ekwall, Karin M. E. Andersson, Anders Nilsson, Li Bian, Mikael Brisslert, Maria I. Bokarewa.
Fms-Like Tyrosine Kinase 3 Ligand Controls Formation of Regulatory T Cells in Autoimmune Arthritis. *PLoS ONE 2013; 8(1): e54884.*

II. Mattias N. D. Svensson, Kersti Månsson, Karin M. E. Andersson, Ing-Marie Jonsson, Mats Bemark, Mikael Brisslert, Maria I. Bokarewa.
Germinal center B cells require Flt3-mediated activation of Stat6 for IgG1 class switch recombination. *Manuscript*

III. Mattias N. D. Svensson, Malin C. Erlandsson, Ing-Marie Jonsson, Karin M. E. Andersson, Maria I. Bokarewa
Impaired signaling through the Fms-Like tyrosine kinase 3 receptor results in increased osteoclast formation and joint destruction during experimental arthritis. *Manuscript*

OTHER PUBLICATIONS

Other publications not included in this thesis

IV. Elisabeth A. Boström, Mattias Svensson, Sofia Andersson, Ing-Marie Jonsson, Anna-Karin H. Ekwall, Thomas Eisler, Leif E. Dahlberg, Ulf Smith, Maria I. Bokarewa
Resistin and insulin/insulin-like growth factor signaling in rheumatoid arthritis. *Arthritis and Rheumatism 2011, 63(10):2894-904*

V. Sofia E.M Andersson, Mattias N.D Svensson, Malin C. Erlandsson, Mats Dehlin, Karin M.E Andersson, Maria I. Bokarewa
Activation of Fms-Like Tyrosine Kinase 3 signaling enhances Survivin expression in a mouse model of Rheumatoid Arthritis.
PLoS One, 2012; 7(10): e47668.

VI. Malin C. Erlandsson, Mattias D. Svensson, Ing-Marie Jonsson, Li Bian, Noona Ambartsumian, Sofia Andersson, ZhiQi Peng, Jukka Vääräniemi, Claes Ohlsson, Karin M. Andersson, Maria I. Bokarewa
Expression of metastasin S100A4 is essential for bone resorption and regulates osteoclast function. *Biochim Biophys Acta, 2013: 1833(12):2653-2663*

CONTENT

ABBREVIATIONS

ACPA	Anti-citrullinated protein antibody
AID	Activation-induced (DNA-cytosine) deaminase
APC	Antigen-presenting cell
Bcl6	B-cell lymphoma 6
BCR	B-cell receptor
Blimp1	B lymphocyte induced maturation protein 1
CCR7	C-C chemokine receptor type 7
CD	Cluster of differentiation
cDC	Classical dendritic cell
CDP	Common dendritic cell progenitor
CSR	Class switch recombination
CTLA4	Cytotoxic T-lymphocyte antigen 4
CXCR5	C-X-C chemokine receptor type 5
DNA	Deoxyribonucleic acid
DC	Dendritic cell
FcγR	Fc-gamma receptor
Flt3	Fms-like tyrosine kinase 3
Flt3L	Fms-like tyrosine kinase 3 - ligand
Flt3LKO	Fms-like tyrosine kinase – 3 ligand knock out
FoxP3	Forkhead box protein 3
GC	Germinal center
GLT	Germ line transcript
GM-CSF	Granulocyte macrophage colony stimulating factor
HLA	Human leukocyte antigen
ICOS	Inducible T-cell costimulator
IFN	Interferon
IRF4, 8	Interferon regulating factor 4 and 8 respectively
IL	Interleukin
JAK	Janus kinase
KL	Kit-ligand
M-CSF	Macrophage colony stimulating factor
MHCI, II	Major histocompatibility complex I and II respectively
mTOR	Mammalian target of rapamycin
NF- κB	Nuclear Factor κ B
NK-cell	Natural killer cell
Pax5	Paired box protein 5

pDC	Plasmacytoid dendritic cell
PDCA1	Plasmacytoid dendritic cell antigen 1
PD-1	Programmed cell death 1
PD-L1	Programmed cell death – ligand 1
PTPN 6,22	Protein tyrosine phosphatase, non-receptor type 6 and 22 respectively
RA	Rheumatoid arthritis
RAG1, 2	Recombination activating gene 1 and 2 respectively
RORγt	Retinoic acid receptor gamma t
RNA	Ribonucleic acid
RTK	Receptor tyrosine kinase
Runx2	Runt-related transcription factor 2
SHM	Somatic hypermutation
SL	Surrogate light chain
SLE	Systemic lupus erythematosus
STAT x	Signal transcducer and activator of transcription (x= number)
T-Bet	T-box transcription factor TBX21
TACE	TNF-α converting enzyme
TCR	T-cell receptor
T_{FH}	Follicular T helper cell
TGF-β	Transforming growth factor beta
Th	CD4+ T helper cell
TK	Tyrosine kinase
TLR	Toll like receptor
TNF-α	Tumor necrosis factor - alpha
SHM	Somatic hypermutation
RANK	Receptor Activator of Nuclear Factor κ B
WT	Wild type
Xbp1	C-box binding protein 1

INTRODUCTION

The mammalian immune system is designed to protect from invading pathogens but also from malformed cells, such as cancer cells and virus infected cells. At the same time as the immune system has to recognize millions of different potential dangers, it needs to discriminate between self and non-self, danger and no danger. The immune system therefore consists of several control mechanism and regulatory cells that help to eliminate dangerous self-reactive cells and limit inflammatory responses. In some severe cases, the control mechanisms fail and the immune system loses tolerance towards it self, which result in an autoimmune syndrome. Why some individuals develops autoimmune diseases is not completely understood but the involvement of genes, environment and chance has been defined. Although it is known that the immune system plays a central part in autoimmune diseases, which control mechanism that fails is still unknown in most cases. The immune system arises from a single stem cell precursor in the bone marrow, the hematopoietic stem cells. Differentiation of stem cells to competent immune cells relies on a series of growth factors and cytokines. Recently, one of these factors, fms-like tyrosine kinase 3 – ligand (Flt3L), has been found in increased levels in the blood of patients suffering from the rheumatic diseases, primary Sjögren´s syndrome (pSS) and rheumatoid arthritis (RA). Further studies have revealed that Flt3/Flt3-ligand signaling can be an important player in these autoimmune diseases, since it has high potential to affect the differentiation of immune cells. In the present thesis we will explore the potential role for Flt3 receptor signaling in affecting and modulating immune cells and immune responses during the development of RA.

Fms-like tyrosine kinase 3 and its ligand

Hematopoiesis is a highly regulated process in which a small population of self-renewing stem cells differentiates into cells that populates the blood and the immune system. This process is controlled through the action of cytokines and growth factors, some of which exert their function through activation of tyrosine kinases (TKs)[1]. TKs are enzymes that induce activation through phosphorylation of proteins and function as an "on" and "off" switch of intracellular signaling pathways, which regulates many critical cellular processes such as proliferation, differentiation, survival and migration [2, 3]. Tyrosine kinases are divided into two classes: receptor tyrosine kinases (RTKs) and cytoplasmic or non-receptor tyrosine kinases (cTKs). Only RTKs have an extracellular ligand-binding domain whereas both cTKs and RTKs have a cytoplasmic domain that contains the tyrosine kinase [3]. Most known RTKs exists as monomers in the cell membrane. Upon ligand binding, RTKs dimerize and undergo autophosphorylation of their cytoplasmic domain. This allows the kinase to associate with downstream substrate proteins and promote signal transduction. Several different classes of RTK exist, where the Class III family has been found to play an important role in hematopoiesis. This class of RTKs contains receptors for the macrophage colony-stimulating factor (M-CSF), the kit-ligand

(KL), the platelet-derived growth factors A and B (PDGFα and PDGFβ) and Fms-like tyrosine kinase 3 ligand (Flt3L)[1].

Fms-like tyrosine kinase 3

In 1991, the murine form of the Class III RTK fms-like tyrosine kinase 3 (Flt3, CD135), also known as fetal liver kinase 2 (Flk2) or stem cell kinase 1 (STK-1), was cloned independently by two groups [4, 5]. Shortly after cloning of the human variant followed, which was found to have an 85% sequence homology to the murine equivalent [6, 7]. The expression of Flt3 is rather limited to the hematopoietic stem cell compartment and the receptor is primarily found on multipotent hematopoietic stem cells and lymphoid progenitors in the bone marrow, where it serves an important function in stem cell development and differentiation [8].

Fms – like tyrosine kinase 3 – ligand

After the cloning of Flt3, mouse Flt3 ligand (Flt3L) was cloned independently by two groups [9, 10] and as with the Flt3 receptor, cloning of human Flt3L followed shortly after [11]. Like the Flt3 receptor, human and murine Flt3L show large sequence homology with a 72% identity at the amino-acid level and the ligand show great cross specie activity [12]. Both human and murine Flt3L are expressed as a type I transmembrane protein, which can be proteolytically cleaved to generate a soluble form. Both the transmembrane and soluble forms of Flt3L are biologically active. However, due to translational differences of the Flt3L gene between human and mouse, the structure of the transcribed transmembrane form of the protein differs slightly between the species. This contributes to differences in how the Flt3L are proteolytic cleavage from the membrane and released into circulation. Although little is known about the enzymes involved in the proteolytic release of Flt3L, a recent study indicated that the TNF-α converting enzyme (TACE) could have an important function in proteolytic processing and release of Flt3L [13].

Production of Flt3L

In contrast to the limited expression of the Flt3 receptor on mainly haematopoietic stem cells, Flt3L mRNA and protein can be found in both haematopoietic and non-haematopoietic tissues [2, 14]. In particular, Flt3L protein have been detected in stromal fibroblasts, endothelial cells, T-cells, NK-cells, B-cells and lately also in mast cells [15-17]. Low levels of Flt3L may be found in the plasma of healthy individuals, although the ligand has been found to increase during hematological diseases such as aplastic anemia [18]. Also, increased serum levels of Flt3L is found in patients with a newly defined syndrome of dendritic cell, monocyte, B- and NK-cell deficiency [19]. It has therefore been suggested that increased levels of Flt3L in serum reflects a compensatory response which aims to restore the hematopoietic stem cell compartment [20]. Furthermore, Flt3L has also been found to increase during inflammatory conditions, such as rheumatic diseases, and during infection, e.g. plasmodium infection [17, 21, 22]. Signaling through the Flt3 receptor on

hematopoietic stem cell compartment is blocked by transforming growth factor beta (TGF-β) and the inflammatory cytokine tumor necrosis factor – alpha (TNF-α), which significantly reduce the ability of Flt3L to stimulate growth of early bone marrow progenitors [23]. Furthermore, TNF suppress hematopoietic stem cell activity in mice, leading to reduced levels of lymphocyte progenitors during inflammation [24]. Together, these results indicate that the increase serum levels of Flt3L seen during inflammation may be a consequence of impaired stem cell activity.

Flt3 signaling

When unstimulated, the Flt3 receptor resides in the plasma membrane as a monomer [2]. After binding of Flt3L, membrane-bound Flt3 form homodimers, which stabilize the receptor and leads to exposure of phosphoryl acceptor sites in the receptors tyrosine kinase domain (TDK) [25, 26]. Activation through phosphorylation occurs within 5 - 15 minutes after ligand binding. Shortly after activation the ligand-receptor complex is internalized and degraded and degradation products can be seen 20 minutes after Flt3L stimulation (Figure 1)[25].

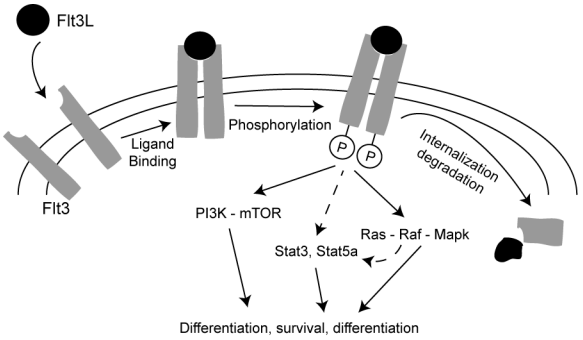


Figure 1. *Flt3 signaling induced by ligand binding.* The Flt3 receptor is expressed as a monomer on the cell-surface. Upon Flt3L binding, the receptor dimerizes and exposes TDKs, which are phosphorylated, activating one of two signaling pathways: the PI3K-mTOR pathway or the Ras-Raf-Mapk. Flt3 also induces activation of Stat3 and 5a.

Binding of Flt3L triggers phosphorylation of the Flt3 receptor, which leads to recruitment intracellular adaptor molecules[20]. These molecules are parts of two different signaling pathways that have been associated with Flt3 signaling, the Ras/Raf/Map kinase pathway and the PI3K - mTOR pathway [2, 27]. Furthermore, Flt3 signaling induces the phosphorylation of Stat3 and Stat5a, both of which are associated with Janus kinase activity (JAK). But Flt3 signaling does not activate JAK kinases by itself, suggesting that the activation of Stat3 and Stat5a by Flt3 might be JAK independent [28-30].

Flt3 signaling in hematopoiesis

Signaling through Flt3 has an important role in early hematopoiesis by inducing survival, proliferation and differentiation of early hematopoietic stem and progenitor cells [5, 31]. Flt3L acts as a weak growth stimulator on its own and usually needs to synergize with other hematopoietic growth factors to stimulate proliferation and differentiation of progenitors [32-34]. The role of Flt3 signaling in hematopoiesis has been clearly identified in mice that have a targeted deletion of either Flt3 or Flt3L. These genetically modified mice have reduced cellularity in lymphoid organs and show reduced numbers of lymphoid progenitors [8, 35]. Accordingly, treatment of mice with Flt3L increases the expansion of hematopoietic stem cells and cause a significant stimulation of hematopoiesis, with increased cellularity in lymphoid organs [31]. Furthermore, deficiency of Flt3 signaling severely affects development of NK-cells and B-cell progenitors [35]. The most striking effect of Flt3 signaling has been seen on the dendritic cell (DC) population, and Flt3L is now considered as the primary differentiation factor for DCs [35-37]. Also, competitive transplant experiment with stem cells from Flt3 deficient or WT mice have shown that Flt3 deficient stem cells do not reconstitute the hematopoietic system efficiently, most notably seen in the T-cell compartment indicating that Flt3 can have a function also in T-cell development [8]. It is now clear that Flt3 have an essential role in early development and differentiation of immune cells that are critical for the formation of a normal immune system. The role of Flt3 signaling in development of DCs, B-cells and T-cells will be discussed in more detail below.

The immune system

The immune system protects the body from harmful invaders such as bacteria, viruses and other microorganisms. This system consists of three “barriers”. The first line of defense is the skin and the mucosal surfaces found in body cavities. If a microbe breaks through this first barrier, they encounter the next two barriers: the innate and adaptive immune system.

The innate immune response

The innate immune system is also known as the non-specific immune system. This means that the cells of the innate system will respond to invading pathogens in the same way every time they are encountered To identify invading pathogens or cellular damage the cells of the innate immune system use a group of receptors known as pattern recognition receptors (PPRs). These receptors recognize pathogen associated molecular patterns (PAMPs) expressed by microbes and danger-associated molecular patterns (DAMPs), which are endogenous danger signals released during cellular stress. The PPR family includes a variety of different receptors such as Toll-like receptors (TLRs), Nod-like receptors (NLRs), C-lectin-like receptors (CLRs) and RIG-I-like receptors (RLRs). The innate immune system consists of cells such as granulocytes, monocytes, macrophages, mast cells and NK cells[38, 39]. One of the most intriguing cells of the innate immune system is the dendritic cell (DC). These

cells constantly engulf extracellular antigens, which they process and present as peptides on major histocompatibility complex (MHC) molecules to T-cells of the adaptive immune system. If DCs are activated by for example bacterial antigens through TLR activation, DCs act as powerful antigen-presenting cells (APC). DCs therefore serve as a powerful link between the innate and adaptive immune response [40].

Dendritic cells – the link between innate and adaptive responses

In 2011, Ralph Steinman was awarded the Nobel Prize in Medicine for his role in the discovery of the DC. Although discovered 40 years before, the initial description of the cell met great skepticism from the scientific community. Today DCs are known as essential mediators of immunity and tolerance [40, 41]. Recent advances in DC research has identified that this population of cells show great heterogeneity. Today, DCs are divided into two major subpopoluations; classical DCs (cDCs) and plasmacytoid DCs (pDCs). Both of these populations originate from a newly defined common DC progenitor (CDP) in the bone marrow and are dependent on Flt3 signaling for their development (Figure 2) [42, 43].

Classic dendritic cells

The cDCs form a small population of immune cells that populate almost all tissues in the body and can be subdivided into lymphoid tissue and nonlymphoid tissue cDCs [44]. Nonlymphoid tissue cDCs represent 1-5% of tissue cells depending on the organ that they are found in and are divided into two major subsets based on their surface markers: CD103+CD11b- DCs and CD11b+ DCs (Figure2). After encounter of antigens, nonlymphoid cDCs can migrate to tissue draining lymph nodes thorough the lymphatic system and are then referred to as tissue-migratory DCs[45].

Lymphoid tissue cDCs refer to cDCs that differentiate in, and spend their entire lives within secondary lymphoid organs, such as the spleen and lymph nodes. The lymphoid tissue DCs can be divided into two major subpopulations: CD8+ and CD11b+ cDCs (Figure 2). The CD11b+ cDCs can be further divided two populations based on their expression of CD4 (CD4+ or CD4-) [46]. CD8+ DCs represent around 20-40% of the spleen and lymph node cDCs whereas the CD11b+ cDCs usually are the dominant population. Although both nonlympohid tissue cDCs and lymphoid tissue cDCs can be found in lymph nodes, only lymphoid tissue cDCs are thought to populate the spleen [47]. All cDCs express high levels of the integrin CD11c and MHCII. They also lack expression of the T-cell lineage marker CD3 and the B-cell lineage marker B220.

The different functions of the lymphoid and non-lymphoid cDCs are not particularly well defined. Most is known about the functions of the lymphoid resident CD8+ cDC and non-lymphoid CD103+CD11b- cDC. It is now realized that these two populations have the same origin, similar phenotype and rely on the same transcriptionfactors for differentiation. Both CD8+ and CD103+ cDCs have increased capacity of cross-presentation of antigens on MHCI molecules and are therefore considered potent activators of CD8+ T-cell responses. These cells are also

the main source of IL-12 and can direct T-cell responses towards Th1. Also, CD8+ cDCs have been found to produce TGF-β under steady state conditions and are therefore suggested to have a more potent regulatory function. The role of CD11b+ cDCs is less well defined but they seem to preferentially present antigens on MHCII and activate CD4+ T-cells[44, 48].

