Differentiation factor Fms-like tyrosine kinase 3 ligand is a modulator of cell responses in autoimmune disease

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ABSTRACT

Fms-like tyrosine kinase 3 (Flt3) is a receptor on common stem cell progenitors and has a crucial role in haematopoiesis, regulating cell proliferation and differentiation in man and mice. The growth factor Flt3 is activated by its soluble ligand, Flt3-L, leading to differentiation of multipotent stem cells and lymphoid progenitors. Flt3-L functions as a differentiation factor for dendritic cells (DC) in the periphery. These properties of the Flt3/Flt3-L system lead us to further investigate the role of the growth factor Flt3-L in rheumatic disease.

In paper I, Flt3-L was found to be strongly expressed at the site of inflammation in human RA, the joint. Levels of Flt3-L were significantly higher in the synovial fluid compared to serum in RA patients and levels of Flt3-L in RA synovial fluid were significantly higher compared to synovial fluid from non-inflammatory joint diseases. Furthermore, intra-articular administration of a B-cell line overexpressing Flt3-L resulted in highly erosive arthritis while inoculation of the same B-cell line without hyper expression of Flt3-L did not induce erosivity and caused arthritis in a minority of cases. Thus, Flt3-L is expressed at the site of inflammation in human RA and facilitates tissue destructive properties in the joint cavity.

In paper II, mice with antigen-induced arthritis, mBSA-arthritis, were treated with the Flt3-inhibitor sunitinib. Treatment was started together with mBSA immunization or together with the induction of arthritis. Abrogation of Flt3 signalling reduced the intensity of synovitis and the frequency of bone
destruction. Inhibition of Flt3 reduced also the number of differentiated 
(mature) dendritic cells concomitant with reduction of antibody production 
and bone metabolism. In addition to this, we investigated the ability of mouse 
bone marrow cells to migrate towards Flt3-L in a migration assay. Flt3-L was 
found to be a potent chemo attractant facilitating mobilization of Flt3+ cells 
from the bone marrow. Thus, the processes of antigen presentation, influx of 
leukocytes into synovial tissue and bone remodelling are mediated by Flt3 
signalling in antigen-induced arthritis.

In paper III, Flt3-L in CSF correlated to levels of T-tau and P-tau of patients 
with Sjögren’s syndrome, fibromyalgia and Alzheimer’s disease, implying 
involvement of Flt3L in brain homeostasis. Furthermore, CSF Flt3-L in pSS 
correlated to a marker for microglia activation, MCP-1. Levels of Flt3-L in 
CSF were significantly decreased in pSS, and AD, compared to FM. Low 
levels of Flt3-L were associated to low levels of amyloid degradation 
peptides in pSS and AD patients. Thus, in CNS of patients with pSS Flt3-L is 
strongly correlated to neuroaxonal plasticity and microglia activation and 
reduced levels of CSF-Flt3-L in pSS are linked to changes in tau and amyloid 
turnover resembling processes ongoing in AD patients.

Taken together, these results indicate that Flt3-L is involved in the 
inflammatory and tissue remodelling processes in joints and neuroaxonal 
structures of the brain. Flt3/Flt3-L signalling is an essential regulator of 
antigen-induced processes in autoimmune diseases.

**Keywords:** Flt3-L, rheumatoid arthritis, Sjögren’s syndrome

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ABBREVIATIONS

Aβ  amyloid-β peptide
AD  Alzheimer’s disease
AML  acute myeloid leukaemia
ANA  anti-nuclear antibody
Anti-CCP  anti-cyclic citrullinated peptide
APC  antigen presenting cell
APRIL  a proliferation inducing ligand
BAFF  B cell activating factor
cDC  conventional dendritic cell
CDP  common dendritic progenitor
CLP  common lymphoid progenitor
CMP  common myeloid progenitor
CNS  central nervous system
CSF-1  colony stimulating factor-1
CSF1R  colony stimulating factor-1 receptor
CSF  cerebrospinal fluid
CTX-I  C-terminal crosslinking telopeptide of type I collagen
CTX-II  C-terminal crosslinking telopeptide of type II collagen
DC  dendritic cell
Erk  extracellular-signal-regulated kinases
Flt3  fms-like tyrosine kinase 3 receptor
Flt3-L  fms-like tyrosine kinase 3 ligand
FLS  fibroblast-like synoviocyte
FM  fibromyalgia
GFAP  glial fibrillary acidic protein
GIST  gastrointestinal stromal cell tumours
GMP  granulocyte-monocyte progenitor
Gab2  Grb2 associated binding protein 2
Grb2  growth factor receptor-bound protein 2
HSC  hematopoietic stem cell
ICAM-1  intercellular adhesion molecule 1
Ig  immunoglobulin
IκB  inhibitory κB protein
IL  Interleukin
JAK  Janus kinase
LPS  lipopolysaccharide
ITAM  immunoreceptor tyrosine-based activating motif
M-CSF  macrophage colony stimulating factor
MAPK  mitogen-associated protein kinase
mBSA  methylated bovine serum albumin
MCP-1  monocyte chemoattractant protein 1
MDP  monocyte-dendritic progenitor
MHC  major histocompatibility complex
MkEP  megakaryocyte-erythrocyte progenitor
MLS  macrophage-like synovial cell
MMP  matrix metalloprotease
moDC  monocyte derived dendritic cell
MSA  multiple systemic atrophy
NFκB  nuclear factor kappa-light-chain-enhancer of activated B cells
NFL  neuro filament light
NK  natural killer cell
non-RTK  non-receptor tyrosine kinases
OPG  osteoproteoglin
p38  p38 mitogen-activated protein kinases
PAMP  pathogen-associated molecular patterns
PB  peripheral blood
pDC  plasmacytoid dendritic cell
PDGFR  platelet-derived growth factor receptor
PD  Parkinson disease
Pep  peptidoglycan
Pi3  phosphoinositide 3 kinase
PRR  pattern recognition receptor
pSS  primary Sjögren’s syndrome
P-tau  phosphorylated tau
RANKL  receptor activator of nuclear factor kappa-B ligand
RA  rheumatoid arthritis
RBC  red blood cell
RCC  renal cell carcinoma
RF  rheumatoid factor
RTK  receptor tyrosine kinases
S  serum
sAPPα  soluble amyloid precursor protein α
sAPPβ  soluble amyloid precursor protein β
SD  standard deviation
SF  synovial fluid
SFK  Src family kinases
SH2  Src homology 2 domain
SH3  Src homology 3 domain
SHP2  Src homology 2 domain containing phosphatases
STAT  signal transducer and activator of transcription
Syk  spleen tyrosine kinase
TACE  TNF-α converting enzyme
TK  tyrosine kinases
TLR  toll like receptor
TNF-α  tumour necrosis factor α
VCAM-1  vascular cell adhesion molecule 1
VEGF  vascular endothelial growth factor
VEGFR  vascular endothelial growth factor receptor
1 INTRODUCTION