Plasmacytoid dendritic cells

When first encountered, the pDC was described as a plasmacytoid T-cell or plasmacytoid monocyte due to the morphological and phenotypic characteristics of these cells. Although these cells were later reported to be a subset of DCs and correspond to the previously described natural type I interferon-producing cell [49]. In contrast to cDCs, pDCs develop in the bone marrow were they represent 1-2% of all bone marrow cells. After development, pDCs usually accumulate in the blood and lymphoid tissues into which they enter from the blood circulation (Figure 2)[44]. In contrast to cDCs, pDCs have low expression of MHCII and the integrin CD11c in steady state and can be identified by the expression PDCA1 (also known as Bst2) and the B-cell associated marker B220, which is not found on cDCs[50]. pDCs are the major source of type I IFN (especially INF-α), which is released upon activation of toll-like receptors (TLR) 7 and 9 by viral antigens. Upon activation, pDCs can differentiate into immunogenic DCs, which results in a reduced production of type I IFN and a upregulation of MHC I and II and co-stimulatory molecules CD40, CD80 and CD86[50].

Other DC subsets

Langerhans cells (LCs) are tissue resident DCs that populate the epidermal layer of the skin. These cells have lower expression of MHCII and CD11c. In contrast to other DCs, LCs self-renew in the skin during steady state and are independent of bone marrow progenitors. Also, LCs development is not dependent on Flt3 signaling, but instead requires M-CSF for development[51].

Monocytes were originally thought to be the precursors for cDCs. However, subsequent studies have shown that monocytes only seem to differentiate into cDCs under inflammatory conditions, and these are therefore today referred to as inflammatory DCs (Figure 2)[52].

Flt3 signaling in dendritic cell development and homeostasis

As mentioned above, the most dramatic effect of Flt3 signaling in lymphoid development has been seen in the DC compartment [36]. Mice lacking Flt3 or Flt3L have severely reduced numbers of both cDCs and pDCs in peripheral lymphoid organs [35, 37]. In line with this, injections of Flt3L in both mice and humans induce great expansion of DCs in blood and lymphoid organs [53, 54]. Furthermore, peripheral cDCs express the Flt3 receptor, where it is essential to maintain DC homeostasis, indicating that Flt3 is also important in the function of peripheral DCs [37]. Flt3L are particularly important for development of pDCs and CD8+ cDCs. Inhibition of mTOR-signaling blocks Flt3L-induced DC development affecting

primarily CD8+ cDCs and pDCs [27]. Also, Flt3L-induced activation of Stat3 is essential for Flt3L-induced DC development [29].

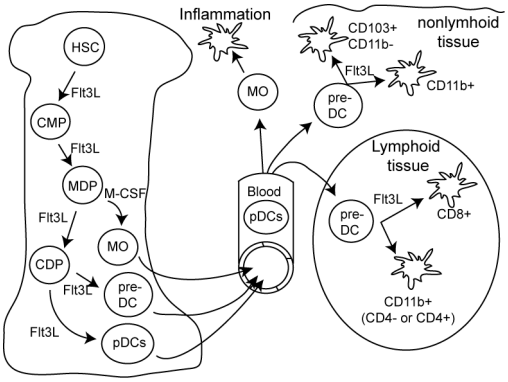


Figure 2. *Development of dendritic cells.* DCs arise from hematopoietic stem cells in the bone marrow (HSC). Under the influence of Flt3L, the HSC differentiate through different progenitors stages which gives rise to common myeloid progenitor (CMP), the macrophage and DC progenitor MDP. At the MDP stage the progenitor can different into either a monocyte (MO), under the influence of M-CSF, or into a common DC progenitor (CDP), under the influence of Flt3L. The CDP only gives rise to pDCs and pre-DCs. The pDCs and pre-DCs leaves the bone marrow and enters the blood circulation. The pDCs circulates in the blood whereas the pre-DC migrates to a lymphoid organ, where it develops into a functional lymphoid resident cDC, or to a nonlymphoid tissue where it develops into a tissue-resident cDC. Monocytes can differentiate into a DC during inflammation. These DCs are referred to as inflammatory DCs.

Dendritic cells in immunity and tolerance

It is now recognized that DCs play an essential part of the immune system. Through the actions of PPRs DCs can recognize invading pathogens and initiate innate and adaptive immune responses. After activation, DCs upregulate costimulatory molecules (e.g. CD80, CD86 and CD40) and produce cytokines that drive T-cell priming and activation of adaptive immune responses. In the absence of activation, DCs present antigens to T-cells without upregulation of costimulatory molecules that might lead to unresponsiveness of the T-cell and promote tolerance[55, 56]. However, due to the potent antigen-presenting function of DCs, changes in DC function might lead to aberrant activation and presentation of self-antigens with the potential to induce self-reactive T-cells and promote autoimmunity. Recently, genetic defects in DCs have been shown to promote autoimmunity in mice. Specific deletion of Stat3 in DCs causes cervical lymphadenopathy and colitis in mice[57]. Furthermore, deletion of the phosphatase SHP1 (PTPN6) in DCs caused a polyclonal immune activation and development of SLE-like disease[58, 59]. Lastly, the deletion of BLIMP1, more commonly known for its function in T and B-cells[60], in mature DCs induced production of autoantibodies and a SLE-like disease[61]. Of interest is that polymorphisms in Blimp1 has recently been identified in systemic lupus erythematosus (SLE) and DCs from healthy individuals carrying the Blimp1 SLE-risk allele showed the same inflammatory phenotype as the Blimp1 deficient DCs from

mice[62, 63]. Recently, Blimp1 was also identified as a risk allele for rheumatoid arthritis patients[64]. These studies suggest that aberrant activation of DCs due to genetic polymorphism can be a potential trigger in autoimmune diseases.

The adaptive immune response

In contrast to the innate immune system, the adaptive immune system is characterized by specificity. Also, after initial encounter of a specific pathogen the adaptive immune system will develop immunological memory, meaning that the next time the pathogen is encountered the immune response will be enhanced. The adaptive immune system consists of B-cells and T-cells, both of which express antigen-specific receptors: the B-cell receptor (BCR) and the T-cell receptor (TCR)[65, 66]. The huge receptor diversity that exists among the TCRs and BCRs is encoded in the mammalian genome by a genomic modification process called the V(D)J recombination. During this process encoded gene segments termed variable (V), diversity (D) and joining (J) are assembled to from the BCR and TCR[67]. This process takes place in the central lymphoid tissues, which is the thymus for T-cells and the bone marrow for B-cells, and is dependent on the two enzymes RAG1 and RAG2[68].

T-cells

T-cells originate from the bone marrow, but the progenitors move at an early stage to the thymus where commitment to the T-cells lineage occurs. When T-cell progenitors reach the thymus they neither express a T-cell receptor (TCR) nor the TCR co-receptors CD4 and CD8, and are therefore refereed to as double negative (DN) cells. During entrance into the thymus, the T-cell progenitor will interact with thymic epithelial cells in the cortex (cTECs) of the thymus and differentiate into a double positive (DP) cell, expressing both CD4 and CD8 and low levels of the TCR. T-cells can express two types of TCRs: α/β, which is expressed by most T-cells, or the γ/δ, which is expressed by a small subset of T-cells. T-cells expressing α/β TCRs only recognize antigens presented as peptides on MHC molecules whereas γ/δ TCR recognize native, non-processed, antigens [69]

Central tolerance of T-cells – positive and negative selection

During the transition from DN to DP the T-cell will rearrange the TCR-β chain followed by the TCR-α chain to complete the α/β TCR. The DP T-cell will then interact with cTECs to get positively selected. This selection is based upon the affinity (binding strength) by which the TCR can bind self-peptide/MHC complexes presented on cTECs. Only T-cells that bind with intermediate affinity to the self-MHC on cTECs will survive and migrate to the medulla of the thymus. Most of the T-cells will not recognize these self-peptides and will undergo apoptosis. Depending on if the TCR on the DP T-cell will bind to MHC I or MHC II on cTECs, the T-cell will develop into a single positive (SP) CD4+ MHCII binding cell or a SP CD8+ MHCI binding cell. This first process is known as *positive selection*. Next, T-cells will enter the thymic medulla were they will scan the surface of medullary thymic

epithelial cells (mTEC) and thymic DCs presenting self-peptides on MHCs. If the T-cells express a TCR that bind to the self-MHC with too high of affinity, the T-cell will undergo apoptosis during a process referred to as *negative selection*[55, 70]. The transcription factor AIRE (autoimmune regulator), expressed by mTECs, allows tissue-restricted antigens such as insulin to be expressed in the thymus and is important in the deletion of autoreactive T-cell[71]. Three subclasses of DCs have been identified in the thymus: thymus resident cDCs (CD8+CD11b-), migratory cDCs (CD8-CD11b+) and pDCs. Both migratory cDCs and pDCs have the ability to migrate to the thymus and induce tolerance by presentation of peripheral self and foreign antigens[44]. None of the DC subsets found in the thymus express AIRE, and the mechanism by which self-antigens are presented by DCs are today not completely understood.

Activation of CD4+ T-cells and differentiation into specific subsets

CD4+ and CD8+ T-cells that survive the thymic selection migrate to peripheral tissues and secondary lymphoid organs, such as spleen and lymph nodes. CD8+ T-cells recognize peptides presented on MHC I, which is expressed on all nucleated cells, and have been found to be important in the killing of infected cells or cancer cells and are therefore often referred to as cytotoxic T-cells. On the other hand, CD4+ T-cells recognize peptides presented on MHCII molecules expressed by APCs (e.g. DCs or B-cells). CD4+ T-cells are called helper T-cells (Th) since they aid other immune cells during immune responses through production of various cytokines[65, 66]. When a naïve CD4+ T-cell interacts with a DC in the spleen or lymph node, the T-cell can differentiate into one of various effector subsets. The decision of which Th subset the CD4+ cell will differentiate into is usually decided by the cytokines in the microenvironment of the interaction, which in turn is regulated by the type of signal (e.g. from an infection) that has activated the DC (Figure 3).

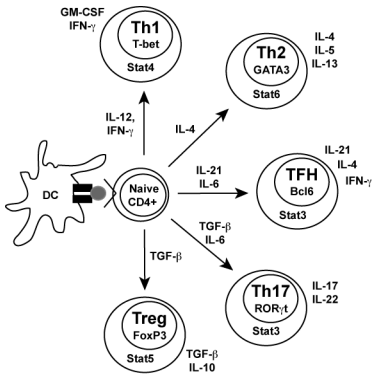


Figure 3. *Differentiation of naïve CD4+ T-cells into different effector subsets.* A naïve CD4+ T-cell can differentiate into one of various effecter subsets depending on the cytokines produced in the microenvironment in which the T-cell is activated. Which type of cytokines that is produced depends on what type of signals (e.g. from a pathogen) that has activated the DC.

DCs activated by intracellular pathogens will induce production of IL-12 and INF-γ that polarizes T-cell differentiation towards Th1. Th1 differentiation is dependent on the signal of transducer and activator 4 (Stat4) and the transcription factor T-Bet. Th1 cells produce GM-CSF and INF-γ that activates CD8+ T-cells and macrophages to aid in the defense against intracellular pathogens. Production of IL-4 during extracellular pathogen infection cause activation of Stat6 in naïve T-cells and the transcription of GATA3 followed by commitment to the Th2 subset. Th2 cells are important in humoral immunity and produce IL-4, IL-5 and IL-13. IL-17 and IL-22 are the two main cytokines produced by Th17 cells and this subset is important in the protection against extracellular pathogens, especially at mucosal surfaces. Th17 differentiation is induced the cytokines IL-6 and TGF-β, which leads to activation of Stat3 and transcription of RORγt[72]. The newly discovered follicular T-cell (T_{FH}) cell has an important role in aiding B-cells in humoral responses through production of its key cytokine IL-21. T_{FH} cells can also produce IL-4 and INF-γ that aid in the effector maturation of antibodies. T_{FH} cells require Stat3 activation and the transcription factor Bcl6 for differentiation and function[73]. Finally, induction of the transcription factor FoxP3 after concomitant stimulation with TGF-β will induce the formation of regulatory T-cells (Treg)[74].

Commitment to a CD4+ T-cell effector population was previously thought to be an irreversible event that involved stable genetic programs that would maintain the cytokine prolife of a differentiated T-cell even during conditions that would promote differentiation into other effector populations. However, it is now clear that there is great plasticity in the T-cell lineage, as Treg cells have been shown to convert to T_{FH} and Th17 cells and similarly Th17 have been show to differentiate into Th1 or Th2 cells[75].

Regulatory T-cells – natural and induced

Since the discovery of the of a CD4+CD25+ regulatory T-cell by Sakaguchi in 1995, it is now well appreciated that this population severs a critical function in immune regulation and maintenance of tolerance [76, 77]. Treg formation occurs both centrally in the thymus and in the periphery. Thymus-derived Tregs (referred to as natural Tregs (nTregs)) are generated during thymic selection although the mechanism is not completely understood. However, in contrast to naïve T-cells, Tregs are already antigen-primed when they leave the thymus[78]. DCs have been proposed to have a role in generation of peripheral Tregs (referred to as induced Tregs (iTregs)) under the influence of TGF-β or retinoic acid[79, 80]. Both iTregs and nTregs are defined by expression of CD25 and the transcriptionfactor FoxP3. FoxP3 defines the transcriptional program for Tregs and is of critical importance for the suppressive activity of these cells. Loss of FoxP3 causes severe autoimmunity in mice and an immune dysregulated polyendocrinopathy enteropathy X-linked (IPEX) autoimmune syndrome in humans[81, 82]. Furthermore, the level of FoxP3 expression has been shown to correlate with the suppressive capacity of Tregs[83, 84]. Tregs can suppress immune responses by different mechanisms. By using the membrane bound receptor cytotoxic T-Lymphocyte Antigen 4 (CTLA4), which blocks interactions between the costimulatory molecules CD80 and CD86, expressed

on APCs, and CD28 on T-cells, Tregs may use contact mediated suppression to inhibit T-cell activation. Tregs may also suppress immune responses through production of anti-inflammatory cytokines (e.g. IL-10, TGF-β) or through consumption of IL-2, which limits its availability to proliferating t-cells [85]. Tregs may also suppress production of antibodies from B-cells. Recently a population of follicular Treg cells that reside inside germinal centers (GC) was described that limits T_{FH} and GC B-cell numbers and loss of these follicular Tregs cause enhanced GC reaction[86, 87]. These follicular Tregs can tune the GC response and possibly prevent unwanted production of autoantibodies and autoimmunity. Tregs have been shown to be potent suppressors of autoimmune arthritis in various animal models[88, 89]. However, although Tregs are present at the site of inflammation in human RA patients, they seem to have lost their suppressive function[90, 91]. This it thought to depend on the inflammatory environment found in the inflamed joints of RA patients, according TNF-α has been shown to suppress the function of Tregs in RA and Treg function is restored after successful anti-TNF treatment[92]. This suggests that Tregs can lose their suppressive function during chronic inflammation, which may be of significant importance in autoimmune diseases such as RA.

Role of Flt3 in T-cell development and function.

Flt3 has a limited role in T-cell development. Flt3L synergize with IL-7 to stimulate proliferation of murine thymocytes and potentiates T-cell development from BM-derived precursors cultured in presence of thymic stroma and IL-12[10, 93]. Flt3-defiecient stem cells also have impaired ability to reconstitute T-cells in peripheral blood and thymus[8]. However, despite having reduced numbers of early T-cell progenitors in the bone marrow, mice with deficient Flt3 signaling have normal numbers of mature functional T-cells in peripheral blood and lymphoid organs[8, 35]. On the other hand, Flt3L has recently emerged as an important regulator of peripheral Treg homeostasis. This effect of Flt3L is mediated indirectly through the effect of Flt3 signaling on DCs. Expansion of DC using Flt3L results in a simultaneous increase in Treg numbers. On the contrary, Flt3 and Flt3L deficient mice have reduced numbers of both DCs and Tregs[94, 95]. Furthermore, depletion of Tregs causes a significant increase in serum Flt3L and expansion of DCs. A feedback relationship between Tregs and DCs, which is mediated through Flt3L, has also been shown in humans. Patients with a newly defined syndrome of DC deficiency have reduced numbers of Tregs in the peripheral blood and highly increased serum levels of Flt3L[19]. Furthermore, humans that have been treated with Flt3L have expansion of both DC and Treg numbers[96]. It is now clear that a feedback loop between DCs and Tregs exists that is regulated by the production of Flt3L.

Peripheral tolerance of T-cells

Not all self-antigens are presented in the thymus and therefore some T-cells bearing self-reactive TCRs escape negative selection in the thymus and enter the periphery. Therefore, peripheral-tolerance mechanism exists that help to prevent activation of self-reactive T-cells in the periphery. Autoreactive T-cell may undergo either anergy or deletion in the periphery. T-cells are activated in the presence of a TCR signal and a costimulatory signal that are mediated through CD28. If the T-cell doesn't receive a costimulatory signal or receives a coinhibitory signal the T-cell will become hyporesponseive, or anergic. Examples of coinhibitory signaling molecules is programmed death 1 (PD-1) and is ligand PD-L1 and PD-L2. The importance of this mechanism in protection of autoimmunity have been shown in mice where lack of PD-1 leads to a lupus-like diseases [97]. PD-1 has also been shown to mediate the conversion of T cells into Tregs. Also CTLA-4 has an important role in peripheral tolerance, since CTLA-4 deficient T-cells are resistant to anergy induction and CTLA4 deficient mice suffers from autoimmunity. Peripheral DCs have been proposed to have an important role in induction of peripheral tolerance. DCs express the ligands for PD-1 and when in an immature state DCs can present self-antigens to T-cells without delivering adequate costimulatory signals [55]. However, the role of DCs in peripheral tolerance have been questioned since constitutive ablation do not seem to cause severe autoimmunity[98]

B-cells

B-cells are the second important actor of the adaptive immune response. Although the main effector function of B-cells is to produce antibodies that help in the elimination of invading pathogens, B-cells also serves as potent antigen-presenting cells and can produce inflammatory cytokines such as TNF-α and IL-6 [99, 100]. Lately, the discovery of regulatory B-cells, which produce vast amounts of IL-10, have been put into focus due to its important role in suppressing immunological responses[101]. As other lymphocytes, B-cells arise in the bone marrow from a common lymphoid progenitor. After commitment to the B-cell fate and initial development in the bone marrow, B-cells leave in an immature state for further maturation in the spleen.

B-cell development and Flt3

B-cell development takes place in the bone marrow in adults. B-cells originate from a common lymphoid progenitor (CLP) and go through multiple maturation stages as pre/pro B-cells, pro-B-Cells, pre-B-cells and immature B-cells. Various factors, such as the chemokine CXCL12, IL-7 and Flt3L, are important in early B-cell development. CXCL12 is important for retaining B-cell progenitors in the bone marrow and is important for their survival and differentiation[102, 103]. IL-7 signaling is vital in early B-cells and promotes expression of early B-cell factor (EBF), which is critical for induction of the transcription factor network needed for B-cell development[104].

Shortly after the cloning of the Flt3 receptor, expression of Flt3 was found in human pre-B-cells and further analysis of the downstream signaling pathway suggested that this receptor could provide mitogenic signals to B-cell progenitors[6, 9, 105]. Accordingly, targeted deletion of either Flt3 or Flt3L revealed an important role for Flt3 in early B-cell development. Mice with non-functional Flt3 signaling showed severe reduction in pre/pro-, pro- and pre-B-cell progenitors (Figure 4) [35, 106, 107]. Later studies have shown that Flt3 and IL-7R signaling synergize in early lymphoid progenitors to promote differentiation of early B-cell precursors [107]. Interestingly, despite the block in B-cell development mice with a genetic deletion of Flt3 or Flt3L have normal numbers of mature peripheral B-cells and normal serum levels of antibodies[8, 35]. Despite the important role for Flt3 in the early stages of B-cell development, Flt3 has to be suppressed to allow for B-cell lineage commitment. This is accomplished by repression of Flt3 transcription by the transcription factor paired box protein 5 (Pax5) (Figure 4) [108]. Pax5 is the major B-cell commitment factor and induces B-cell lineage-specific genes such as CD19 and repress lineage inappropriate genes such as M-CSFR and Notch1 [109, 110].

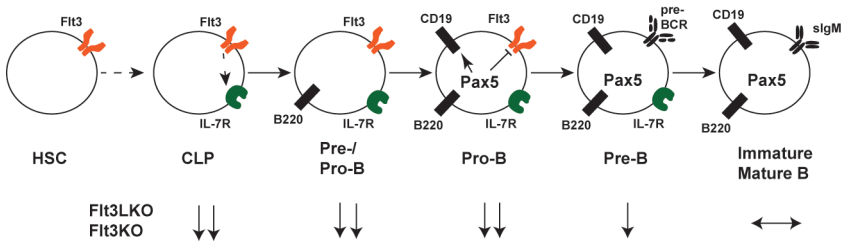


Figure 4. *Flt3 in early B-cell development*. Signaling through Flt3 is involved in the differentiation of early hematopoietic stem cells (HSC) into common lymphoid progenitors (CLP). At this stage, Flt3 is thought to aid in expression of the IL-7R. Signaling through the IL-7R and Flt3 synergize to promote differentiation of pre-/pro B-cells into pro-B-cells. At this stage, commitment to the B-cell lineage occurs by the expression of Pax5. Pax5 suppress the expression of Flt3 and induce expression of B-cell associated genes such as CD19. B-cell continues to develop and finally reach an immature stage and leaves the bone marrow for further development in the periphery. Mice lacking the Flt3 receptor (Flt3KO) or the Flt3L (Flt3LKO) have significantly reduced numbers of CLPs, Pre-Pro B-cells, Pro B-cells and slightly reduced numbers of Pre-B-cells. Interestingly, these genetically modified mice do not have any differences in immature B-cells or mature B-cells.

The B-cell receptor – a membrane-bound antibody

During B-cell differentiation the B-cell receptor (BCR) is assembled. The B-cell receptor can be simply explained as a membrane-bound antibody, which consists of two identical pairs of heavy and light chains, each containing a variable and a constant region. The variable region determines the specificity (Fab-part) whereas the constant region determines the effector function (Fc-part) of an antibody. This specificity is determined at the genomic level through the process of V(D)J recombination, where variable (V), diversity (D) and joining (J) gene segments are recombined and encoded together with the exon that encodes the constant region.

The process of V(D)J recombination is critically dependent on the two recombination activated gene products known as RAG1 and RAG2, whose expression is restricted to lymphocytes during developmental stages [111]. The constant region of the heavy chain defines the class and subclass of the antibody. The heavy locus contains five different constant regions (μ, γ, α, ε, δ), which all code for a different antibody class (IgM, IgG, IgA, IgE, IgD). The first constant region to be transcribed is always μ and the first antibody receptor on immature B-cells is therefore IgM [112].

The V(D)J recombination of the heavy chain starts at the pro-B-cell stage. At the pre-B-cell stage, the B-cell has completed the heavy chain V(D)J recombination and expresses a heavy chain which assembles with a surrogate light chain to form the pre-BCR. The pre-BCR associates with Igα and Igβ, two molecules that aid in the signaling through the pre-BCR receptor. As the pre-B-cell progresses through differentiation the surrogate light chain is lost and subsequently replace by the light chain. The synthesis of the light chain, either in the form of κ or λ, leads to the assembly and expression of the BCR on the cell-surface as a membrane-bound antibody[111]. The B-cell has now reach the immature B-cell stage and may leave the bone marrow for further differentiation in the periphery.

Central tolerance of B-cells

Due to the incredible diversity of receptor specificities that arise during V(D)J recombination of the BCR, there is a great risk of creating antigen receptors which react to self proteins, i.e. autoreactive BCRs. It is therefore essential that B-cells that express autoreactive BCRs are removed. Therefore, as for T-cells, there are various checkpoints that B-cell progenitors have to pass through before becoming a mature B-cell. The first checkpoint occurs at the pre-B-cell stage and test for the ability to from a functional pre-BCR though binding of the surrogate light chain. Signals form a functional pre-BCR will induce proliferation and subsequent down-regulation of the surrogate light chain. If the pre-BCR cannot not pair with the surrogate light no proliferation will be induced. However, despite an non-functional pairing these pre-B-cells still proceed in differentiation but the contribution to developing B-cell pool are thought to be significantly lower compared to those pre-B-cell with a working pre-BCR [111, 113]. If an immature B-cell present an autoreactive BCR in the bone marrow a series of cellular events are directly triggered. If the receptor binds a self-antigen with high affinity, the immature B-cell will rapidly internalize the BCR, halting the maturation process. This will reduce expression of homing receptors on the immature B-cell needed for entering lymphoid organs. Furthermore, the receptor for B-cell activating factor (BAFF), a cytokine required for B-cell survival, is poorly induced leading to reduced survival potential. Third, the RAG1 and RAG2 genes are re-expressed allowing the light chain to be rearranged and replaced in the BCR. This process, refereed to as *receptor editing*, usually only involves the light chain (and less commonly the heavy chain) and is an attempt to acquire a useful non-self reactive BCR. Today receptor editing is thought to be the predominant mechanism of central tolerance[114]. If the B-cell retains its self-reactivity after receptor editing, the B-cell will undergo clonal deletion. If the immature B-cell survives this initial control,

which is the case for around 10% of the newly generated B-cells, they will leave the bone marrow and migrate to the spleen for further development[115, 116].

Peripheral B-cell tolerance

Immature B-cells that arrive in the spleen will pass through two initial differentiation states transitional (T)1 and T2. Upon entering the spleen immature B-cells may encounter self-antigens not present in the bone marrow. If a T1 B-cell binds a self-antigen with high affinity, it will undergo rapid deletion. On the other hand, if the binding to self-antigen is of low or very-low avidity, this will result in induction of anergy or ignorance, respectively. Anergic B-cells desensitize the BCR, resulting in reduced responsiveness and antigen presentation capabilities and an impaired ability to compete for BAFF, and will therefore undergo cell death. Recognition of self or foreign antigens will induce relocation of B-cells to the T-cells zone, called peri-arteriolar lymphoid sheath (PALS), where they search for T-cell help. If the B-cell does not receive T-cell help or TLR dependent co-stimulatory signals, the B-cell will die within two-three days. B-cell tolerance is further controlled during the germinal center reaction, under which B-cells undergo hyper mutations of the BCR that can result in an autoreactive receptor. Selection during the GC reaction will be discussed more in the section below[116].

After maturation some of the B-cells will become splenic marginal zone (MZ) B-cells whereas most of them will become follicular (FO) B-cells. MZ B-cells will remain in the splenic marginal zone whereas FO B-cells will circulate the follicles in the spleen, to the lymph nodes and to the bone marrow until they encounter an antigen or die.

Antigen activation – the humoral immune response

When dendritic cells (DCs) are activated by antigen (e.g. bacterial antigen or a protein antigen), they increase expression of MHCII and the co-stimulatory molecules CD40, CD80 and CD86 and start producing co-stimulatory cytokines. DCs will activate and induce proliferation of naïve CD4 T-cells specific for the presented antigen. The T-cell will also stimulate and increase the maturation of DCs through production of cytokines (e.g. IFN-γ) and interaction between CD40L on T-cells and CD40 on DCs. The activated T-cells will also start to differentiate into a specific effector subset (e.g. Th1 or Th2), which is decided by the cytokine milieu in which the T-cells are activated. After activation the T-cell can aid B-cells to induce a humoral response.

In contrast to T-cells, B-cells can be activated by non-processed "native" antigens and do not need presentation on MHCII molecules. B-cell responses are usually divided into T-independent and T-dependent. T-independent antigens are usually polysaccharides (e.g. LPS) or bacterial flagellin, which binds the B-cell receptor (BCR) and at the same time provide activation signals through binding to TLRs expressed on B-cells. These responses are usually initiated in the MZ of the spleen and induce fast formation of antibody secreting B-cells, i.e. plasma cells[117]. On the other hand, most antigens can't stimulate B-cell activation without the help of

T-cells, therefore referred to as T-dependent antigens. These responses are usually initiated from FO B-cells and results in the formation of germinal centers (GCs), in which T-cells, B-cells and follicular dendritic cells collaborate to induce a high affinity humoral response against the activating antigen.

The germinal center

Secondary lymphoid organs (e.g. spleen and lymph nodes) are segregated into T-cell and B-cell (follicle) zones. The localization of B-cells and T-cells to these zones depends on the chemokine receptors CXCR5 (follicle) and CCR7 (T-cell zone). When activated, FO B-cells reduce the expression of CXCR5 whilst increasing expression of CCR7, which induces migration towards the T-cell zone. Once at the T-cell – B-cell border, B-cells will interact with previously activated T-cells, inducing proliferation at the outer part of the follicle[118]. At this point, the B-cell can undergo one of three faiths; 1) differentiate into a short lived plasma cell which produce low-affinity antibodies, 2) differentiate into a early memory B-cell that enter the blood circulation or 3) return to the center of the follicle to form a GC[119].

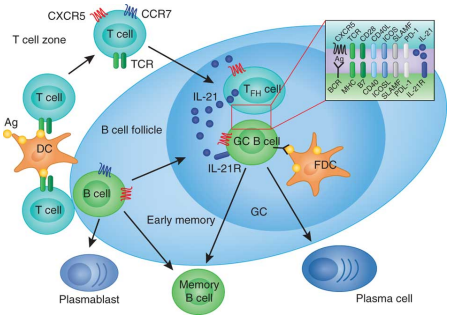


Figure 5. *The germinal center reaction.* When activated, follicular B-cells reduce the expression of the CXCR5 receptor whilst increasing expression of the CCR7 receptor. This will induce migration towards the T-cell zone were they interact with pre-activated T-cells. After this interaction, the B-cells can differentiate into a short-lived plasma cell or a memory B-cell. The third option is to re-enter the follicle to form a GC. At the same time, a population of T-cells will upregulate the CXCR5 receptor and differentiate into follicular T-cells (T_{FH}). These will migrate into GC where they will interact with activated B-cells through co-stimulatory receptors. Through production of cytokines such as IL-21 and IL-4, T_{FH} cells promote survival and differentiation of GC B-cells. GC B-cells also interact with follicular dendritic cells, which present antigens to the B-cell. During the GC reaction the B-cell will undergo affinity and effector maturation of its BCR, through the processes of SHM and CSR. After the GC reaction, the B-cell may leave the GC as a plasma cell or a memory B-cell. Adopted with permission from Nature publishing group. (Nutt, 2011)

GCs are areas inside the B-cells follicle where B-cells undergo rounds of proliferation and induce affinity and effector maturation of antibodies through the process of somatic hypermutation (SHM) and class switch recombination (CSR), respectively. The GC is usually divided into two zones, the light zone (LZ) and the dark zone (DZ). In the DZ, B-cells proliferate and undergo SHM, whereas in the LZ B-cells undergo CSR and are selected based on their ability to bind antigens presented by follicular dendritic cells and receive help from follicular T-cells (T_{FH}). The role of T_{FH} in the GC reaction is now well established. T_{FH} arise from naïve T-cells that are activated outside the follicle. T_{FH} express the CXCR5 receptor that allows them to migrate into the follicle and the GC to interact with activated B-cells.

The interactions between CD40-ligand and inducible T-cell costimulatory (ICOS) on T_{FH} cells and CD40 and ICOS-Ligand on B-cells, as well as production of cytokines such as IL-21 and IL-4 by T_{FH} cells are of significant importance for GC function and establishment. Follicular dendritic cells are non-hematopoietic cells that reside inside the follicle expressing high levels of complement receptors, which capture and present antigens to activated B-cells inside the GC. Expression of the transcription factor Bcl6 by B-cells is of critical importance for GC development, and Bcl6 is specifically expressed in GC B-cells. Mice lacking Bcl6 have normal B-cell development, but do not form GCs and lack affinity maturation. Bcl6 acts as transcriptional repressor and is thought to allow the vigorous proliferation of GC B-cells while they at the same time undergo genomic remodeling during SHM and CSR. Bcl6 accomplishes this through repression of various pro-apoptotic genes such as the tumor suppressor gene p53. A second major function of Bcl6 in GC B-cells is to inhibit genes involved in T-cell activation, such as CD80, thereby preventing premature exit from the GC before completion of SHM. To arrest cell proliferation, Bcl6 needs to be downregulated. This is achieved by BCR signaling and signaling through CD40 [119-121].

Somatic hypermutation – affinity maturation of antibodies

During activation and proliferation in the GC, the B-cell receptor undergoes what is known as somatic hypermutation (SHM). SHM occurs in the variable region of the immunoglobulin results in increased binding strength (affinity) of the antibody. SHM is associated with DNA strand breaks and requires the activity of activation-induced cytidine deaminase (AID), an enzyme that aids in the initiation of this process through deamination of cytidines directly in the DNA[121].

Class switch recombination – effector maturation of antibodies

During class switch recombination (CSR), the B-cell undergoes an irreversible recombination of their immunoglobulin class from IgM and IgD to other classes with specific effector functions. During CSR the constant (C) exons of the heavy chain Ig heavy chain (IgH) coding for IgM (Cμ) are deleted and replaced with the C exons coding for either IgG (Cγ), IgE (Cε) or IgA (Cα). This process is accomplished by joining two nonhomologous DNA sequences, switch regions, which are located upstream of each C gene. As for SHM, CSR also requires the expression of AID, which deaminates deoxycytosines in the switch region DNA, yielding deoxyuracils. During the removal of deoxyuracil bases, double-stranded DNA breaks occur in the upstream (donor) and downstream (acceptor) switch regions. This activates a DNA damage response, which promotes long-range recombination. Eventually the double stranded breaks in μ switch region and the downstream target switch region are joined through a non-homologous end joining mechanism, resulting in the formation of a new antibody isotype (Figure 6) [122].

CSR is initiated by sterile transcription from isotype-specific intronic promoters that continues through the intronic exon, the adjacent switch region and the C exons creating a germ-line transcript (GLT). GLTs are non-coding but are thought to initiate CSR by rendering the switch region accessible for AID. Both

primary and secondary stimuli are necessary for induction of CSR in B-cells. Whereas T dependent (i.e. CD40L) or T independent (i.e. TLR) primary stimuli induce expression of AID, secondary stimuli such as IL-4 (IgG1, IgE), IFN-γ (IgG2c) and TGF-β (IgA) are needed for directing the class switch to a specific antibody isotype through the induction of GLT[122]. CSR within GC is T-cell dependent and usually gives rise to IgG1 or IgG2c subclasses[120, 123]. To visualize the importance of GC for T-dependent CSR, one can consider production of IgG1. CSR to IgG1 are highly dependent on GC formation, since mice lacking GCs have severely reduced levels of IgG1[120]. Furthermore, IgG1 production is dependent on the type I cytokine IL-4 which is produced by T_{FH} cells during a GC reaction. As with lack of functional GCs, deficiency of IL-4 or Stat6, the major signaling molecule downstream of the IL-4R, leads to significantly reduced levels of IgG1. IL-4 induces phosphorylated Stat6, which dimerize and translocate to the nucleus where it binds to the promoter region of γ1, thereby inducing transcription of GLT and subsequent CSR to IgG1 [73, 124, 125]

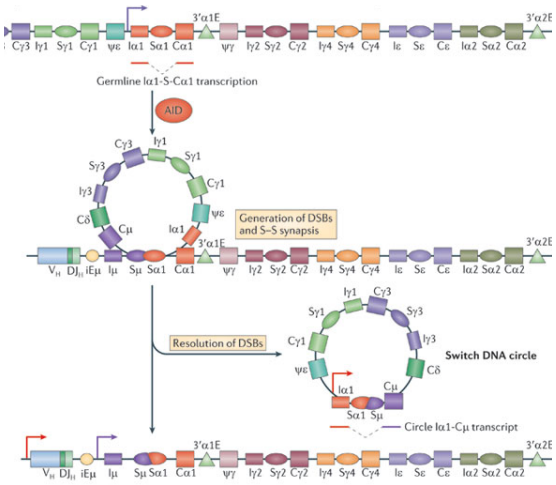


Figure 6. *Class switch recombination.* CSR is initiated by sterile transcription from an isotype-specific intronic (I) promote continuing through intronic exon, the adjacent switch (S) region and the constant C exon creating a germ-line transcript (GLT). GLTs are thought to initiate CSR by rendering the switch region accessible for AID. AID deaminates deoxycytosines in the S region DNA, yielding deoxyuracils. During the removal of deoxyuracil bases, double-stranded DNA breaks occur in the upstream (donor) and downstream (acceptor) S regions. This activates a DNA damage response, which promotes long-range recombination. Eventually the double stranded breaks in μS region and the downstream target S region are joined through a non-homologous end joining mechanism, resulting in the formation of a new antibody isotype. Figure adopted with the permission of Nature publishing group: Nature Reviews Immunology. (Casali, 2012)

Selection in the germinal center reaction

GCs have an important role in regulating the development of the B-cell repertoire. After hypermutation B-cells are either positively or negatively selected based on the affinity of their B-cell receptor, local competition for antigens presented on follicular dendritic cells and ability to receive help from T_{FH} cells[126]. GC B-cells are prone to undergo apoptosis due to a pro-apoptotic gene expression, indicating that if the B-cell does not receive proper survival signals from T_{FH} cells or follicular dendritic cells, the B-cell will undergo apoptosis. This is usually the case for self-reactive B-cells that are generated during SHM[121]. B-cells that are positively selected after the GC reaction may leave the GC as a memory B-cell or an antibody-producing plasma cell.