1.1 Autoimmunity

It is of utmost importance for the immune system to discriminate between foreign and self. The immunological unresponsiveness to self is termed self-tolerance. Autoimmune disease is defined as breakdown of self-tolerance such that the adaptive immune system responds to self-antigens and mediates cell and tissue damage. The following three conditions must be fulfilled for autoimmune disease to occur. The existence of MHC molecules that can present self antigens, the T-cells with receptors that can recognize the presented antigens and finally the environmental factors leading to breakdown of normal tolerance mechanisms designed to eliminate self-reactive lymphocytes.

There are several mechanisms in the immune system to preserve self-tolerance. Central tolerance, the deletion of self-reactive T-cells in thymus aims at providing T-cells that recognize MHC with a self-antigen with low avidity and eliminate T-cells that recognize MHC with self-antigen with high avidity. While central tolerance is induced in the maturing immune system, peripheral tolerance is an ongoing process and can be viewed as a backup system for preventing autoimmunity where central tolerance has been incomplete. A mature T-cell recognizing self-antigens in the periphery can be inactivated in three different ways: anergy, apoptosis or suppression by regulatory T-cells.

In spite of this, T-cells with high avidity for self-antigens do become activated in the periphery and autoimmunity develops in a not negligible number. The great diversity of different autoimmune conditions reflects the complexity of the immune system and the genetics involved.

1.2 Rheumatic diseases

1.2.1 RA

Rheumatoid arthritis (RA) is an autoimmune inflammatory joint disease affecting approximately 1 % of the world population, affecting all ethnic groups although with different prevalence. Females are 2.5 times more likely to be affected compared to males. Onset of disease can occur at any age but peak incidence occurs within the fourth and fifth decades of life. RA patients
Differentiation factor Fms-like tyrosine kinase 3 ligand is a modulator of cell responses in autoimmune disease express autoantibodies such as RF and anti-CCP antibody. Many factors contribute to the risk of developing RA and the most known being genetic factors, sex, and tobacco \(^1\). The disease may also engage extra-articularly affecting inner organs developing e.g. noduli, pulmonary fibrosis, vasculitis, and amyloidosis.

The primary target organ in RA is the synovial membrane which is subjected to increased cellularity, pannus formation, angiogenesis, and infiltration with immune inflammatory cells and osteoclastogenesis leading to destruction of cartilage and bone following joint deformities and disability. Components from both the innate and the adaptive immune system are implicated in onset and maintaining of the disease. The importance of both B- and T-cells for pathogenesis has been emphasized by the good clinical effect observed on both B-cell eradication \(^2\) as well as inhibition of T-cell co-stimulation \(^3\) and the presence of autoantibodies.

The greatly improved pharmacological treatment possibilities combined with the observation that prognosis of rheumatoid arthritis is improved if treatment is provided early \(^4\), \(^5\), have created an urge for improved prognostic factors allowing individualisation of treatment. In addition to autoantibodies and genes, bone erosions upon diagnosis predicts poor prognosis.

### 1.2.2 pSS

pSS is a systemic autoimmune disease affecting approximately 0.1-0.6 % of the world population\(^6\) according to the American European consensus criteria for pSS \(^7\) although it shall be stated that there is a lack of reliable indicators for epidemiologic studies of the disease. 90 % of patients are female. Onset of disease can occur at any age but peak incidence occurs within the fourth and fifth decade of life. The aetiology to pSS is unknown but many factors are considered and the most well known being genetic factors, sex, and virus infections. Enhanced activity of the type 1 IFN system is the reply of the innate immune system upon viral infection. Patients with pSS exhibit an "interferon-signature", they display a pattern of overexpressed genes that are inducible by interferons and this has been shown locally in the salivary gland\(^8\) and in the blood\(^9\). The IFN-inducible genes correlate to production of antinuclear antibodies of Ro- and La-specificity. pDCs have been identified as the main source for IFN-production \(^9\).

The primary target organ in pSS are exocrine glands exhibiting a particular pattern of periductal focal lymphocytic infiltration in otherwise normal-appearing glands leading to progressive lacrimal and salivary gland
dysfunction. Formation of ectopic germinal centres in the pSS salivary gland has been described\textsuperscript{10} and this may promote loss of salivary gland tissue as well as acting as a tertiary lymphoid organ.