Memory B-cells

Memory B-cells are usually found after an immune response and have an increased ability respond to activating antigens. These cells also have an increased ability to form GC upon antigen re-challenge and differentiate faster into antibody secreting cells, indicating that these cells have an important protective function in the immune system. Memory B-cells are characterized in having mutated immunoglobulins, although memory B-cells without mutations have been described. Generation of memory B-cells are highly dependent on GC formation, and lack of GC formation results in reduction in memory formation.[127]

Plasma cells

Plasma cells can be characterized as the true effector population of B-cells. These antibody-secreting cells produce vast amounts of antibodies that have an important function in the protection of the body.

After antigen activation both marginal zone (MZ) and follicular (FO) B-cells can differentiate into plasma cells. MZ B-cells are the first to meet blood-borne antigens in the spleen and have the ability to quickly form short-lived plasma cells independently of T-cell help. MZ B-cells that have been activated by antigens will undergo a burst of proliferation and at the same time differentiate into antibody producing cells. These plasma cells usually do not have any somatic mutations and are short-lived. On the other hand, plasma cells formed after the GC reaction are usually long-lived and migrate to the bone marrow were they provide long-lasting immunity even after an infection has been cleared[118].

The commitment of a B-cell to terminal differentiation into an antibody secreting plasma cell is regulated by a mutual antagonism between two sets of transcription factors; Pax5 and Bcl6, which promote the B-cell gene program, versus Blimp1 and IRF4 that supports the plasma cell program. These genes function as transcriptional repressors for each other's expression[118]. Pax5, the major B-cell commitment factor, sustains expression of genes essential for B-cell identity and initiate transcription of AID in the GC. At the same time, Pax5 suppresses the expression of plasma cell-associated genes such as Bimp1 and Xbp1. Therefore, Pax5 needs to be downregulated to allow for plasma cell differentiation[128, 129]. When the B-cell gene program is downregulated, plasma cell genes must be upregulated to

induce differentiation. This is achieved by the transcriptional repressor Blimp1, which represses the expression of Pax5, Bcl6 and AID and promotes the exit from the cell-cycle by repression of genes such as c-myc. Repression of B-cell associated genes allow the expression of genes essential for plasma cell development and function, such as Xbp1[118, 130].

Although Blimp1 is critical for establishing and stabilizing plasma cell differentiation, it seems to have a redundant role during the initiation of plasma cell differentiation [131]. Before Blimp1 function is initiated the repressive function of Pax5 is reduced, initiating transcription of genes that are normally repressed by Pax5. One transcription factor that is initiated early and is required for plasma cell differentiation is IRF4[132]. However, IRF4 also serves an important role in CSR during the GC reaction through induction of AID and is required for the initial proliferative burst of activated B-cells[132]. Interestingly, one of the target genes for Pax5 that is reexpressed upon B-cell activation *in vitro* is Flt3. This has been associated with the reduced repression of Pax5 and initiation of plasma cell differentiation [131].

Effector functions of antibodies

Antibodies usually do not exert their effects on their own, but rather associate with other effectors molecules to promote removal of infectious agents. Many of the effector functions exerted by antibodies are mediated through binding to receptors specific for the Fc fragments on IgG, Fc-gamma receptors (FcγR). Three activating, (FcγRI, FcγRIII and FcγRIV), and one inhibitory (FcγRIIB), FcγR exist in mice. These receptors are expressed on most cells of the innate immune system (e.g. DCs, monocytes and macrophages) and ligation of the receptors by IgG antibodies leads to increased activation of the responding cell. The FcγR provide a further link between the specific adaptive immune system and the unspecific innate immune system. Different IgG subclasses bind FcγR with different affinities. IgG1 binds to the inhibitory receptor FcγRIIB with highest affinity, although also to the activating FcγRIII. In contrast, the IgG2a/c and IgG2b have a much higher affinity for the activating FcγR receptors. IgG3 binds only weakly or if at all to FcγRs, but can activate the immune system through other pathways. B-cells also express FcγR, but only the inhibitory FcγRIIB, which regulates positive signals from the B-cell receptor. The importance of FcγRIIB on B-cells was demonstrated in mice deficient for the receptor, which developed a severe systemic lupus erythematosus (SLE) like syndrome[133]. A role for FcγR in other autoimmune disease such as rheumatoid arthritis have also been shown in animal models, where mice deficient for FcγR signaling are protected from bone destruction[134].

Autoimmunity – failure of self tolerance

The immune system is designed to protect us from invading pathogens, such as bacteria, viruses and parasites. Therefore, the immune system needs to discriminate between millions of different microorganism that are constantly surrounding the body, posing a potential treat. Some of these microbes might have epitopes (foreign-antigens) that are closely related to proteins (self-antigens) found in our body. It is therefore of great importance that the cells of the immune system can discriminate between antigens expressed on our own tissue and antigens expressed by pathogens. T- and B-cells that are reactive towards self-tissues are eliminated during central and peripheral tolerance, as describe above. Furthermore, regulatory cells of the immune system (e.g. Tregs), further controls immune responses towards antigens. However, sometimes the control mechanism fail and autoreactive cells are allowed to attack self-tissues. This breach of self-tolerance results in inflammation and finally autoimmunity, which is a highly destructive situation. Autoimmunity is a complex condition that seems to rely on multiple synergistic mechanisms. The great question is what triggers the immune system to react towards self-tissues. Activation of self-reactive B-cell and T-cell are a hallmark of autoimmunity. Although B-cells and T-cells expressing self-recognizing receptors may be found in healthy individuals, they are not allowed to cause autoimmunity under normal circumstances. It is now clear that autoimmunity conditions reflects a complex involvement of genes, environment and the immune system.

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a common autoimmune disease that today affects around 0.5-1% of the world population, although the incidence have declined over the years. The disease is a chronic systemic inflammatory disease that is characterized by inflammation of synovial membranes in flexible joints, leading to hyperplasia and pannus formation and subsequent cartilage and bone destruction. If RA is left untreated, the disease causes great disability, systemic complications and early death [135]. Production of autoantibodies, such as rheumatoid factor and anti-citrullinated protein antibodies (ACPA), is an early sign and a sensitive diagnostic characteristic of the disease. Today, great advances in treatment of RA, such as cytokine antagonists and T-cell regulating and B-cell depleting therapies, have significantly improved the outcome for patients. However, despite the advances in RA treatment, therapies sometimes fail or produce only partial responses. Furthermore, there is still a lack of predictive and reliable biomarkers for prognosis and therapeutic response [136].

Pathogenesis of rheumatoid arthritis

The pathogenesis of RA is still relatively unknown. Although it is clear that the disease involves interplay between genetic factors, environmental triggers and chance. Several genetic risk factors that involve immune regulatory functions have been identified for RA [137]. The most established genetic association it the presence

of the human leukocyte antigen (HLA)-DRB1 locus, which is associated with autoantibody production. Alleles that contain a common amino acid motif (QKRAA), more commonly known as the shared epitope, show even higher susceptibility [138]. Many other risk alleles have been found to be associated with RA, primarily ACPA-positive RA. Mutations in genes involved in T-cell stimulation, activation and functional differentiation (e.g. PTPN22, CD28, CTLA4), which may contribute to failure in intrinsic regulation of T-cell activation [64, 139, 140]. Furthermore, polymorphisms in genes important in the effector function and terminal differentiation of leukocytes (e.g. Blimp1) have also recently been identified [64].

Autoantibodies

Production of autoantibodies is a common feature of RA. Although not found in all patients, almost 80% of RA patients have production of Rheumatoid factor (RF). RF was originally described as an IgM antibody directed towards the Fc-region of IgG. The binding of RF to the Fc-region of IgG is thought contribute to the formation of immune complexes in inflamed joints, which leads to activation of the immune system through complement factors. However, RF is not only found in RA patients but can also be found in healthy individuals and in other inflammatory conditions and after infections.

In 1998 Schelleknes *et al* identified antibodies in the sera from RA patients that were reactive towards a synthetic peptide that contained the amino acid citrulline [141]. Citrulline arises from a post-translational modification of the amino acid arginine, which leads to a change in the chemical nature of the amino acid. This process takes place during cell-death and inflammation and several citrullinated proteins, such as α-enolase and fibrinogen have been identified in the joint of RA patients. These antibodies directed against citrullinated proteins are referred to as anti-citrullinated protein antibodies (ACPA). Further studies have shown that ACPA antibodies can be found in the sera from RA patients several years before symptoms arise and are currently used as prognostic markers predicting development of RA and disease severity, joint damage, development of extra-articular manifestations and poor responses to treatment [142, 143].

Immune system in RA

The genetic profile of RA and the presence of autoantibodies place the adaptive immune system in the center of pathogenesis. Also, effective treatment with B-cell depleting and T-cell regulating therapies used in RA further supports the fundamental role of these cells in the pathogenesis [144, 145] However, adaptive immune response initiated by T and B-cells is thought to be a "late" response that occurs after activation of the innate immune system. This propose that that innate cells such as DCs can play an important part in the initiation phase of RA.

Dendritic cells

The genetic association of HLA-DR in RA proposes a role for antigen-presenting cells such as DCs in the pathogenesis of RA. DCs are highly regulatory cells that have a role in both the induction of peripheral and central tolerance and the initiation of immune responses towards invading pathogens. However, DCs may also present self-antigens and due to the potent role for DCs in initiating T-cell activation, these cells can be of significant importance in RA pathogenesis. Accordingly, these cells are found in the synovial tissue and blood of RA patients[146]. Mouse models of RA have further supported a role for DCs in RA, although here they have been found to have both a protective and an instigating role in the disease [147, 148]. These reasons may be due to the heterogeneity of the DC population and the different functions associated with the maturation state of the DC.

T-cells

T-cells may be abundantly found in the inflamed joints of RA patients clearly providing evidence for an important role of these cells in the pathogenesis of the disease. However, direct targeting of T-cells using cyclosporine or anti T-cell therapy have not been successful in RA. This may be due that both regulatory and effector populations of T-cells are removed with this therapy. RA is traditionally thought to be a Th1-driven disease although today attention has been increasingly focused on Th17 cells. Th17 may drive the inflammatory response in the knee joint through production of pro-inflammatory IL-17 [136]. Interestingly, although Tregs are found in the circulation and the synovial membrane of RA patients, they seem to have lost their suppressive capacity. This has been most commonly associated with an inhibitory effect of TNF-α on Treg function [92, 149]. Another important function of T-cells is to aid B-cells in maturation and antibody production.

B-cells

Recent advances in RA treatment using B-cell depleting therapies, which depletes mature B-cells expressing CD20, has identified B-cells as an important players in the RA pathogenesis[145]. B-cells are found both in the synovial membrane and synovial fluid of RA patients and through production of inflammatory cytokines, such as IL-6 and TNF-α, B-cells contribute to the inflammatory environment in the affected joints[150]. Also, B-cells may serve as potent antigen-presenting cells, sustaining T-cell activation. Finally, the development of germinal centers-like structures in the synovial membrane or RA patients and the presence of antibody producing cells with the potential of producing autoantibodies such as rheumatoid factor and ACPA further supports the role for B-cells in the disease[136]. The presence of ACPA usually correlate with a more aggressive disease and these autoantibodies has been found in the synovial fluid of RA patients[151]. Furthermore, a recent study identified that around 25% of IgG-expressing B-cells in the inflamed synovium are specific for citrullinated auto-antigens[152], supporting an important role in RA pathogenesis. Successful B-cell depleting therapies result in reduced levels of autoantibodies and alleviation of RA symptoms. However, when B-cells have

repopulated, autoantibody levels return to the same level as before treatment indicating that the underlying mechanism is still present[153].

Flt3 and Flt3-ligand in rheumatic disease

The role of Flt3 signaling in autoimmune diseases is relatively unexplored, although in recent years Flt3L has been linked to the rheumatic diseases primary Sjögren's syndrome (pSS) and RA. In pSS patients, Flt3L is increased in the serum and B-cells expressing the Flt3 receptor can be found in both blood and salivary glands of these patients. Furthermore, Flt3L was found to potentiate the survival and proliferation of anti-IgM stimulated B-cells isolated from both pSS patients and healthy individuals [22]. The level of Flt3L in pSS patients has been correlated with lymphoma development and is associated with lymphocytopenia [154]. In RA, Flt3L has been found to accumulate in the synovial fluid of patients and also to be increased in the serum when compared to healthy individuals [21, 155]. Furthermore, serum levels of Flt3L are reduced after 2 weeks treatment with corticosteroids and correlate with clinical improvement, indicating that Flt3L is related to inflammatory status in RA [21]. Flt3L was recently listed in a panel of pre-clinical cytokine markers found in association with development of RA in a large study[156]. This supports a possible role for Flt3L in RA pathogenesis. Association between Flt3 and RA has also been proposed in animal models of arthritis. Local knee exposure of Flt3L aggravates severity of peptidoglycan-induced arthritis and blockade of Flt3 signaling reduced severity of arthritis in an antigen-induced arthritis model [147, 155]. The increased levels of Flt3L found in synovial fluid of RA patients might also contribute to differentiation and survival of local DCs, which can sustain the inflammatory process through activation of T-cells. Accordingly, a recent study identified $Flt3^+$ cells in the synovial membrane of RA patients, implicating that Flt3L can be involved in the pathogenesis of arthritis [21].

Bone and structural damage in rheumatoid arthritis

Bone makes up the skeleton that is the foundation of our body. Consisting of a fine bony network, the trabecular bone, and a dense bony shell, the cortical bone, the skeleton provides structure for muscles and protection to internal organs. Furthermore, the bone serves as a reservoir for calcium and provides the home for the hematopoietic stem cells. Both the cortical bone and the trabecular bone are constantly undergoing remodeling, a function that is mainly carried out by two different cell types: osteoclasts and osteoblasts[157].

Osteoclasts

Osteoclasts are large multinucleated cells that derive from the monocytic lineage of the hematopoietic stem cell pool. These cells are the only cells found in the body that have the potential to degrade bone. Osteoclasts adhere tightly to the bone surface, creating a tight seal between the osteoclast and bone surface thereby separating the surrounding extracellular space from the bone. Osteoclasts then use proton pumps to

create an acidic milieu that supports the release of calcium from bone. The bone matrix is degraded using enzymes such as cathepsin K, matrix metalloproteinases (MMP) and tartrate-resistant acid phosphatase type 5 (TRAP), synthesized by the osteoclast[158]. Two factors have been found to be essential for osteoclast formation: macrophage colony stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANK-L). M-CSF stimulates the proliferation of pre-osteoclasts, whereas RANK-L induce proliferation, differentiation and survival of pre-osteoclasts and osteoclasts. The importance of these cytokines in osteoclasts differentiation and function has been shown in various mouse models [159, 160].

Osteoblasts

Osteoblasts are bone-building cells that counteract the bone degrading function of osteoclasts through production of proteins and collagen that makes new bone matrix. The osteoblast originates from mesenchyme stem cells in the bone marrow[161]. Osteoblasts are defined by expression of the transcription factors Runx2 and Sp7/Osterix, which are of critical importance for the development and function of these cells since genetic deletion cause a complete absence of osteoblasts [162, 163]. Furthermore, LPR5, a co-receptor for Wnt signaling, has important roles in osteoblast function and differentiation. Osteoblast differentiation is supported by the growth factor cytokine TGF-β, which have been shown to increase Runx2 expression. Furthermore, osteoblast function is regulated by the two molecules bone morphogenetic protein (BMP) and the wingless (Wnt) family proteins. Osteoblasts support osteoclast function by production of M-CSF and RANK-L and thereby promoting the local bone remodeling[161].

Bone remodeling and the immune system

The role of the immune system in regulation of bone remodeling is now well established. Through production of various cytokines, immune cells can contribute to stimulation or inhibition of bone remodeling[164]. Dendritic cells (DCs) serve as an important link between the innate and adaptive immune system by regulating and initiating T-cell responses to foreign antigens. In steady state, DCs do not seem to contribute to bone remodeling since DC deficient mice show no signs of skeletal defects. However, under the influence of inflammatory cytokines or M-CSF, DCs have the ability to transdifferentiate into bone-degrading osteoclasts [165-168]. Furthermore, DCs are potent activators of T-cells, which have a known role in regulating osteoclastogenesis (Figure 7). Activation of T-cells, during infection or inflammation, increases the production of RANK-L, TNF-α and IL-6, essential triggers of osteoclastogenesis[169, 170]. However, resting T-cells have been found to suppress osteoclastogenesis, and osteoclastogenesis is increased in mice lacking T-cells or after T-cell depletion[171, 172]. These results indicate the importance of T-cells in the regulation of bone homeostasis. Although T-cells produce cytokines that potentiate osteoclast differentiation, the two major Th1 and Th2 cytokines, IFN-γ and IL-4 respectively, both inhibit osteoclastogenesis. Recent evidence suggests that Th17 cells are the osteoclastogenic T-cell population, by production of RANK-L and TNF-α and also through the actions induced by IL-17 [173, 174]. IL-17 induces

expression of RANK on osteoclast precursors and RANK-L on stromal cells and osteoblasts, thereby potentiating osteoclast development [174, 175]. Also, IL-17 promotes local inflammation by recruiting and activating immune cells, which further increase the production of inflammatory cytokines such as TNF-α[174]. The regulatory role of osteoclastogenesis found for T-cells can be attributed to the Treg population. Tregs have been shown to inhibit the differentiation and function of osteoclast *in vitro* and to suppress bone erosions in animal model of arthritis *in vivo*[176-178]. Tregs suppress osteoclastogenesis by direct cell contact (e.g. CTLA4) or by production of cytokines (e.g. IL-10 and TGF-β)[179, 180]. The action of CTLA4 may be both through directly acting on osteoclast precursors or indirect through reducing T-cell activation (Figure 7)[180, 181].

B-cell development in the bone marrow is under the influence of growth factors and cytokines produced by stromal cells and osteoblasts. Although B-cells have key roles in bone destructive disease such as RA, their role in bone remodeling is not completely understood. Activated B-cells produce the osteoclast driving factors TNF-α and RANK-L, and have the potential to induce activation of T-cells[182, 183]. However, B-cells produce OPG, a soluble RANK-L decoy receptor that block RANK-RANK-L interactions, and IFN-γ and TGF-β, which all inhibit osteoclastogenesis[171, 184, 185]. Activation of Fcγ-receptors (FcγR) on osteoclast precursors by autoantibody cross-linking potentiates osteoclast differentiation [186]. FcγR activation is also important for cartilage and bone destruction in animal models of arthritis [134, 187, 188]. Therefore, although the role of B-cells in bone remodeling is not as established as for T-cells, they may promote osteoclastogenesis during inflammatory and autoimmune conditions through production of osteoclast-promoting cytokines and antibodies (Figure 7).