Furthermore, pSS is characterized by polyclonal T and B lymphocyte activation, production of antinuclear antibodies of Ro- and La-specificity as well as polyclonal IgG and cryoglobulins. Patients with pSS have a substantially increased risk, 16-fold, of developing non-Hodgkin (B-cell) lymphoma. Identified risk factors are hypocomplementemia, lymphocytopenia, prolonged salivary gland enlargement and palpable purpura\textsuperscript{11} but also the presence of ectopic germinal centres in the salivary gland\textsuperscript{12}. The role of B-cells in the pathogenesis of pSS is emphasized by the pronounced antibody production, differences in distribution of mature B cells in pSS compared to other autoimmune diseases as well as healthy controls with pSS patients displaying increases of less differentiated IgD\textsuperscript{+} B-cells and decreases in the more mature forms\textsuperscript{13}. Furthermore, B-cell depletion has shown significant effects on salivary gland function and extraglandular manifestations\textsuperscript{14}.

The disease may also engage extraglandularly in forms of arthralgias, thyroiditis, pulmonary, interstitial nephritis, cutaneous vasculitis, CNS and PNS.

Outcome in pSS patients with neurological traits is frequently severe, especially in patients with CNS involvement\textsuperscript{15}. CNS involvement in pSS varies, frequencies from 20\% to <5\%\textsuperscript{16} have been reported, and is characterized by asymptomatic MRI findings to focal brain and spinal cord processes presenting clinically by a broad spectrum of CNS symptoms including spinal cord involvement, neuromyelitis optica, seizures, dementia with cognitive dysfunction and encephalopathy\textsuperscript{16}.

The CNS pathogenesis is not well delineated. There are however signs of activation of both the adaptive and the innate immune system. Analysis of CSF points to lymphocytosis, raised IgG index, oligoclonal IgG production suggesting migration of B-cells into CNS and intrathecal Ig-production\textsuperscript{17}. Intrathecal activation of terminal complement, C5b-9, have been shown in pSS patients with CNS involvement\textsuperscript{18}. Histopathological examination of brain tissue has showed inflammatory vasculopathy and in line with this cerebral angiography have showed small vessel cerebral angiitis\textsuperscript{19}.

Diagnosing CNS involvement in pSS is in many cases difficult and there are great needs for better diagnostic tools. Apart from imaging of the CNS the
main diagnostic efforts are directed towards CSF analyses. Cellularity, cytology, intrathecal Ig-production, levels of pro-inflammatory cytokines and markers of CNS-damage do in many cases not provide sufficient information for a good clinical diagnosis.

1.3 Flt3 / Flt3-L

1.3.1 Flt3

The Fms-like tyrosine kinase 3 receptor (Flt3) was cloned in 1991\textsuperscript{20, 21} and described as a member of the PDGFR/CFS1-R tyrosine kinase family. Shortly thereafter, its ligand, (Flt3-L) was discovered. Flt3 is a membrane bound tyrosine kinase receptor which has a crucial role in haematopoiesis, regulating cellular differentiation, proliferation and apoptosis. Physiologically, it is mainly expressed on early myeloid and lymphoid progenitors\textsuperscript{22} but also on more mature cells of myeloid lineage such as DC progenitors in secondary lymphoid organs.\textsuperscript{23} Furthermore, its expression on B-cells in the salivary glands of patients with Sjögren’s syndrome has recently been shown\textsuperscript{24}. Flt3 is also expressed in the CNS, mostly in differentiated post mitotic neurons in the hippocampus, olfactory bulb, cerebellum and in dorsal root ganglion in the spine\textsuperscript{25}.

1.3.2 Flt3-L

The activation of Flt3-mediated signalling is achieved by interaction between Flt3 and its ligand, Flt3-L, resulting in differentiation and proliferation of early hematopoietic progenitors, both of myeloid\textsuperscript{26} and lymphoid origin.\textsuperscript{27} Human Flt3-L is a type 1 trans membranous protein consisting of 235 amino acids. The dominating isoform is the full-length trans membrane isoform but there are also soluble forms which consist of different sizes of the extracellular domain. All isoforms are biologically active. Little is known about the enzymes involved in the proteolytic release of Flt3-L into its soluble form. Previous studies have shown TNF-\alpha converting enzyme (TACE) to be involved in the shedding of the ligands for the closely related type III tyrosine kinase receptors KIT and CSF-1\textsuperscript{28, 29}. TACE has been shown critical of processing Flt3-L in cell based assays\textsuperscript{30} and serum Flt3-L levels are decreased in conditional TACE-deficient mice\textsuperscript{31} implying a role for this enzyme in regulating soluble Flt3-L in vivo. Flt3-L is also present in a soluble, intracellularly stored, form\textsuperscript{32}. The mouse and human Flt3-L proteins are 72\% identical at the amino acid level and do not have species specificity, hence murine Flt3-L can activate human Flt3 and vice versa\textsuperscript{33}. In contrast to the Flt3 receptor, Flt3-L is expressed in most human tissues (spleen, thymus,
bone marrow, prostate, kidney, and brain) but the highest levels are seen in PB leukocytes. Serum levels of Flt3-L are low in healthy individuals but markedly elevated levels are seen in patients with secondary leukopenia\textsuperscript{34} and leukaemia\textsuperscript{34}.

Mutations in Flt3, internal tandem duplications, leaving the TK in what is considered a constitutively activated state has been found in haematological malignancies, especially acute myeloid leukaemia (AML) where as much as 30\% of patients display this rendering them poorer prognosis\textsuperscript{34}. Interestingly, these patients also display increased levels of the ligand, Flt3-L, \textsuperscript{34}.