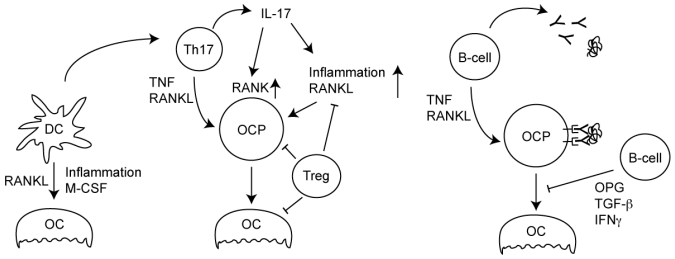


Figure 7. *Contribution of different immune cells to bone remodeling.* Immune cells have a profound impact on bone remodeling. DCs have been shown to transdifferentiate into osteoclasts (OCs) during inflammatory conditions. Furthermore, DCs can activate T-cells to differentiate into Th17 cells. Th17 cells promote osteoclastogenesis through production of IL-17, which potentiates local inflammation and the production of RANK-L. IL-17 also induces expression of RANK on osteoclast precursors (OCP). Furthermore, Th17 cells can produce TNF and RANK-L and directly contribute to OC development. Tregs have an important role in suppressing osteoclastogenesis, either by directly acting on osteoclast or osteoclast-precursors or indirectly by reducing inflammation. Finally, B-cells may also promote osteoclastogenesis through production of TNF and RANK-L and through production of IgGs, which can potentiate osteoclast differentiation and function through FcγR activation. However, B-cells can also suppress OC development through production of OPG, TGF-β and IFN-γ.

Involvement of Flt3 in bone remodeling

The role of Flt3 in bone remodeling is relatively unexplored, although recent finding have implicated a function for Flt3L in osteoclast differentiation. A study by Lean *et al* showed that Flt3L had the potential to substitute for M-CSF during osteoclast differentiation both in an *in vitro* culture assay and in osteopetrotic op/op mice, which lack functional M-CSF, but the effect of Flt3L on osteoclast differentiation was significantly lower than that of M-CSF [189]. DCs, which are dependent of Flt3 signaling for development, have the potential to transdifferentiate into functional osteoclasts when subjected to inflammatory conditions or under the influence of M-CSF [165-168]. The receptor for M-CSF (Csf-1R) is expressed on DC progenitors, however although the expression of Csf-1R is lost on CD8+ and CD103+ cDCs it is maintained on a population of CD11b+ cDCs [36]. This suggests that there might be differences in which subset of DCs that are susceptible for osteoclast transdifferenitation.

Structural damage and bone remodeling in rheumatoid arthritis

Bone erosions are a hallmark in the RA pathogenesis. Erosions normally occur rapidly after RA diagnosis and have been associated with prolonged and increasing inflammation. The increased destruction is supported by the presence of RANK-L and M-CSF in the synovial tissue, which together promotes osteoclast differentiation [159, 190, 191]. Osteoclast development and function is further potentiated by the increased production of the inflammatory cytokines TNF-α, IL-6 and IL-17 [173, 192-194]. In addition, the inflammatory environment found in the arthritic joint reduces the function of bone-building osteoblasts [195]. Together, the reduced function of osteoblasts and the increased formation of bone degrading osteoclasts lead to an increased risk of developing osteopenia and osteoporosis, a common feature among patients suffering from RA.

AIM

The evidence of an involvement of Flt3/Flt3L signaling in the pathogenesis of rheumatic diseases is accumulating. Also, Flt3 signaling regulates the development of many of the immune cells that have been postulated to pose key roles in RA. Therefore, the overall aim of this thesis was to further investigate how signaling through the Flt3 receptor may affect and modulate immune responses during experimental arthritis. The specific aims for each paper was as follows:

Paper I

Flt3L is a key cytokine in DC development, and is also found to be involved in Treg homeostasis. Due to the potential role of DCs and Tregs in RA pathogenesis, the aim of this paper was to investigate how Flt3L treatment affects formation of DCs and Tregs during development of experimental arthritis and how this affects the outcome of the disease.

Paper II

Flt3 signaling is involved in early development of B-cells while its role in peripheral B-cell responses is relatively unexplored. Due to recent findings that Flt3 is upregulated on activated B-cells, the aim of the second paper was to investigate the role of Flt3 signaling on peripheral B-cells activation and terminal differentiation.

Paper III

Flt3 signaling has been implied in osteoclast development and bone destruction. The aim of the third paper was to investigate how signaling through Flt3 might affect bone homeostasis during arthritis.

METHODS

In this section an overview of the main methods used in this thesis will be presented.

Animal mode of human rheumatoid arthritis

In all three papers included in this thesis we used an animal model of antigen-induced arthritis (AIA). Dumonde and Glynn first developed the AIA model in rabbits in the early 1960s[196]. Later, Ian R. Mackay and colleagues developed this model in mice in the late 1970s [197]. Classical antigens used have been ovalbumin, bovine serum albumin (BSA) and fibrin. An important factor in this model is to achieve antigen retention in the joint tissue, which can be accomplished by using cationic antigens in mice. Therefore methylated BSA (mBSA) is usually used in mouse models of AIA. The positive charge achieved after methylation of BSA, allows firm adherence of the antigen to the negatively charged ligaments and cartilage surfaces in the injected joint[198, 199]. This model is today a well-established arthritis model where morphological changes within the inflamed joint show great similarity to the human RA with presence of immune infiltration in synovial tissue, immune complex deposition in the cartilage and progressive destruction of cartilage and bone erosion[200]. The mBSA model also shows immunological similarity to human RA being highly dependent on CD4+ T-cells with subsequent activation of B-cells and antigen-specific antibody production[201, 202]. Although the role of B-cells and antibody production is not that well established in the mBSA model, the use of FcγR knockout mice have revealed that activation of these receptors is a crucial component of development of cartilage and bone erosions in this model[134, 187, 203]. One of the advantages of the mBSA model is that susceptibility to mBSA it not dependent on MHCII genetics, which is the case in the collagen induced arthritis model[200, 204].

In paper I, II and III, mice were immunized subcutaneously with 200 μg mBSA (mBSA) emulsified in Complete (C57Bl/6) or Incomplete Freund's (Balb/C) adjuvant on day 0. On day 7 mice were given a booster immunization with 100 μg mBSA emulsified in incomplete Freund's adjuvant (IFA). Mice were left until day 21 when arthritis was induced by an intraarticular injection of 30 μg of mBSA dissolved in PBS. Mice were sacrificed one week after arthritis induction and injected knee joints were taken for morphological evaluation. Experimental setup and representative pictures of arthritic knee joints can be seen in figure 8.

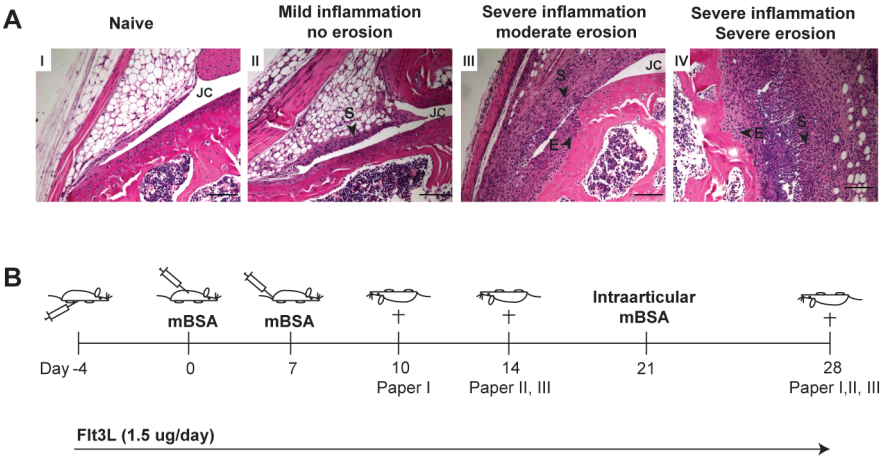


Figure 8. *Representative images of joint destruction and inflammation in mBSA arthritis and experimental layout.* **A)** Representative images for naïve an arthritic knee joints with varying severity. Synovitis was defined by the membrane thickness of more than two cell layers, and scored as follows: 1, mild; 2, moderate; and 3, severe synovitis. The presence of destructions was scored by severity as 1, mild; 2, moderate and 3, severe destruction. **B**) Experimental layout used in the three included papers. Mice were sacrificed at day 10 and day 28 in paper I and day 14 and day 28 in paper II and III. In treatment experiments, mice received daily intraperitoneal injections starting 4 days before first immunization with mBSA.

Flow cytometry (Paper I, II and III)

Flow cytometry is a powerful tool based on the detection of emitted light from fluorochromes that have been excited by lasers. These fluorochromes can for example be conjugated to antibodies or be coupled to compounds that bind intracellular structures, or incorporated into the cell membrane. Today this a common method used in most laboratories that can be used for assessing the phenotype of immune cell population, determining level of cytokines in serum and supernatants, studying intracellular signaling pathways or following proliferation of cells. Flow cytometry was one of the main methods used in all three papers included in this thesis. We used a FACS Canto II equipped with a HTS plate reader, three lasers (red, green and violet) and eight filters. A maximum of eight different fluorochromes were used in one panel. To address for spectral overlap between different fluorochromes, compensations were performed before each run. Single cell suspensions were prepared from isolated spleens, lymph nodes, bone marrow cells or *in vitro* stimulated cells. Prior to staining cells were blocked with FC-block (anti-CD16/CD32 antibody) to avoid Fc-mediated adherence of antibodies.

In paper II, flow cytometry was used for following *in vitro* proliferation of splenocytes and purified B-cells. Before stimulation cells were stained with CellTrace™ Violet, which is an intracellular dye that is diluted during proliferation of cells. Cells where harvested after 48 and 72h and stained with surface markers to identify proliferating cells. Due to the high degree of cells that die during *in vitro*

stimulation, cells were always stained with a viability marker to allow for the exclusion of dead cells before analysis.

In paper II, a flow cytometry-based assay was used to determine the level of phosphorylated Stat6 (pStat6) after IL-4 stimulation. Isolated cells where stimulated *in vitro* with LPS or LPS+IL-4 for 72h. Fixating solution was added directly to the stimulated well to avoid loss of phosphorylation. Cells were then permeabilized and stained for the surface markers B220 and CD4 and pStat6 (pY461). This protocol and antibodies were obtained from BD Bioscience.

Finally, flow cytometry was used to determine the level of cytokines produced in splenocyte cultures in paper I. This was done using a Th1/Th2 and Th17 cytometric bead array (CBA) kit purchased from BD Bioscience.

In vitro stimulations

[^{3}H]-Thymidine proliferation assay (Paper I, III)

For measurement of leukocyte proliferation in response to mBSA, LPS and anti-CD3 (αCD3) stimulation a [^{3}H]-thymidine incorporation assay was used. Splenocytes were seeded in a concentration of 1x10^6 cells/ml and stimulated with either mBSA (25µg/ml), LPS (10µg/ml), plate-bound αCD3 (1µg/ml) or medium (unstimulated). After 48h of stimulation, radiolabeled [^{3}H]-Thymidine was added to the cultures and cells were left overnight. Cells were then harvested and radioactivity was measured using a β-counter. [^{3}H]-Thymidine is incorporated into the DNA of proliferating cells and by measuring the radioactivity the proliferation can be assessed. When total splenocytes are stimulated, this culture do not allow determination of which leukocyte population that is proliferating.

In vitro plasma cell generation (Paper II)

Plasma cells were generated *in vitro* by stimulation of isolated splenocytes with LPS (10 µg/ml) for 3 and 5 days. Plasma cell generation was analyzed by flow cytometry, using surface markers B220 and CD138, and by qPCR, measuring the expression of Blimp1, IRF4 and XBP-1. This protocol was adopted from Klein, U *et al* [205]

Class switch recombination (Paper II)

CSR may be induced *in vitro* through stimulation of B-cells with an activating cytokines such as LPS. CSR to specific antibody isotypes may be achieved by using simulations stimulation with cytokines. To initiate CSR to IgG1 we used IL-4 (50 ng/ml) in combination with the B-cell activating mitogen LPS (10 µg/ml). This method was used to study CSR to IgG1 in isolated splenocytes from Flt3LKO and WT mice. To assess the relationship between Flt3 and IgG1 during CSR, we isolated WT B-cells and stained these with a CellTrace™ Violet marker. The isolated B-cells were stimulated for 48h and 72h with LPS+IL-4, after which the cells were analyzed using flow cytometry.

Enzyme-linked immunosorbent assay (Paper I, II & III)

Enzyme-linked immunosorbent assay (ELISA) is a powerful tool commonly used for detection of cytokines and antibodies in serum samples and supernatants. We used for ELISA for determination of serum and supernatant levels of immunoglobulins and Flt3L. Measurements of Flt3L were performed using a commercial kit available from RnD Systems. Measurements of total and antigen-specific antibodies were performed using an inhouse ELISA. For detection of mBSA antibodies plates were coated with mBSA (10ug/ml) and for detection of total Ig levels plates were coated with goat $F(ab')_2$ fragments to mouse Igs (5 ug/ml). Binding of Igs to antigen was detected using antibodies specific for IgM, IgG, IgG1, IgG2b, IgG2c and IgG3 conjugated to biotin followed by extravidin-HRP (Sigma) and TMB substrate. Plates were blocked using 2% skim milk to avoid cross reactivity between BSA and mBSA. For the mBSA ELISA, naïve serum was used as a negative control. Measurements were performed using serial dilutions of serum and supernatants. Absorbance was read at 450 nm. Antibody levels are presented wither as OD values of the dilution in the linear part of a reference dilution curve. Titer values are calculated as 50% of the max absorbance value of a reference dilution curve.

Gene expression analysis (Paper I, II and III)

For measurement of mRNA expression in stimulated cells and spleen samples we used quantitative polymerase chain reaction (qPCR), or real-rime PCR). In contrast to standard PCR, were only a PCR end product can be measured, qPCR allows one to follow the PCR reaction in "real-time" using fluorescents dyes. For detection we used the fluorescent dye SYBR Green that intercalates into double stranded DNA. After each run a melting curve was run to ensure specific amplification. RNA was isolated from stimulated cells and total spleen tissues using an Rneasy mRNA isolation, kit commercially available from Qiagen. Concentration and quality of RNA was evaluated with Nanodrop spectrophotometer and Experion (Bio-Rad). 100 – 400 ng or RNA was used for cDNA analysis. In house designed primers and probes were used for gene expression analysis. The results are expressed as the fold change compared to a relevant control and was calculated using the ddCt-method. GAPDH was used a reference gene except for measurement of germ-line transcripts when Igβ was used as a reference.

Bone mineral density and trabecular bone formation (Paper III)

Evaluation of bone mineral density (BMD) of cortical and trabecular bone and trabecular bone formation were performed using a micro-computed tomography (micro-CT) scan. Non-arthritic femurs were removed from WT and Flt3LKO mice at day 28. At day 14, one femur and the spine were removed from WT, Flt3LKO and Flt3LKO mice treated with Flt3L. Samples of spine and femurs were fixated in 4% formaldehyde for 48h and then transferred to 70% EtOH for long-term storage. Before micro-CT measurements, samples were transferred into PBS for 48 hours. Scanning was performed on a Skyscan1176 micro-CT with a pixel size of 9 μm.. The projection images were reconstructed into 3D images using NRECON software and CT-Analyzer. For measurements of BMD, micro-CT BMD calibration phantoms with known mineral densities were scanned together with the samples to allow for accurate BMD measurements. Trabecular bone was measured in the metaphysis of the femur and cortical bone was measured in the diaphysis area of the same femur. Trabecular bone of the spine was measured in four vertebraes in the thoracic curve of the spine.

RESULTS AND SUMMARY

In the following section I will summarize the major findings from each paper included in this thesis.

Paper I – *Fms-Like Tyrosine Kinase 3 Ligand Controls Formation of Regulatory T Cells in Autoimmune Arthritis*

Today, Flt3L is known as the primary differentiation factor for dendritic cells (DCs). Flt3L have also been found to support the expansion of Tregs, a mechanism that is indirectly mediated via DCs. Prior to this study, our group had shown that Flt3L accumulates in the synovial fluid of RA patients and that local knee exposure to Flt3L aggravated peptidoglycan-induced arthritis in mice. Also, we had shown that blocking Flt3 signaling using a tyrosine-blocking agent (Sunitinib) reduced the severity of mBSA-induced arthritis. In this study we explored the role of increased serum levels of Flt3L on the formation of DCs and Tregs during arthritis development. We could show that Flt3L treatment increase formation of both cDCs and Tregs, which leads to a reduced inflammatory and humoral response along with reduced bone destruction.

Results

Flt3L treatment increases the formation of immune regulatory cells during mBSA arthritis

To increase serum levels of Flt3L, mice were treated with recombinant Flt3L starting 4 days before first immunization with mBSA. As expected, mice treatment with Flt3L had increased the serum level of the ligand. To evaluate the effect on immune responses during arthritis, mice were sacrificed at day 10 and day 28. Increased Flt3L levels in treated mice were associated with the expansion of the two major DC populations, cDCs and pDCs, in both spleen and draining lymph nodes at both time points. Further examination of the three cDC subpopulations, revealed that Flt3L preferentially promoted development of the CD8+ cDCs over CD4-CD8- and CD4+ cDCs populations

In accordance with the increased frequency of DCs, we found that Flt3L treatment increased the splenic gene expression of Flt3. Interestingly, Flt3L also increased the expression of Blimp1, a transcription factor recently found to have an important function in development and tolerogenic function of DCs [61, 206]. The gene expression of Blimp1 was found to have high correlation to the gene expression of Flt3. As Flt3 is highly expressed on peripheral DCs, this correlation suggests that the increased Blimp1 expression was associated with the increased DC population.

Recent reports have suggested an important role for Flt3L in maintaining Treg homeostasis [95]. Accordingly, we found that Flt3L treated mice had increased frequency of Tregs in spleen and lymph nodes, an effect that was most prominent at day 10. Flt3L treatment also increased the splenic gene expression of FoxP3 and

FoxP3+ Tregs from Flt3L treated mice were found to have increased expression of FoxP3 protein.