\textbf{Figure 1.} The crystal structure of soluble Flt3L - a homodimer of two short chain alpha-helical bundles. Thanks to http://www.ebi.ac.uk/ for figure.

\textbf{1.3.3 Tyrosine kinase}

Tyrosine kinases (TK) are key regulators of many critical cellular processes such as proliferation, differentiation, cell survival and cell migration. They comprise of two classes of molecules: cell surface bound receptors named receptor tyrosine kinases (RTK) and intra-cellular enzymes called non-receptor tyrosine kinases (non-RTK).

There are about 60 known human RTKs divided into 20 subfamilies which all share a similar molecular architecture including a ligand binding region in the extracellular domain, a single trans membrane helix, and a cytoplasmatic region that contains the protein tyrosine kinase.

Most non-RTKs are located in the cytoplasm and lack receptor-like features such as extracellular ligand binding region and trans membrane spanning region. However, some non-RTKs are anchored to the cell membrane. Non-RTKs possess domains that allow interaction between protein and protein, lipids and DNA. The most commonly found protein-protein interaction domains are the SH2 and SH3 domains. The non-RTKs are integrated in the
Differentiation factor Fms-like tyrosine kinase 3 ligand is a modulator of cell responses in autoimmune disease.

Intracellular signalling cascades triggered by RTKs and other cell surface receptors, such as G protein coupled receptors and immune system receptors.

In general, ligand (hormone or growth factor) binding induces receptor oligomerization leading to auto phosphorylation of intracellular motifs inducing conformational changes that allow binding of ATP and substrate. The activated RTK then phosphorylates tyrosine residues creating docking sites on the receptor for downstream signalling or activation of substrate proteins, both promoting signal transduction. Adaptor proteins, lack enzymatic activity but with the capacity of interacting with several proteins at the same time, bind to the phosphorylated tyrosine residues stepwise enhancing signal transduction. Adaptor proteins activate transcription factors. When transcription factors are activated they translocate to the nucleus, bind to the promoter regions of DNA initiating gene transcription. The effects exerted involve proliferation, differentiation, apoptosis, migration and metabolic changes.

Figure 2. Schematic picture of RTK activation and i.c. signalling. Ligand binding induces receptor oligomerization leading to phosphorylation of tyrosine residues. This creates docking sites for adaptor proteins activating transcription factors translocating to the nucleus modulating effects on gene transcription such as proliferation, differentiation, apoptosis, migration and metabolic changes.
Or in more concrete terms, binding of the ligand insulin to the insulin RTK leads to recruitment of adaptor proteins, one being Pi3 which activates the transcription factor Akt. Activated Akt exerts metabolic effects through glycogen synthesis; effects on cell survival through inhibition of pro-apoptotic signals; effects on T-cell migration through expression of adhesion molecules; effects on cell cycle and proliferation.

1.3.4 Intracellular signalling – Flt3 and RA

It is necessary for the cell to respond to extracellular, environmental stimuli. Signal transduction occurs when an extracellular signalling molecule activates a cell surface receptor, e.g. a RTK. In turn, this receptor alters intracellular molecules creating an intracellular response. An activated RTK can be thought of as a node in a complex signalling network that transmits information from the exterior to the interior of the cell.

The ways of intracellular signalling through Flt3 are today not fully mapped. Ligand-activated Flt3 dimerizes which leads to concomitant juxtapositioning of the cytoplasmatic domains followed by trans phosphorylation of specific intracellular tyrosine residues such as 589, 591, 768, 955 and 969. This provides binding sites for adaptor proteins such as Grb2, SFKs, Pi3 and SHP2. The cytoplasmatic domain of Flt3 sterically holds the kinase in an inactive conformation which is changed upon phosphorylation of the tyrosine residues 589 and 591. The constitutively activated state of Flt3 seen in AML occurs due to internal tandem duplications in the Flt3 gene and could be explained by insertions in the cytoplasmatic domain abolishing the sterically achieved auto inhibition. The adaptor proteins regulate and enhance different signal transduction pathways leading to transcription factors such as Akt, MAPK (Erk, p38) and STAT achieving alterations in transcription of DNA and translation of proteins. The effects result in proliferation, survival and differentiation processes of cells.
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Figure 3. Intracellular signalling of Flt3. Ligand activated Flt3 dimerizes leading to trans phosphorylation of specific tyrosine residues providing binding sites for adaptor proteins, such as SFKs, SHP2, Grb2 and Pi3. These activate transcription factors such as Akt, Erks, p38 and STAT. The latter is not activated in “healthy” conditions. The transcription factors influence DNA transcription exerting effects on proliferation, differentiation and survival of cells.

Many of the intracellular responses seen in RA involve tyrosine kinases. The main pathways recognized are JAKs, NFκB, MAPKs and Syks. These pathways share features with the ways of intracellular signalling through Flt3. NFκB complexes are located in the cytoplasm. In response to proinflammatory cytokines, such as TNFα and IL-1β, the inhibitory protein IκB becomes phosphorylated resulting in rapid proteolysis thereby allowing the liberated NFκB to translocate to the nucleus. There it regulates the expression of cytokines central to RA, such as IL-1, TNFα and IL-6, which has rendered it a great deal of attention in the RA field. JAKs bind to the cytoplasmatic region of cytokine receptors resulting in activation of STATs which are phosphorylated, dissociate and dimerize via their SH2 domains and translocate to the nucleus. JAKs are considered attractive targets for treatment of RA due to their role in transduction of IL-6 signalling. JAK 3 is involved in development of NK and T cells and function and proliferation of
B-cells. So far, JAK inhibitors are promising for treatment of RA. The MAPK are activated by signalling through TLR as well as inflammatory cytokines and act by transcription factors Erks and p38. Syk is a non-RTK and it is activated through binding of tandem SH2 domains to immunoreceptor tyrosine-based activating motif (ITAM) of the cytoplasmatic region of immune receptors, such as the B- and T-cell receptor. Syk activates downstream MAPKs and PI3 leading to expression of IL-6 and MMP production. Trials with Syk inhibition on RA patients have achieved significant effects.