Increased formation of Tregs is associated with reduced production of inflammatory cytokines, proliferative response and immunoglobulin production

The expansion of Tregs induced by Flt3L treatment could be associated with reduced spontaneous production of IL-6 and TNF in cultured splenocytes isolated at day 10. At day 28, the inhibitory effect on IL-6 was lost in Flt3L treated mice, whereas TNF levels was similar between the groups. Furthermore, Flt3L treatment was associated with reduced production of antigen-specific antibodies and a reduced proliferative response towards LPS and mBSA stimulation at day 28.

Plasmacytoid dendritic cells and Tregs are found in the synovial membrane of mice with mBSA arthritis

Tregs and pDCs have the ability to migrate to inflamed areas, whereas lymphoid resident cDCs reside in lymphoid organs. We could show that both FoxP3+ Tregs and pDCs were present in the inflamed synovia of arthritic mice. However, we found no difference in the number of FoxP3+ cells between Flt3L and PBS treated mice, Flt3L treated mice had an increased influx of pDCs in the inflamed knee joint. Since pDCs were found to express the co-inhibitory molecule PD-L1, important in immune tolerance, this suggests that pDCs and Tregs might cooperate to reduce the inflammatory response.

Dendritic cells cooperate with T-cells to predispose naïve mice for arthritis

To investigate if DCs are potent antigen-presenting cells during mBSA arthritis we transferred enriched DCs and purified T-cells from mBSA-immunized mice into naïve recipients. Eleven days after transfer, arthritis was induced in recipient mice. We found that only when antigen-primed DCs and T-cells were co-transferred to naïve recipient, was a humoral response against mBSA induced. Furthermore, only mice that were transferred with both antigen-primed DCs and T-cells developed synovial inflammation after injection with mBSA. This indicates that splenic DCs are potent antigen-presenting cells in the model of mBSA arthritis.

Reduced severity of mBSA arthritis in mice treated with Flt3L

Finally, to evaluate if prolonged exposure to elevated serum levels of Flt3L would affect arthritis development morphological evaluation of arthritic joints were performed. Mice treated with Flt3L were found develop a less severe arthritis, with reduced signs of bone destructions, compared to PBS treated controls. No differences were seen in synovial inflammation.

Summary of Paper 1

In paper I we show that Flt3L has potent immunoregulatory properties during development of mBSA arthritis (Figure 9). The increased serum levels of Flt3L led to an increased frequency of cDCs, preferentially CD8+ cDCs, and pDCs in peripheral lymphoid organs. This was associated with an expansion of Tregs with the ability to suppress antigen induced proliferation and the production of antigen-specific antibodies. Tregs could also contribute to the suppressed production of the inflammatory cytokines IL-6 and TNF. pDCs and Tregs were found in the inflamed synovial tissue where they potentially contribute to reducing joint destruction. The results from this first paper indicate a protective role for Flt3L in development of arthritis.

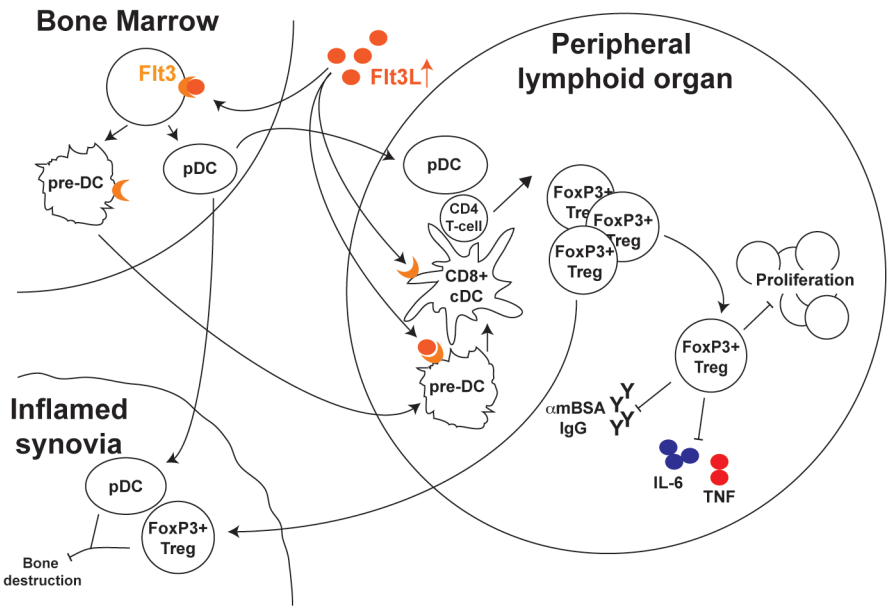


Figure 9. *Proposed model of action of Flt3L during mBSA induced arthritis.* Increased levels of Flt3L leads to an expansion of cDC, preferentially CD8+, and pDC, both through actions in the bone marrow and directly on progenitor cells in the periphery. This is further associated with an expansion of Tregs with the ability to suppress antigen induced proliferation, production of antigen-specific antibodies and production of inflammatory cytokines. Finally, pDCs and Tregs migrate to the inflamed synovial tissue were they can help to suppress bone destruction.

Paper II - *Germinal center B cells require Flt3-mediated activation of Stat6 for IgG1 class switch recombination*

B-cells are important participants in the pathogenesis of RA. Through the production of autoantibodies, by production of inflammatory cytokines and by functioning as antigen-presenting cells, B-cells contribute to the inflammatory environment found in the joints of RA patients. It has long been known that Flt3 plays an important role in early B-cell development, but a function in late antigen-dependent B-cell responses remains unexplored. In paper II we characterized the $Flt3^+$ B-cell population *in vivo* and *in vitro* aiming to explore the function of Flt3 signaling through this receptor.

Results

Phenotypic characterization of $Flt3^+$ B-cells

We could show that Flt3 is re-expressed on peripheral B-cells after *in vitro* activation with LPS and *in vivo* in the spleen and lymph nodes of antigen challenged mice. Further analysis revealed that Flt3 is expressed during proliferation of activated B-cells. Expression of Flt3 on B-cells was associated with increased levels of co-stimulatory CD80, CD86, CD40 and elevated levels of MHCII. Together, these results indicated that Flt3 is upregulated during proliferation and activation of B-cells and may have an important function in late B-cell responses.

Impaired Flt3 signaling in activated B-cells results in enhanced plasma cell differentiation and IgM production

Activation of B-cells is an essential prerequisite for terminal differentiation into antibody-secreting plasma cells and we therefore explored if signaling through Flt3 might be involved in this process. First we identified that Flt3 is expressed on activated B-cells before differentiation into plasma cells, supported by the fact that Flt3 and CD138 was not co-expressed on the surface of lymphocytes. Using mice lacking production of Flt3L (Flt3LKO) we could show that plasma cell differentiation was enhanced in the absence of functional Flt3 signaling. This was associated with an increased accumulation of IgM producing plasma cells in both spleen and bone marrow of Flt3LKO mice, and increased splenic gene expression of IRF4, Blimp1 and XBP-1. Whereas levels of IgM were increased in Flt3LKO mice IgG levels remained similar to WT controls, suggesting that the increased population of plasma cells was mainly IgM producing.

Mice deficient for Flt3L have a severely impaired IgG1 production.

The increased IgM production seen in Flt3LKO mice caused us to question if this could be due to an IgG subclass deficiency. We could show that Flt3LKO mice had significantly reduced serum levels of both total and antigen-specific IgG1. This reduction was found to be specific for IgG1 since other IgG subclasses (IgG2b, IgG2c, IgG3) were found at normal or even increased levels in the serum of Flt3LKO mice.

In combination with reduced numbers of IgG1 expressing splenic B-cells this suggests that Flt3LKO mice have impaired antibody CSR specifically for IgG1.

Flt3 is expressed on B-cells during the germinal center reaction.

CSR to IgG1 mainly occurs in GCs during T-cell dependent responses. We found normal formation of splenic and nodal GC and normal induction of T_{FH} cells and IL-21 production in Flt3LKO mice. This suggested that a defect in GC formation could not explain the reduced IgG1 production seen in Flt3LKO mice. Due to the activated phenotype of GC B-cells we explored if Flt3 would be expressed on these cells. Accordingly, we found that Flt3 expression on a subpopulation of GC B-cells in both spleen and lymph nodes. Flt3 expression on splenic GC B-cells were only in Flt3LKO mice, and consequently GC B-cells from these mice showed a more activated phenotype when compared to WT GC B-cells. Since CSR to IgG1 is initiated in GCs we hypothesized that Flt3 may be involved in the CSR process to IgG1.

Impaired IL-4 induced activation of Stat6 in Flt3LKO B-cells results in a reduced ability to initiate CSR to IgG1

To explore the relationship between Flt3 and IgG1 during CSR we stimulated WT B-cells *in vitro* with LPS+IL-4. We found that IgG1 was expressed on the surface of $Flt3^+$ B-cells. Importantly, expression of Flt3 preceded IgG1 expression, suggesting that Flt3 signaling can be involved in the initiation of CSR to IgG1.

Accordingly, analysis of the initiating GLT in the spleen of Flt3LKO and WT mice revealed a significantly reduction in mRNA expression of GLT for γ1 in Flt3LKO mice. This defect in initiation of CSR was also found after *in vitro* stimulation with LPS+IL-4, indicating that B-cells from Flt3LKO mice have a reduced ability to initiate CSR to IgG1.

This observation led us to question if this could be due to a defect in IL-4 signaling, known to be important in CSR to IgG1. Flt3LKO did not show any differences in mRNA expression for IL-4 or the IL-4R, neither *in vivo* nor *in vitro* after stimulation. However, the IL-4R was found to be co-expressed with Flt3 on *in vitro* activated B-cells, suggesting a possible synergy between Flt3 and IL-4R signaling. In line with this hypothesis, Flt3LKO B-cells were found to have a significantly reduced ability to initiate activation of Stat6 after IL-4 stimulation. Flt3L was produced during *in vitro* activation of WT B-cells with LPS+IL-4, further supporting a possible link between IL-4 and Flt3L signaling.

This implied that the reduced ability for Flt3LKO B-cell to initiate IgG1 production is associated with a reduced ability activate Stat6 and subsequent transcription of GLT for γ1.

Summary of Paper II

In paper II we describe a novel and previously unacknowledged role for Flt3 signaling in peripheral B-cell responses. We could show that Flt3 was re-expressed on mature B-cells during LPS activation *in vitro* and after antigen activation *in vivo*. Expression of Flt3 defined an activated B-cell population with increased co-stimulatory and antigen-presenting properties. Absence of functional Flt3 signaling in mature B-cells caused an increased accumulation of IgM-producing plasma cells in both spleen and bone marrow. The increased production of IgM could be associated with severely reduced levels of IgG1. This was found to be due to a reduced ability for B-cells of Flt3LKO mice to initiate CSR to IgG1, as evident by the low levels of GLT for γ1. Flt3LKO B-cells were found to have reduced ability to induce activation of Stat6 after IL-4 stimulation, a possible explanation for the deficiency in IgG1 production.

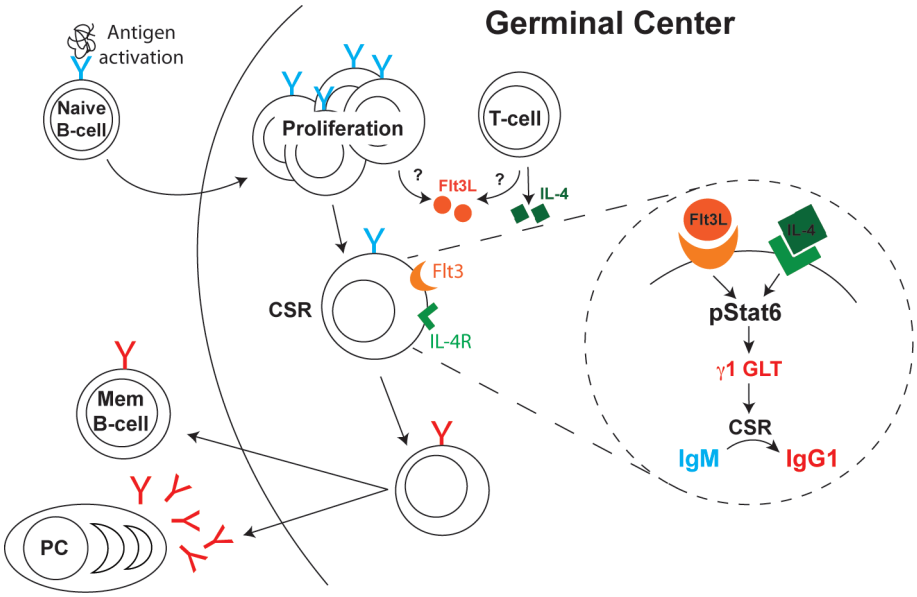


Figure 10. *Proposed role for Flt3 during class switch recombination to IgG1.* Antigen activation of B-cells induces germinal center formation. Inside the GC, B-cells up-regulate Flt3 during proliferation and undergo CSR with the help from T-cells. During initiation of CSR for IgG1, Flt3L and IL-4 needs to synergize for proper activation of Stat6. Activated Stat6 induce transcription of GLT for γ1 and thereby CSR to IgG1. IL-4 is produced by T-cells in the GC, whereas Flt3L may be produced by either proliferating B-cells or T-cells. After CSR to IgG1, B-cells may differentiate into memory B-cells or antibody secreting plasma cells.

Paper III - *Impaired signaling through the Fms-Like tyrosine kinase 3 receptor results in increased osteoclast formation and joint destruction during experimental arthritis*

In RA, the chronic inflammation in synovial joints is associated with accumulation of osteoclasts, which leads severe bone erosions, a hallmark in RA pathogenesis. Flt3L has been suggested to have the ability to substitute for M-CSF in osteoclast differentiation. Also, DCs have the potential to transdifferentiate into osteoclasts when subjected to inflammatory environments. In paper III, we explored the role of Flt3 signaling in bone metabolism during arthritis development and present experimental evidence that Flt3L acts as a negative regulator of osteoclast development during mBSA arthritis.

Results

Increased bone destruction in Flt3L deficient mice is associated with increased formation of osteoclasts

To evaluate the role of Flt3L on bone homeostasis, arthritis was induced in Flt3LKO and WT mice using mBSA-immunization. Morphological evaluation of arthritic joints revealed a tendency of increased joint destruction in Flt3LKO mice, whereas the synovial inflammation was similar. This increased bone destruction was associated with an increased accumulation of osteoclasts (defined as cathepsin K+ cells) and osteoclast precursors (RANK+ cells) in the periarticular area of the femur and tibia in arthritic knee joints. This accumulation was specific for the periarticular area of the joint, indicating that differences in the inflamed joint environments between Flt3LKO and WT mice might cause differences in osteoclastogenesis.

Reduction in Tregs cells in Flt3LKO mice cause loss of control over T-cell proliferation and a reduced Treg/Th17 ratio.

Treg cells regulate osteoclast differentiation in animal models of RA. Also, Treg homeostasis is dependent on Flt3L. In accordance with previous reports, we found a reduction of Tregs in the spleen of Flt3LKO mice. A reduction of Tregs was also seen in the bone marrow of these mice, in agreement with the important function of Flt3L in Treg homeostasis. Furthermore, Tregs from Flt3LKO mice had reduced expression of FoxP3 protein when compared to WT Tregs. A reduction in Treg function was shown by reduced ability to inhibit αCD3 induced T-cell proliferation and increased frequencies of activated CD4+ T-cells.

Th17 cells promote osteoclastogenesis and are thought to have a central role driving joint damage in RA. We found that Th17 cells were significantly increased in the spleen during mBSA arthritis when compared to naïve mice, in accordance to their important role in this model. The frequency of Th17 cells was similar between WT and Flt3LKO mice. This was somewhat surprising since Th17 and Treg are thought to arise from the same precursor and Tregs show high dependence on Flt3L signaling. Due to this fact, the Treg/Th17 balance was significantly reduced in Flt3LKO, which might support the increased osteoclastogenesis.

Skewed antibody production may contribute to increased osteoclastogenesis in Flt3LKO mice.

Antibody mediated activation of FcγRIV, specific for IgG2b and IgG2c, can potentiate osteoclast differentiation. We found a skewed antibody response against mBSA in Flt3LKO mice towards more potent IgG2b and IgG2c subclasses. This also reflected the presence immunoglobulins in the synovial tissue of arthritic joints. Flt3LKO mice had low, if any staining for IgG1, whereas staining for IgG2b was similar to WT controls. This skewed antibody response towards mBSA can contribute to the increased osteoclastogenesis and bone degradation seen in these mice.

Flt3L can act directly on osteoclast precursors to regulate osteoclast differentiation

Since previous reports have described that Flt3L might support osteoclast differentiation, we sought to investigate if Flt3 is expressed on osteoclast precursors. Indeed, Flt3 was expressed on a population of *in vitro* derived osteoclast progenitor cells, indicating that Flt3L can impact osteoclast differentiation directly.

We therefore analyzed if genes involved in osteoclast differentiation and function may be changed in the bone marrow of Flt3LKO mice. Flt3L did not affect the transcription of RANK, CD61, cathepsin-K or RANK-L. On the other hand, Flt3L positively regulated transcription of IRF8, a repressor of osteoclast differentiation. Furthermore, Flt3L treatment of Flt3LKO mice reduced the expression of M-CSF, an important cytokine in osteoclast generation. These results indicate that Flt3L might act as a negative regulator of osteoclast development.

To test this hypothesis, WT mice were treated with Flt3L starting 4 days before first immunization and continuing throughout the experiment. Histomorphometric analysis of trabecular bone showed reduced osteoclast numbers and osteoclast surface in the femur of Flt3L treated mice compared to PBS controls. We observed no effect of Flt3L treatment on osteoblast formation. This supports the theory that Flt3L can act directly on osteoclast progenitors to inhibit osteoclast differentiation.

Flt3L regulates trabecular bone formation during arthritis

In accordance with the proposed role for Flt3L as a negative regulator of osteoclast formation, the BMD of femoral trabecular bone was significantly reduced both at day 14 and day 28 in Flt3LKO mice, suggesting increased osteoclast activity. This was supported by a reduction in trabecular thickness, trabecular number and bone volume in Flt3LKO mice. Trabecular bone formation was restored in Flt3LKO mice after 18 days of Flt3L supplementation during mBSA arthritis. Furthermore, Flt3L treatment significantly increased the formation of trabecular bone in the vertebrae of Flt3LKO mice when compared to WT and non-treated Flt3LKO mice, which presents further support of a positive function for Flt3L on trabecular bone formation.