Figure 4. Intracellular signalling pathways in RA. For more detail see text above. Liberated NFκB translocate to the nucleus. There it regulates the expression of cytokines central to RA, such as IL-1, TNFα and IL-6. JAKs bind to cytokine receptors and activate STATs which translocate to the nucleus. The MAPK include Erk and p38 and are activated by signalling through TLR as well as inflammatory cytokines. Syk is a non-RTK activated through binding to the cytoplasmatic region of immune receptors, such as the B- and T-cell receptor.
1.4 The immune system

The immune system can be divided into innate and adaptive subsystems whose inappropriate activation leads to auto inflammatory and autoimmune diseases, respectively.

1.4.1 The innate immune system

The phagocytic cells of the innate immune system recognize pathogens and are activated by pattern recognition receptors (PRRs), such as toll-like receptors (TLR), identifying pathogen-associated molecular patterns (PAMPs). The PAMPs, such as viral RNA and lipopolysaccharide (LPS), are essential for microbial survival which restricts major changes in their structure. Upon PPR activation, inflammatory pathways are upregulated leading to production of pro-inflammatory cytokines. The innate immune system is the first line of defence, it reacts fast, non-specifically, in the same way for every stimulus and does not improve over time.

1.4.2 The adaptive immune system

In contrast, the adaptive immune system reacts slower but more specific and improves over time generating memory. One major difference between innate and adaptive immune cells is the ability of the latter to express unique, highly antigen-specific receptors.

The adaptive immune system comprises of humoral and cell-mediated immunity. Immunoglobulins, secreted by activated B cells, recognizing antigens eliciting various effector mechanisms participating in deletion of the antigen constitutes the humoral system. T-lymphocytes provide cell-mediated immunity by activating macrophages, NK-cells, cytotoxic T-cells, regulatory T-cells and B-cells, enhancing their antibody production. To do this, the T cell needs to be activated through interaction with an antigen presented to the T cell receptor by a professional antigen presenting cell (APC) on a certain peptide display molecule, the major histocompability complex (MHC). MHC exists in two forms, class 1 is present on all nucleated cells except neurons and red blood cells while MHC class 2 is expressed only on the professional APCs and activated T cells. Expression of different MHC alleles correlates with susceptibility to develop autoimmune disease.

However, the majority of rheumatic diseases depend on both the innate and the adaptive part of the immune system for their development and progress.
Figure 5. Innate and adaptive immune response exemplified by induction of antigen-induced arthritis with mBSA. Immunization is performed with mBSA in Freund's adjuvants, the latter used to strengthen the reaction. APCs are activated through Pattern Recognition Receptors and mBSA is phagocytized, processed into peptide fragments which are complexed with MHC I and II and presented on the surface of the APC. In draining lymph nodes, the antigen is presented to T-cells activating them and further differentiation occurs into effector and memory cells. Furthermore, B cells are activated and mBSA-specific antibodies are produced. Figure adapted with permission from Sofia S. Lindblad.

The APCs of the innate immune system form a link to the adaptive immune system by presenting antigen and activating it.

1.4.3 Antigen presenting cells

Dendritic cell

The dendritic cell is the main professional APC spread throughout the body, both in peripheral and lymphoid tissue. Most DCs originate from bone marrow myeloid progenitors and can be classified into three different subpopulations. The conventional DC (cDC) (B220^- CD11c^+) with a short lifespan in comparison to the plasmacytoid DC (pDC) (B220^+ CD11c^-) which is specialized in type 1 IFN secretion in response to virus. The third form, the monocyte derived DC (moDC) appears to exist only under inflammatory conditions. The pDC is formed in the bone marrow while cDC precursors leave the bone marrow and acquire a mature cDC surface phenotype and morphology in secondary lymphoid organs.

Upon activation, the DC increases its expression of MHC class 2 and migrates to lymphoid tissue presenting antigen to the naive T cell and possibly activating it. Shared features of the DCs are the surface marker CD11c and the expression of MHC class 2. DC progenitors also express the tyrosine kinase Flt3 which when activated by its ligand leads to clonal...
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DCs display a dual role in autoimmune conditions. They are necessary for inducing (auto) immunity but also regulating immune tolerance. DCs are crucial for maintaining peripheral tolerance. DCs with tolerogenic properties down regulate co-stimulatory molecules (CD80/86) and on interaction with T cells, induce anergy or activation induced cell death. They can also produce TGFβ, a cytokine necessary for differentiation of naïve T cells to regulatory T cells \(^{44}\). The constitutive ablation of DCs in mice leads to break in self-tolerance and development of severe autoimmune conditions \(^{45}\).

On the other hand, DCs are implied in the pathogenesis of rheumatoid arthritis \(^{46}\) as well as autoimmune CNS disease \(^{47}\). The joint and synovium in RA is infiltrated with DCs in great numbers at the expense of PB \(^{46}\). They can contribute to the inflammatory process through auto antigen presentation or production of proinflammatory factors, such as IL-1, IL-6 and TNF-α. Indeed, anti-TNF-α targeted therapies ameliorate clinical symptoms in RA and also reduce frequencies of peripheral activated DC \(\text{in vivo}^{48}\) and \(\text{in vitro}^{49}\) diminish their maturation and their ability to produce pro-inflammatory cytokines and chemokines.

**Macrophage**

The macrophage is also a professional APC. Proinflammatory or metabolic stimuli recruits monocytes into the tissue where they differentiate into macrophages which display tissue specific properties. Examples are Kupffer cells in the liver, alveolar macrophage in the lung and osteoclasts in bone.

The macrophage release inflammatory cytokines and chemokines (TNFα and IL-1β) that amplify the inflammatory reaction by recruiting more cells to the site. They phagocytize and present antigen but in contrast to the dendritic cell it does not leave the tissue for secondary lymphoid organs instead presenting at place to the arriving T cells.

The macrophage has been highlighted for its role in the early innate immunological response in arthritis and plays a crucial role in the rheumatoid synovium. The healthy synovium is sparsely cellular and made up of macrophage-like synovial cells (MLS) and fibroblast like synoviocytes (FLS) in a two-cellular layer. The macrophage-like synovial cells express markers of hematopoietic origin most consistent with the monocyte–macrophage
lineage (CD11\textsuperscript{b}, CD68, MHC class II and Fc\textgamma). There is a clear correlation between synovial macrophage infiltration and subsequent radiographic joint destruction \textsuperscript{50} as well as degree of clinical improvement \textsuperscript{51} this further emphasizing the crucial role of the macrophage in RA.

**Microglia**

The professional APCs of the CNS are the microglia cells. It is the principal immune cell of the CNS with a dual role, amplifying the effects of inflammation and mediating cellular degeneration as well as protecting the CNS. Microglia are considered the resident macrophages of the CNS. They migrate to the active site releasing proinflammatory cytokines and chemokines recruiting more cells.

There is evidence supporting a common origin of dendritic cells, macrophages and microglia. Mouse bone marrow cells expanded in vitro in the presence of Flt3-L can give rise to all different cell types depending on which other stimulus added \textsuperscript{52}.

Microglia may contribute to autoimmune CNS disease in the same manner as DCs. Whether dendritic cells in areas of inflammation within the CNS are derived from resident microglia or DCs migrating to CNS is an unsolved issue.

**B-cell**

The B-cell recognizes antigen through the B cell receptor, a membrane associated form of immunoglobulin. Binding of antigen to this receptor causes the B-cell to expand into a clonal population all directed against the present antigen. The role of auto reactive B cells in autoimmune disease is emphasized by the good effect of B-cell depletion seen in RA \textsuperscript{53} and pSS \textsuperscript{14}.

### 1.5 Flt3(-L) and the immune system

Flt3 is expressed on stem cells, early lymphoid progenitor cells but also in more mature myeloid monocytic lineage cells. Experiences from targeted gene disruption in mice further emphasizes the role for Flt3/Flt3-L in haematopoiesis. Knockout mice lacking Flt3 exhibit reductions in early multipotent stem cells and lymphoid progenitors whereas no evident myeloid phenotype has been reported \textsuperscript{54}. However, genetic Flt3 fate mapping have established that most cells of the granulocyte-monocyte lineage are derived from Flt3-expressing progenitors \textsuperscript{55}. The common activating mutations of Flt3 in AML leads to ligand-independently dimerization and i.c. tyrosine residue phosphorylation leaving the low differentiated myeloid blasts in a
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constitutively activated state. This emphasizes the role for Flt3 in the early stages of the myeloid lineage. Flt3 is also essential for DC development in peripheral lymphoid tissues.

Figure 6. Flt3 and Flt3-L mediated effects on hematopoiesis. The Flt3 receptor is predominantly expressed in more immature hematopoietic cell populations. It is crucial for development of B-lymphoid progenitors. The majority of progenitors of the granulocyte-monocyte lineage are derived from Flt3 expressing progenitors although not crucial for their development. Furthermore, Flt3 expression is essential for regulation of DC development. Flt3-L is has an essential role in development of myeloid and B-lymphoid progenitors but also DCs and NK-cells. Abbreviations: HSC=Hematopoietic Stem Cell, MPP=MultiPotent Progenitor, CMP=Common Lymphoid Progenitor, CLP=Common Lymphoid Progenitor, GMP=Granulocyte-Monocyte Progenitor, MDP=Monocyte-Dendritic cell Progenitor, CDP=Common Dendritic cell Progenitor, pDC=plasmacytoid Dendritic Cell, cDC=conventional Dendritic Cell, NK=Natural Killer cell, MkEP=Megakaryocyte Erythrocyte Progenitor, RBC=Red Blood Cell.

Stimulation of mice in vivo with Flt3-L gives rise to bone marrow hyperplasia, splenomegaly, enlargement of lymph nodes and liver in mice and increases the number of early lymphoid and myeloid progenitors accompanied by increases in number of peripheral granulocytes, monocytes and clonal expansion of dendritic cells. This effect has been used in
generating DCs as immunostimulatory agents in anti-cancer trials with significant effects. Furthermore, Flt3-L has been shown to support osteoclastogenesis by replacing M-CSF. Mice lacking the gene for Flt3-L exhibit reductions in both lymphoid and myeloid progenitors but also reduced levels of lymphocytes, NK-cells and dendritic cells in the periphery.

In human, Flt3-L treatment gives rise to increase in number of neutrophils, CD14+ monocytes and clonal expansion of dendritic cells in PB while no effect is seen on circulating lymphocytes.