Summary paper III

In paper III we provide evidence for a direct role for Flt3L in bone metabolism. During mBSA arthritis the lack of Flt3L results in an increased accumulation of osteoclasts in the periarticular area of the arthritic joint which is associated with increased bone destruction. Furthermore, Flt3L deficiency results in reduced formation of femoral trabecular bone that is restored by Flt3L treatment. Restoration of BMD was associated with increased expression of IRF8, a transcriptional repressor of osteoclast differentiation. Accordingly, long-term Flt3L treatment resulted in a reduction in osteoclast formation. Furthermore, Flt3L could act indirectly on osteoclast differentiation by regulating homeostasis of Tregs, which reduce osteoclast differentiation and inflammatory responses.

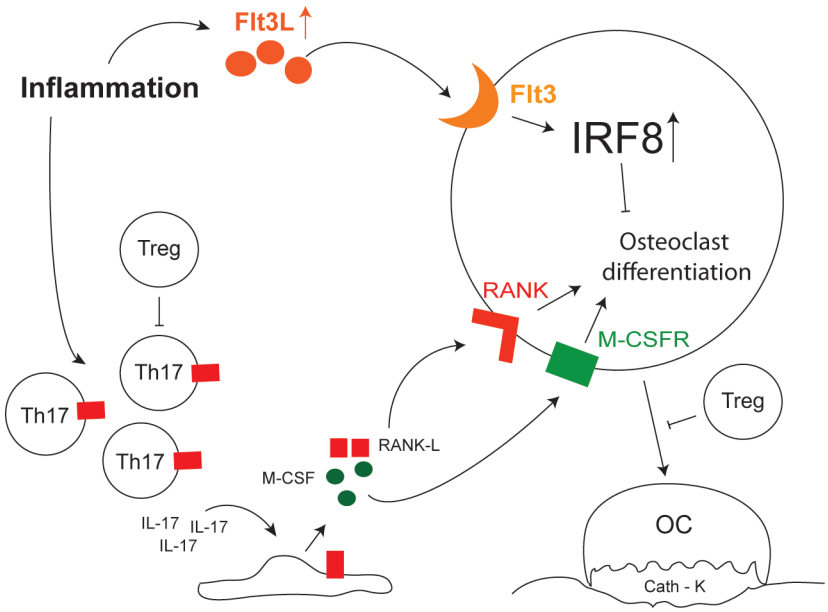


Figure 11. *Proposed model of the role of Flt3L in bone metabolism during arthritic inflammation.* Inflammatory responses lead to an expansion of Th17 cell that produce IL-17 that induce M-CSF and RANK-L expression from synovial cell. Infiltrating osteoclast precursors respond to M-CSF and RANK-L and induce differentiation into osteoclasts (OC). Flt3L induced by the inflammatory response may affect OC differentiation in two ways: directly by inducing IRF8 expression in osteoclast precursors that blocks OC differentiation or indirectly through induction of Treg cells which inhibit inflammatory responses and osteoclast differentiation

DISCUSSION

Rheumatoid arthritis (RA) is a degenerative autoimmune disease. RA is a result of a break of tolerance towards self-antigens, which cause the immune system to react towards it self. The recent years advance in treatment of RA, such as cytokine agonists and T-cell regulating and B-cell depleting therapies have significantly improved the outcome for patients diagnose with the disease. However, the pathogenesis of the disease remains largely unknown.

Flt3 is a tyrosine kinase receptor that is primarily expressed on hematopoietic stem cells and lymphoid progenitors in the bone marrow. Signaling through Flt3 has been proposed to play a role in the pathogenesis of RA since the ligand (Flt3L) is increased in both blood and synovial fluid of RA patients. Also, Flt3L has recently been listed in a panel of pre-clinical markers with predictive value for future development of RA.

The main focus of this thesis was to investigate how Flt3 may modulate immune responses and how different immune cell populations are affect by the signaling during experimental arthritis.

A regulatory role for Flt3 derived DCs during development of arthritis.

The potent antigen-presenting function of DCs has led to the belief that these cells have a central role in the pathogenesis of RA. Accordingly, DCs are found in the synovial membrane and fluid of RA patients, and inhibition of DC development, by blocking of Flt3 signaling, has been shown to reduce the severity of experimental arthritis. Also, intraarticular administration of Flt3L aggravates peptidoglycan-induced arthritis in mice[147, 155]. This suggests that Flt3-driven DCs could be of significant importance in the pathogenesis of arthritis. The presence of Flt3L and $Flt3^+$ cells in the synovial fluid and tissue of RA patients further support a role for Flt3 derived DCs in the pathogenesis of the disease[21, 155]. On the other hand, results provided in this thesis indicate that Flt3 derived DCs can have a regulatory role during arthritis development. Expansion of DCs using Flt3L (paper I) was associated with a reduction in the severity of arthritis. Furthermore, when arthritis was induced in mice lacking production of Flt3L (paper III), and thereby have severely reduced numbers of Flt3 derived DCs, the severity of arthritis was increased. A protective role for Flt3 driven DCs in inflammatory conditions is supported by recent findings in animal models of atherosclerosis. In these studies, Flt3 dependent DCs are found to be atherosclerosis protective [207, 208]. These results could be of vital importance also in RA patients, since arthritis patients have a significantly increased risk of developing cardiovascular diseases such as atherosclerosis[209].

Does treatment with Flt3L induce a more tolerogenic DC profile during arthritis?

Our results on Flt3 driven DCs in RA and the results found in arteriosclerosis models suggest that the DCs induced by Flt3L might have a more tolerogenic function.

Previous reports have shown that Flt3L preferentially induce expansion of CD8+ cDCs and pDCs[44, 48]. This is in line with our findings presented in paper I where Flt3L induced an almost selective expansion of CD8+ cDCs and pDCs in lymphoid organs. Both CD8+ cDC and pDCs has previously been found to improve the outcome of experimental arthritis[210, 211]. Also, depletion of pDCs has been shown to aggravate autoimmune arthritis in mice. These DC populations have also been found to improve the outcome of allograft rejection and autoimmunity [212, 213]. This suggests that Flt3L might expand DC populations with a more tolerogenic phenotype. However, pDCs have been suggested to play a central role in the pathogenesis of the rheumatic disease systemic lupus erythematosus (SLE) and psoriasis[56]. Also, CD8+ cDCs have the potential to activate CD8+ T-cells during diabetes and promote destruction of β-cells, indicating that pDCs and CD8+ cDCs might also have roles driving autoimmunity. Could Flt3L treatment alter the phenotype of DCs? Flt3 is expressed on cDCs in peripheral lymphoid organs indicating that DCs have the potential to respond to Flt3L in the periphery. Expression of Blimp-1 in peripheral DCs has recently been shown to be important for retaining a tolerogenic function of these cells. Specific deletion of Blimp1 in DCs results in an autoimmune SLE-like syndrome with production of autoantibodies and increased production of IL-6[61]. Furthermore, deletion of Blimp-1 in hematopoietic stem cells revealed that this transcription factor is a positive regulatory for differentiation of CD8+ cDCs, indicating that Flt3L might be involved in the induction of Blimp1 in DCs[206]. Accordingly, we found increased expression of Blimp1 in Flt3L-treated mice in paper I, which corresponded to increased formation of CD8+ cDCs. Although, we could not show that the Blimp1 expression was specifically upregulated in this DC population, there was a high correlation between the mRNA expression of Blimp1 and Flt3 suggesting that it was an effect of Flt3L treatment and DC expansion. Blimp1 has an important role in the effector function of both T- and B-cells, where its expression is regulated by the transcription factor Stat3[60]. Stat3 has an indispensible role during Flt3L induced development of DCs, in which Flt3L induce phosphorylation and activation of Stat3 [29, 30]. Specific loss of Stat3 in peripheral DCs cause hyperactivation and increased production of inflammatory cytokines, and autoimmunity in mice [57]. This suggests that Flt3L can induce activation of Stat3 in peripheral DCs, which in turn induce transcription of Blimp-1 and a tolerogenic DC phenotype. Polymorphisms in the Blimp1 gene in patients with RA and SLE have recently been reported, indicating that a malfunction could exist in human patients[63, 64]. Accordingly, DCs isolated from human Blimp1 SLE-risk allele carriers showed the same inflammatory phenotype as Blimp1 deficient DCs isolated from mice[62].

Flt3L regulates formation of Treg cells during development of arthritis.

In addition to its role in DC development, Flt3L has recently been found to have an important function in maintaining Treg homeostasis [94, 95]. This function is suggested to be mediated through the expansion of DCs induced by Flt3L. In line with this, we could show in paper I that Flt3L treatment during arthritis development increases the formation of Tregs. On the contrary, we found a significant reduction of Tregs cells in Flt3LKO mice in paper III. A difference was not only found in Treg frequency but also in Treg phenotype, Tregs isolated from Flt3LKO mice had significantly reduced expression of FoxP3 protein, whereas Tregs from Flt3L treated mice had increased expression of FoxP3 protein. The level of FoxP3 protein has been shown to correlate with the suppressive activity of Tregs [83, 84]. In line with this, we found a reduced suppressive capacity on T-cell proliferation in Flt3LKO mice whereas Flt3L treated mice had increased suppressive capacity on antigen-specific and B-cell proliferation. A protective role of Tregs in arthritis has been proposed using various animal models of RA[89, 178]. However, even though Tregs are present in the synovial fluid and tissue of RA patients, these cells show severe impairment in inhibitory function[90, 91]. This was recently shown to be due to a reduced function of the FoxP3 protein in these cells, induced by the inflammatory environment[92]. Our results indicate that Flt3L induced DCs may be of significant importance in maintaining Treg numbers and function during inflammatory conditions. Accordingly, DCs have been shown to sustain FoxP3 expression of Treg cells and both CD8+ cDCs and pDCs have the potential to induce formation of Tregs [48, 212, 214]

The role of dendritic cells in priming humoral responses during mBSA arthritis

DCs activate naïve T-cells that can differentiate into T_{FH} cells, which in turn will assist B-cells during the germinal center reaction to differentiation into antibody-secreting plasma cells [215]. We could show that transfer of antigen-primed DCs and antigen primed T-cells could initiate antigen-specific antibody responses in naïve recipients (paper I). This is in line with previous results from our group, where inhibition of DC development was associated with reduced production of antigen-specific IgG suggesting that DCs are involved in the initiation of antibody production during mBSA arthritis[147]. However, in paper II we could show that Flt3LKO mice induce formation of germinal centers and antigen-specific antibody production to the same extent as WT mice, despite a severe reduction in DCs. This suggests that antigen presentation and activation of T-cells is still functional despite absence of Flt3L, indicating that Flt3L induced DCs are indispensible for the production of antibodies directed against mBSA. This is in line with previous results that show that Flt3LKO mice have no defects in T-cell priming and respond normally to T-dependent immunizations [35, 216].

Flt3 as a new marker for activated B-cells found in the germinal centers of antigen-challenged mice

Flt3 signaling has an important role in early B-cell development, but the expression of the receptor needs to be downregulated for B-cell lineage commitment. This is achieved by repression of Flt3 transcription by the major B-cell commitment factor Pax5 [108]. During *in vitro* activation of peripheral B-cells, Flt3 is re-expressed due to reduced expression of Pax5 [129, 131]. In paper II we could show that Flt3 is re-expressed on peripheral B-cells after both *in vitro* activation with LPS and antigen-induced activation *in vivo*. Further analysis revealed that Flt3 was upregulated during B-cell proliferation and that B-cells expressing Flt3 have a more activated phenotype when compared to B-cells not expressing the Flt3 receptor. This suggest that B-cells expressing Flt3 represents an activated and proliferating B-cell population. In accordance with their proliferative and activated phenotype, we found expression of Flt3 on a population of germinal center (GC) B-cells in both lymph nodes and spleen of immunized mice.

Flt3 expressing B-cells has recently been shown to be present in the blood of healthy individuals and the frequency of these cells is increased in patients with primary Sjögren's syndrome (pSS). The increased population of Flt3 expressing B-cells in pSS patients correlate with an increased serum level of Flt3L, and Flt3L support proliferation and survival of anti-IgM stimulated B-cells. This support the fact that Flt3 is upregulated during proliferation and that Flt3 expression on B-cells is increased during immune activation [22].

Flt3 signaling is involved in the class switch recombination to IgG1

The upregulation of Flt3 on activated B-cells has previously been associated with reduced Pax5 expression and initiation of plasma cell differentiation, as evident by the finding that a subpopulation of $Flt3^+$ B-cells expresses Blimp1 [131]. In line with this, we could show that Flt3 is not co-expressed with CD138 on peripheral lymphocytes indicating that Flt3 is expressed on an activated B-cell population prior to plasma cell differentiation (paper II). We used Flt3L-deficient (Flt3LKO) mice to explore the function of Flt3 signaling of activated B-cells. Flt3LKO mice were found to have an increased splenic population of $Flt3^+$ B-cells. Furthermore, we could show that plasma cell generation was accelerated in the absence of functional Flt3 signaling. This was associated with an increased IgM production although normal production of IgG. Increased level of IgM is seen in patients suffering from CSR deficiencies [217], proposing that Flt3LKO mice could have an IgG subclass deficiency. Although Flt3LKO mice showed normal levels of total IgG, these mice had a specific reduction in both total and antigen-specific serum levels of IgG1. This low production of IgG1 in Flt3LKO mice was found to originate from an impaired initiation of CSR to IgG1, as evident by low germ-line transcript mRNA for γ1. Neither a lack of germinal center (GC) formation nor impaired IL-4 production could explain the reduced levels of IgG1, suggesting that maybe Flt3 is involved in the CSR process. The identification that Flt3 is expressed on a population of GC B-cells and that Flt3 expression precedes IgG1 expression during *in vitro* CSR further supported the idea that Flt3 might be involved in the initiation of CSR to IgG1. Flt3

have previously been described to induce the expression of the IL-7R and synergies with IL-7 signaling in B-cell progenitors [107, 218]. Since the IL-4R and the IL-7R belongs to the same receptor family [125], Flt3 might also control expression of the IL-4R; however this was not the case. Instead we could provide evidence for a functional synergy between the IL-4 receptor and Flt3 on activated B-cells. B-cells from Flt3L-deficient mice had significantly reduced ability to induce activation of Stat6 after IL-4 stimulation, despite normal expression of the IL-4R, implying a possible explanation for the reduced ability to initiate CSR to IgG1. This proposes a new and previously unacknowledged function for Flt3L in peripheral B-cell responses, and suggests a vital role for Flt3L in CSR to IgG1. Although the function of Flt3 on peripheral B-cells is rather unexplored, studies in pSS patients have shown that Flt3L promotes survival and potentiates anti-IgM induced proliferation of mature B-cells [22]. CSR to IgG1 is proliferation dependent and IL-4 is known to induce proliferation of activated B-cells[125]. Flt3L might act in synergy with IL-4 to promote proliferation and CSR during the GC reaction *in vivo*. GC B-cells are also prone to apoptosis due to the high degree of DNA damage during CSR and SHM [121], indicating that Flt3L might regulate the survival of GC B-cells during IgG1 CSR. However, if this were the case, levels of germ-line transcript mRNA for γ1 would probably be similar since this process occurs before DNA damage.

Flt3L acts as a regulator of bone homeostasis during arthritis through multiple pathways.

Bone erosions are a hallmark of RA pathogenesis and normally occur shortly after diagnosis. During RA, bone-resorbing osteoclasts accumulate in the inflamed joints of patients and contribute to the severe bone destruction[157]. Flt3L have been suggested to support osteoclast differentiation and Flt3L accumulation in the synovial fluid or RA patients [155, 189]. Also, $Flt3^+$ cells can be found in the synovial membrane of RA patients [21]. Together these results suggest that Flt3L might contribute to the bone destruction seen in the joints of RA patients. However, in this thesis we provide evidence indicating that Flt3L has a bone-protective effect during arthritis. Mice deficient in Flt3L (Flt3LKO) have previously been suggested to lack defects in bone formation [164], although during arthritis we could show that deficiency of Flt3L caused significant loss of trabecular bone. Supplementation of Flt3L to Flt3LKO mice restored trabecular bone formation, suggesting that Flt3L have a positive effect on bone homeostasis. Accordingly, Flt3L treatment was found to suppress osteoclast development during mBSA arthritis, whereas absence of Flt3L caused an accumulation of osteoclasts in arthritic joints. The function of Flt3L on osteoclasts development was found to be associated with a positive effect on the transcription factor IRF8, which has recently emerged as a negative regulator of osteoclast development [219]. The factor that induces IRF8 expression in osteoclast precursors has not yet been identified, although IFN-γ has been suggested as one[220]. IRF8 deficient mice suffer from osteoporosis due to increased osteoclast activity and interestingly, Flt3LKO and IRF8 deficient mice show similar bone phenotype. Together with the finding presented in paper III that Flt3 is expressed on a subpopulation of osteoclast precursors, this suggests that Flt3L might be an important factor in inducing IRF8 expression in osteoclast precursors. Flt3L induces

IRF8 transcription during DCs development and IRF8 is critical for in the development of pDCs and CD8+ cDCs, both of which are highly dependent on Flt3L [221-223]. These results suggest that Flt3L has the ability to suppress osteoclast differentiation directly, which may be of significant importance in bone destructive diseases such as RA.

Tregs are important in the regulation of bone homeostasis and block osteoclast development during arthritis [176, 177, 224]. Since, Flt3L is reported to have an important role in Treg homeostasis this implies that the regulatory function seen by Flt3L on bone homeostasis in papers I and III could be an indirect effect mediated through Tregs. Accordingly, Flt3L induced Tregs (paper I) were found to be associated with reduced production of TNF and IL-6; both key cytokines in osteoclastogenesis and in joint destruction during mBSA-induced arthritis [202, 225-227]. Th17 cells are now considered as the osteoclastogenic T-cell population and have been postulated to have a central role in the RA pathogenesis. As shown in paper III, deficiency of Flt3L alters the balance between Th17 and Tregs cells during mBSA arthritis, which may be contributing to the increased bone loss seen in Flt3L-deficient mice since Th17 promote joint inflammation and destruction in mBSA arthritis [228, 229].

The role of antibodies in osteoclast differentiation and function has recently been highlighted in a study by Seeling *et al* [186]. Activation of FcγRIV, expressed on inflammatory monocytes, was found to potentiate osteoclast differentiation. Furthermore, expression of FcγRIV was found to be sufficient to drive joint destruction in a model of autoantibody-induced arthritis[188]. FcγRIV has been reported to be specific for IgG2a/c and IgG2b and to only be expressed on neutrophils and monocytes/macrophages. In paper II and III we showed a skewed antibody response against mBSA in Flt3LKO mice towards IgG2 subclasses. This also reflected the presence of immunoglobulins in the synovial membrane of Flt3LKO and WT mice. This skewing in antibody response could be an explanation to the increased bone destruction seen in Flt3LKO mice. Accordingly, FcγR have been reported to be important in joint destruction during mBSA arthritis, although do not influence synovitis[134, 187, 203], which is similar to what is seen in Flt3LKO mice. In line with this, the reduced signs of joint destruction seen in paper I was associated with a reduced production of anti-mBSA total IgG antibodies. The role of autoantibodies in osteoclast differentiation may be of significant importance in RA, since patients that are positive for ACPA autoantibodies usually have worse prognosis compared to ACPA negative patients. It was recent identified that 25% of IgG expressing B-cells in the synovial tissue of RA patients are specific for citrullinated proteins and the presence of autoantibody producing B-cells in the synovial joint might therefore contribute to an increased formation of osteoclast and increased bone destruction [152].