Hence, Flt3/Flt3-L provides crucial components for an immune response. The APCs for the early, innate immune reaction followed by antigen presentation and the adaptive immune response with lymphocytes, B- and T-cells. The immune response is the same regardless of the origin, it doesn’t tell the difference between bacteria or autoimmune disease. Thus, Flt3/Flt3-L has a role in autoimmune disease adjuvating the immune response.
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1.6 RA pathogenesis

The presence of autoantibodies is a hallmark of RA. Rheumatoid factor, an antibody directed towards the constant Fc-region of IgG, is expressed in approximately 80% of patients with RA and it’s possible role in the pathogenesis of the disease is debated. Anti-cyclic citrullinated peptide (anti-CCP) antibody was discovered 1998, and has become a valued marker in the diagnosis and prognosis of RA. The precise pathogenic role of anti-CCP is not well known. Antigen presentation can occur locally in the synovium but also by DCs migrating to lymph nodes activating T-cells followed by B cell activation. Flt3-L supports this process by providing DCs and lymphoid progenitors facilitating antigen presentation.

Another feature of RA is the rheumatoid synovium displaying an almost tumour like growth infiltrating cartilage and bone exhibiting an abundance of highly differentiated cells of hematopoietic origin. Cells are recruited to the joint by chemotaxis and expression of adhesion molecules. Bone and cartilage destruction is a severe manifestation of joint inflammation in RA leading to joint deformities and disability. The intimal synovial layer, facing the joint cartilage and bone, consists of MLS secreting pro-inflammatory cytokines (TNF-α, IL-1) activating FLS which in turn secrete IL-6, MCP-1 and MMPs and a paracrine/autocrine network is formed perpetuating synovitis. A neovascularized synovial front, pannus, develops and produce villous projections that grow invasively and destruct cartilage and bone. Cartilage is broken down by FLS and chondrocytes producing huge amount of proteases under the influence of IL-1, 17 and TNF. Bone erosion is handled by osteoclasts differentiated in the joint from monocytic progenitors.
under the influence of RANKL and M-CSF/Flt3-L. Flt3-ligand have been shown to support osteoclastogenesis by mobilizing osteoclastic progenitors and replacing M-CSF\(^9\). The synovial milieu is profoundly hypoxic and angiogenesis is a characteristic feature of the RA joint. These processes are supported by VEGF and TNF. The joint contains high levels of IL-6, 12, 23 and TGFβ which promotes the differentiation of TH1 and TH17 cells. Factors for B cell activation and survival (APRIL, BAFF) are also locally produced in the joint.

**Figure 8. The rheumatoid joint.** Cells are recruited to the joint by chemotaxis and expression of adhesion molecules. They arrive through blood but the underlying bone marrow also exhibit inflammatory infiltrates with T- and B-cell aggregates directing a bidirectional insult on the bone and maybe transferring into the joint. Bone and cartilage degradation is performed by osteoclasts, FLS and chondrocytes. Osteoclasts are differentiated from monocytic progenitors under the influence of RANKL and M-CSF/Flt3-L. Cytokines, such as IL-1 and IL-17, induce a switch in the synthesis pattern of chondrocytes from matrix molecules to matrix degrading enzymes, MMPs. In addition, synovial fibroblasts start producing matrix degrading enzymes and invade cartilage when activated by cytokines such as TNFα and IL-1. The synovial milieu contains IL-6, 12, 23 and TGFβ which promotes the differentiation of TH1 and TH17 cells. APRIL is produced by DCs and macrophages and FLS secrete BAFF to promote B cell activation and survival. The RA joint is hypoxic and angiogenesis is a characteristic feature supported by VEGF and TNF.
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1.7 pSS pathogenesis

pSS is characterized by polyclonal T and B lymphocyte activation, pronounced antibody production where IFN-inducible genes correlate to production of autoantibodies. Furthermore, there is a substantially increased risk of developing B-lymphocyte malignancies in pSS. Flt3 has a crucial part in all of these processes.

Precursor lymphocytes are dependent on Flt3 for their development and Flt3-L has been shown increased in pSS as well as in lymphoid malignancies. The main source for IFN-production inducing IFN-signature in pSS are DCs and they rely on Flt3-L for their differentiation and proliferation. A second crucial task for DCs in pSS is presenting antigen preceding the pronounced antibody production.

**Figure 9.** pSS pathogenesis. The salivary gland in pSS is infiltrated by B- and T-cells, DCs and the formation of germinal centres has been observed. Flt3-L may support this by chemotaxis, which is supported by the recent observation that B-cells infiltrating the gland express the Flt-3 receptor. Flt3-L supports antibody production by generating B- and T-cell progenitors and DCs. Furthermore, Flt3-L provides microglia for CNS.
1.8 Inhibition of Flt3 with sunitinib

Tyrosine kinases have emerged as a treatable target not only in rheumatic disease but also in cancer. Sunitinib is a small molecule inhibitor of Flt3. Inhibitory effects on other TKs have been described \(^{63,64}\).

Sunitinib inhibits Flt3 by binding to the ATP binding pocket of the kinase and thereby blocking activation. Sunitinib is used in the clinical practice for the treatment of gastrointestinal stromal cell tumours (GIST), advanced renal cell carcinoma and neuroendocrine pancreas tumours \(^{65}\). The Flt3/Flt3-L system is a tempting target in rheumatic diseases due to effects on dendritic cells and B-cells (antigen presentation and production) and osteoclastogenesis. No studies are performed on humans with rheumatic disease.
1.9 Markers of inflammation and degeneration

1.9.1 Tau / pTau

Tau is found in CNS neurons where it stabilizes microtubule and is essential for axonal transport of cytoplasmatic organelles. Tau possesses a large number of potential phosphorylation sites and the phosphorylation of Tau to pTau in physiological levels is a way in which Tau function is regulated in regards of microtubule dynamics, neurite outgrowth and axonal transport. Under normal conditions there is a balance between phosphorylation and dephosphorylation. In tau related diseases, for instance Alzheimer’s disease (AD), abnormally hyperphosphorylated Tau loses its biological activity and becomes resistant to degradation forming paired helical filaments. Tau is generally considered a marker for axonal degeneration.