Does the inflammatory environment in the synovial membrane of rheumatoid arthritis patients inhibit the Flt3 receptor?

RA is a highly complex disease, involving most cells of the immune system. As shown in the three papers included in this thesis, Flt3L may have protective functions during arthritis development by reducing formation of osteoclast, affecting effector maturation of antibodies as well as reducing inflammation and reestablishing self-tolerance through induction of antigen-specific regulatory T-cells. But Flt3L and $Flt3^+$ cells are already present in the synovium and blood of RA patients, so why is this beneficial function not seen in humans?

Increased levels of Flt3L are observed in mice deficient for Flt3 or after inhibition of the Flt3 receptor[95, 147]. Furthermore, increased levels of Flt3L have been shown in mice after depletion of Treg cells[230]. Humans suffering from a syndrome that is associated with total absence of blood DCs and pDCs, have severely reduced numbers of Tregs as well as highly elevated levels of Flt3L[19]. If these findings are compared to the situation seen in RA patients, this might suggest that the increased levels of Flt3L is a response to deficiencies in Flt3 derived DCs or functional Tregs, which may be a result of a non-functional Flt3 signaling. However, genetic studies have never associated RA with a non-functional or mutated Flt3 receptor. On the other hand, the cytokines TNF, TGF-β, and IL-2, which are highly expressed in the synovial tissue of RA patients, have been shown to inhibit signaling through the Flt3 receptor [23, 231]. Therefore, the cytokine environment in the RA joint can potentially cause an inhibition of Flt3 receptor signaling, which will results in a reduction of Flt3-driven DCs and functional Tregs. Accordingly, successful treatment of RA patients is associated with an increase in blood pDCs that have the potential to induce IL-10 producing Tregs [148].

Is inhibition or stimulation of Flt3 a potential treatment for rheumatoid arthritis? – My thoughts

Reducing inflammation, bone destruction and reestablishment of self-tolerance are the main goals for treatment of RA. Using Flt3L as a therapeutic drug may therefore seem promising due to the beneficial functions presented in this thesis. Treatment of other autoimmune diseases such as diabetes, graft versus host disease and colitis with Flt3L has shown promising results in animal models[94, 95, 232]. Expansion of endogenous dendritic cells presenting circulating self-antigens using Flt3L, would potentially induce regulatory T-cells specific for the RA-eliciting antigen, and promote reestablishment of self-recognition. Furthermore, the beneficial effect seen for Flt3L on bone formation further supports that this could be a promising treatment. However, one should also consider the possible side effects. As described above, inflammatory cytokines inhibit Flt3 signaling, which might explain why this signaling possibly is not working in RA. Furthermore, DCs might go from tolerogenic to pathogenic if Flt3 signaling is not functional, leading to increased activation of effector T-cells directed towards the presented antigen. Since DCs have the potential to transdifferentiate into osteoclasts during inflammation, the expansion of these cells using Flt3L could potentially promote increased osteoclast development.

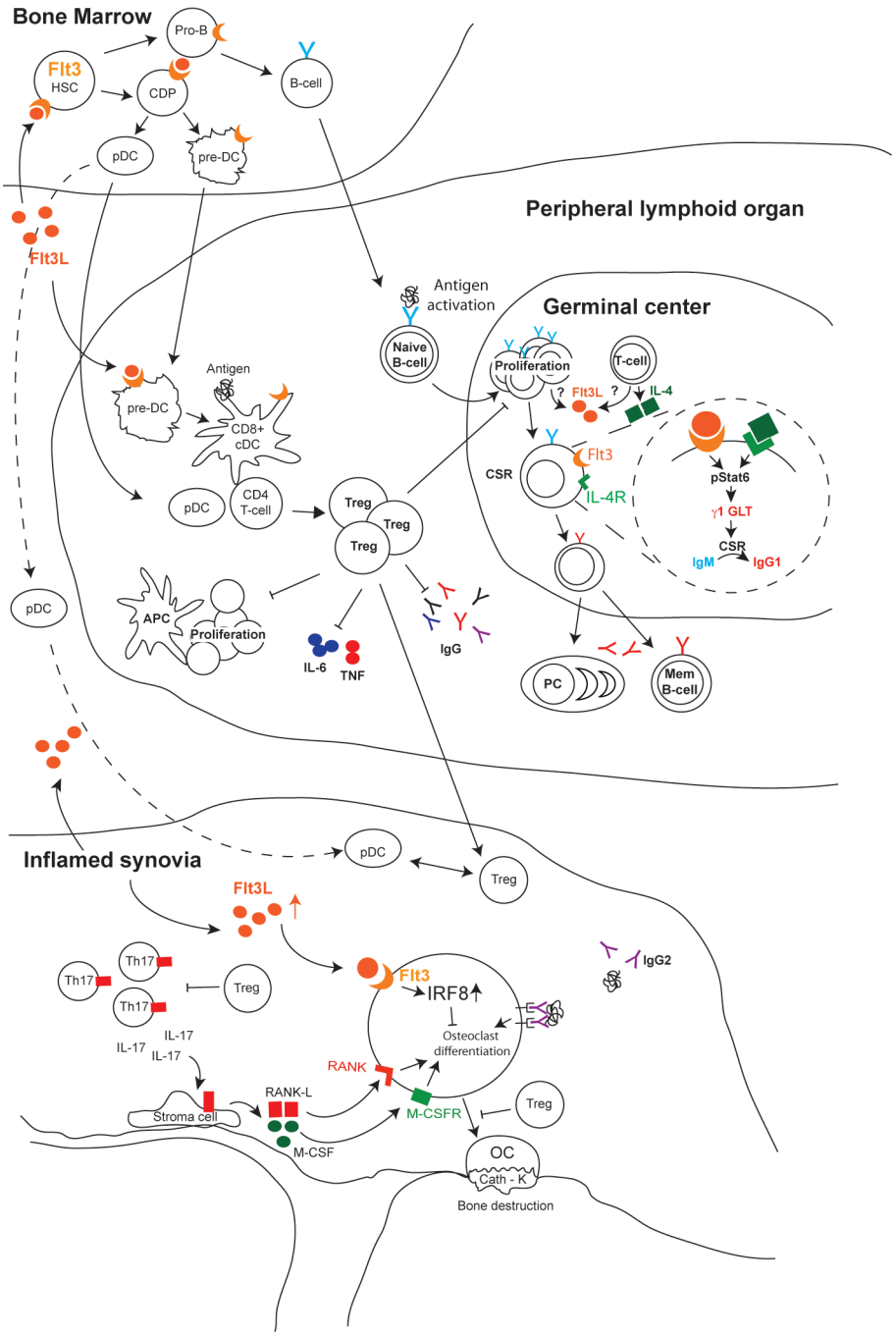
DCs are potent antigen-presenting cells with great capacity to present self-antigens and induce activation of autoreactive T-cells. Therefore, inhibition of DC formation by blocking the Flt3 receptor has recently been proposed as a possible treatment target for RA and other autoimmune diseases[233]. Inhibition of Flt3 using tyrosine kinase inhibitors reduces the severity of arthritis in animal models[147, 233, 234]. Although due to the important function of Flt3L in regulation of Treg homeostasis, bone metabolism and antibody CSR proposed in this thesis, great caution should be taken before initiating such a therapy. Importantly, one has to consider the inhibitors that have been used for blocking Flt3 in these studies. These compounds, such as Sunitinib or SB1578, do not show selective inhibition of Flt3. In contrast, these compounds also inhibit receptors for growth cytokines such as the M-CSF, VEGF, KL and PDGF, all of which have been described to be involved in RA pathogenesis. For example, inhibition of M-CSF would drastically reduce formation of osteoclasts in the arthritic joint, and may also be one of the explanations to the beneficial effect seen for "Flt3" inhibitors.

Flt3L might provide as a new biomarker for successful treatment in RA. If considering that the increased levels of Flt3L is consequence of a non-functional receptor due to chronic inflammation, then reduced serum levels of Flt3L might act as a positive marker for successful treatment of RA. In line with this, a recent study showed that when RA patients were treated with corticosteroids for 2 weeks, levels of Flt3L in serum was significantly reduced possibly as a consequence of reduced inflammation[21]. Also, patients responding successfully to RA treatment have increased numbers of circulating pDCs that induce IL-10 producing Tregs with the potential to suppress autologous T-cell proliferation[148]. This suggests restoration of functional Flt3 signaling in responding RA patients.

CONCLUSION

The results presented in this thesis give new insight into the potential role of Flt3 signaling in the pathogenesis of arthritis. Flt3L seems to have a regulatory function that acts on multiple levels during rheumatoid arthritis (RA), from the initiating cells of the innate immune to the effector cells of the adaptive immune system (Figure 12). The functions presented for Flt3/Flt3L signaling in Treg homeostasis, peripheral B-cells and in osteoclast differentiation and bone remodeling shed light on new and previously unacknowledged functions for Flt3 in RA. Although, lack of Flt3L do not seem to cause adverse effects in steady state, the loss of functional Flt3 signaling during inflammatory conditions may cause severe complications. Therefore, I can conclude that Flt3L is a versatile cytokine that have significant importance for maintenance of immune regulation and bone homeostasis during arthritis.

Figure 12. *Conclusion figure of the multiple actions of Flt3 signaling on the immune system during arthritis development.* Flt3L acts locally in the bone marrow to promote development of plasmacytoid dendritic cells (pDCs) and pre-DCs and support the development of early pro-B-cells. Immature B-cells, pre-DCs and pDCs migrate to peripheral lymphoid organs, where pre-DCs differentiate into cDCs under the influence of Flt3L. Flt3L preferentially promote differentiation into CD8+ cDCs which, after antigen-activation can induce the formation of regulatory T-cells (Tregs) from naïve CD4 T-cells. Splenic pDCs aid in this process through interactions with naïve T-cells. The induced Tregs reduce production of inflammatory cytokines (TNF and IL-6), antigen-specific proliferation and antibody production and proliferation of B-cells. At the same time, B-cells are activated by circulating antigens and with the help of T-cells established germinal centers in the lymphoid organ. During proliferation in the GC, B-cells start expressing Flt3 and induce class switch recombination (CSR) to IgG. Under the influence of IL-4 (from T-cells) and Flt3L (produced by proliferating B-cells or T-cells) B-cells induce CSR to IgG1, which results in formation of IgG1 producing plasma cells (PC) and memory B-cells. At the same time, pathogenic IL-17 producing Th17 cells accumulate in the arthritic knee joint. IL-17 induces production of RANK-L and M-CSF from synovial stroma cells which promotes differentiation of bone degrading osteoclasts from osteoclast-precursors. Cross-linking of IgG2 bound to Fcγ receptors on osteoclast precursors further potentiate osteoclast development. Tregs that have migrated to the inflamed synovium can suppress osteoclastogenesis directly by acting on osteoclast precursors or indirectly by suppressing Th17 cells. The influx of pDCs into the inflamed joint potentially aid in maintaining Treg function. The inflammation in the knee joint will also cause release of Flt3L. Flt3L can act directly on osteoclast precursors, inducing expression of IRF8 and suppressing osteoclast differentiation. Altogether, this leads to reduced bone destruction and reduced severity of arthritis.


Bone Marrow
Pro-B
Flt3
HSC
CDP
B-cell
pDC
pre-DC
Flt3L
Peripheral lymphoid organ
Antigen
activation
Germinal center
Naive
B-cell
Proliferation
T-cell
Flt3L
IL-4
Antigen
pre-DC
CD8+
cDC
CSR
Flt3
IL-4R
pStat6
γ1 GLT
CSR
IgM
IgG1
pDC
CD4
T-cell
Treg
Treg
Treg
APC
Proliferation
IL-6
TNF
IgG
PC
Mem
B-cell
pDC
Inflamed synovia
pDC
Treg
Flt3L
IgG2
Th17
Th17
Th17
Treg
IL-17
IL-17
IL-17
Flt3
IRF8
Osteoclast
differentiation
RANK
RANK-L
Stroma cell
M-CSFR
Treg
M-CSF
OC
Cath - K
Bone destruction

POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Ledgångsreumatism, även kallad reumatoid artrit (RA), är en inflammatorisk sjukdom som drabbar leder i händer, knä och fötter. Till skillnad från den vanligaste formen av reumatiska sjukdomar, artros, uppkommer ledgångsreumatism genom att kroppens immunförsvar börjar attackera den egna vävnaden som om den vore främmande, s.k. autoimmunitet. Autoimmunitet vid RA tros bero på att kroppen tappar tolerans mot vissa delar av den vävnaden som sitter runt våra leder (ledvävnad). Varför vissa människor drabbas av RA är idag inte fullständigt känt men samband mellan bakomliggande orsaker som t.ex. att tidigare släktingar haft besvär (arv) eller rökning (miljö) har bekräftats som riskfaktorer. Idag drabbas ca 0,5 – 1 % av Sveriges befolkning av RA och de flesta är ungefär 50 år gamla när sjukdomen bryter ut. Sjukdomen kan drabba både kvinnor och män, men är vanligare hos kvinnor. RA leder ofta till kraftig värk och stelhet i kroppens leder men även andra organ i påverkas såsom hjärta och lungor. Behandlingen av RA har förbättrats de senaste åren och i dagsläget är prognosen för patienter som drabbas av sjukdomen mycket bättre än vad den var för bara 20 år sedan. Däremot finns det i dagsläget ingen botande behandling. Således är orsaken till varför vissa personer drabbas av RA inte kartlagd, men vad man vet är att immunsystemet har en viktig roll vid uppkomst och utveckling av sjukdomen.

De flesta av våra immunceller (s.k. vita blodkroppar eller lymfocyter) härstammar från en och samma ursprungscell, en s.k. stamcell, i benmärgen. Flt3 är en receptor (signalmottagare) som sitter på våra stamceller i benmärgen. Denna receptor aktiveras av en specifik signalmolekyl (ligand), kallad Flt3-ligand. Aktivering av Flt3-receptorn har en viktig funktion i utvecklingen av ett flertal av våra immunceller, i synnerhet hos dendritiska celler och B-celler. Dendritceller är en viktig cell i immunförsvaret som agerar som medlare till andra celler i immunsystemet. Dendritcellen plockar upp främmande ämnen som kommer in i kroppen, s.k. antigen, och presenterar för en annan grupp av immunceller, T-cellerna. På detta sätt kan de dendritiska cellerna reglera och starta immunsvar mot främmande föremål. Dendritceller finns i både ledvätska och ledvävnad hos patienter med RA. På grund av deras förmåga att presentera antigen (som kan vara delar av den egna kroppen) för andra lymfocyter så anses de ha en viktig roll vid utvecklingen av sjukdomen. En särskild grupp av T-celler, s.k. regulatoriska T-celler, har en viktig roll i att bromsa immunförsvaret och se till att kroppen inte börjar attackera sig själv (se till att toleransen bibehålls). Förlusten av detta skydd (tolerans), vilken tros ligga till grunden för utveckling av RA, kan dels förklaras av en förlust av eller en bristfällig funktion hos dessa regulatoriska celler. B-celler är en annan typ av immuncell som hjälper till i immunsystemets försvar mot bakterier och virus genom produktion av immunoglobuliner (antikroppar). Även B-celler bidrar till sjukdomsförloppet vid ledgångsreumatism. I patienter med RA kan man ofta hitta så kallade autoantikroppar i blodet. Dessa antikroppar är riktade mot proteiner som finns i vår egen vävnad och kan hjälpa till att felaktigt aktivera immunsystemet. Förekomst av dessa autoantikroppar ses ofta långt innan en person drabbas av RA och är därför en bra markör för att någon kommer drabbas av sjukdomen i framtiden. Hos patienter

med ledgångsreumatism ser man ofta en successiv nedbrytning av ben och brosk i de leder som påverkats av sjukdomen. En av orsaken till denna undergång är förekomsten av celler som bryter ner ben, s.k. osteoklaster.

Vi har tidigare visat att patienter med RA har förhöjda nivåer av Flt3-ligand i ledvätskan och att lokal injektion av Flt3-ligand in knäet förvärrar undergången av ben hos möss. Vidare har en studie utförd i USA visat att analys av Flt3-ligand kan vara av värde för att hitta friska personer med risk för att utveckla RA. Även om det finns klara indikationer på att Flt3-ligand kan vara en bidragande orsak till utvecklingen av RA, är dess funktion fortfarande inte känd.

In denna avhandling, som inkluderar tre arbeten, ville vi studera hur signalering via Flt3- receptorn påverkar immunsystemet vid ledgångsreumatism. För att göra detta använde vi oss av en väletablerad musmodell där mössen utvecklar en ledsjukdom som har likheter med den ledgångsreumatism som drabbar människor. Våra studier visar att Flt3-ligand har en viktig roll i att främja bildandet av dendritiska celler och regulatoriska T-celler vid utvecklingen av artrit. Behandling med Flt3-ligand ökar antalet regulatoriska T-celler och resulterar i en minskad bildning av inflammatoriska signalmolekyler (cytokiner) och minskad nedbrytning av ben. Vi kan visa att denna effekt på bennedbrytning beror på att Flt3-liganden har en viktig roll vid bildandet av osteoklaster.

Signalering via Flt3-receptorn har även visat sig vara viktig för bildandet av B-celler i benmärgen. I denna avhandling kan vi visa att signaler från Flt3- receptorn är viktig också för de B-celler som finns utanför benmärgen i de organ som tillhör vårt lymfatiska system (mjälten och lymfkörtlar). Vi visar att när B-celler aktiveras av främmande ämne så började de uttrycka Flt3-receptorn i samband med att de började dela sig för att bli fler (s.k. proliferation), och att signaler via denna receptor är viktig vid bildandet av immunoglobuliner.

Sammantaget visar denna avhandling att Flt3-liganden har en viktig roll vid i bildandet av regulatoriska T-celler, dendritiska celler och de skadliga, benätande osteoklasterna. Därmed drar jag slutsatsen att signalering via Flt3-receptorn spelar en viktig roll i kontrollen av immunförsvaret och den bennedbrytning som sker vid ledgångsreumatism

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