Figure 12. Tau metabolism. Tau stabilizes microtubule and is essential for axonal transport of cytoplasmatic organelles. Tau possesses a large number of potential phosphorylation sites. Phosphorylation (P) at crucial sites detaches tau from microtubules leading to it’s degradation. In AD, abnormally hyperphosphorylated Tau forms paired helical filaments.
1.9.2 Neuro filament light
NFL are structural filaments of neurons, mostly in large myelinated axons, with the purpose of maintaining axon calibre. The levels of NFL reflect axonal degeneration and have been shown to correlate to degree of white matter lesions in the brain in small vessel disease.\(^6\)

1.9.3 Glial Fibrillary Acidic Protein
GFAP builds the glial intermediate filament which is the major cytoskeletal structure of astrocytes. A hallmark of neuroinflammation is astrogliosis where levels of GFAP increases. An injury to CNS may be the onset of neuroinflammation and levels of GFAP reflects the degree of damage.

1.9.4 MCP-1
Microglia are considered the resident macrophages of the CNS. Microgliosis is considered another hallmark of neuroinflammation. MCP-1/CCL-2 has chemotactic properties acting on macrophages, microglia and monocytes. MCP-1 is produced in CNS by astrocytes, microglia and infiltrating macrophages leading to autocrine regulation. MCP-1 is expressed in increased levels in RA serum and SF. PB monocytes and SF macrophages in RA express the receptor, MCP-1R. Thus, MCP-1 promotes influx of monocytes/macrophages into the joint. Inhibition of MCP-1R has been performed in experimental arthritis and the timing and method of blocking produce diverse outcomes. In human, it has been shown that levels of MCP-1 decreases upon RA treatment although inhibition of MCP-1R did not lead to clinical improvement in human RA.

1.9.5 IL-6
IL-6 regulates immune responses, haematopoiesis, acute phase response and bone metabolism. The dysregulation of IL-6 production seen in RA leads to activation of T- and B-cells followed by hypergammaglobulinemia and increased titres of autoantibodies such as RF. IL-6 also exerts effects on osteoclast differentiation implying a role in erosive course of RA. The importance of IL-6 in RA is furthermore emphasised by the good clinical effect on IL-6 inhibition in RA.

IL-6 is produced at low basal levels in CNS in normal conditions and up regulated in pathology. Various cell types can produce IL-6 but in CNS the main source comes from astrocytes and microglia. IL-6 exerts multiple effects in the CNS and displays dual roles participating in neuroinflammation as well as playing a substantial role in homeostasis and development of the
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nervous system. In neuroinflammation, IL-6 expands astrocytes and microglia and controls blood-brain barrier integrity.\[75\]

### 1.9.6 IL-8

The chemokine IL-8 is a lymphocyte and neutrophil chemoattractant and activation factor. Its levels are increased in RA recruiting cells to the inflamed joints. Serum levels of IL-8 decreases upon anti-TNF treatment in RA. IL-8 is produced at low basal levels in CNS in normal conditions and up regulated in pathology. Sources of IL-8 in CNS are activated microglia, astrocytes, endothelial cells and infiltrating neutrophils. IL-8 has chemotactic properties acting on neutrophils and naive T-cells recruiting them into CNS and once inside directing them towards the place of inflammation.\[76\]

### 1.9.7 Amyloid

Numerous physiological properties have been attributed to the amyloid precursor protein (APP) including neuronal migration, neurite outgrowth, synaptogenesis and maintenance of synaptic structures in the developing and adult brain. However, the best elaborated role of APP lies in the generation of the amyloid-β peptide (Aβ) which is highlighted as one major contributor to AD. Different metabolic pathways cleave APP into different amyloid precursor protein metabolites such as APPα, APPβ and Aβ-oligomers.

![Amyloid metabolism](ipeltan_wikimedia_commons)

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**Figure 13. Amyloid metabolism.** Trans membranous APP is cleaved e.c. by α-secretase or β-secretase generating APPα or APPβ. The remaining e.c. APP is cleaved at different sites by γ-secretase generating Aβ-oligomers of different length. (With permission from Ipeltan, Wikimedia Commons)
Extracellular deposition of amyloid precursor protein degradation products and abnormally hyperphosphorylated Tau in the hippocampus, an area of the brain important for memory and spatial navigation, are hallmarks of AD\textsuperscript{81}. 
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2 AIMS

To study the role for Flt3-L in human and experimental rheumatoid arthritis

To study the role of Flt3/Flt3-L signalling in experimental arthritis using a synthetic inhibitor of Flt3

To study the place for Flt3-L in autoimmune processes in the CNS
3 PATIENTS AND METHODS

The purpose of this section is to provide a brief overview of the materials and methods used in the work of this thesis. In more detail this is described in the papers of the thesis.

3.1 Patients

3.1.1 RA

Paired serum (S) and synovial fluid (SF) samples were collected from 130 RA patients who attended the rheumatology clinics at Sahlgrenska University Hospital, Göteborg for acute joint effusion. RA was diagnosed according to the American College of Rheumatology criteria. Blood samples from healthy individuals (n=70; mean age 52 and age range 18-73; 54 females and 16 males) were used as controls. In addition, synovial fluids from patients with traumatic knee joint injuries and osteoarthritis were used as controls (n=37; mean age 47 and age range 22-88; 17 females and 20 males).

<table>
<thead>
<tr>
<th></th>
<th>RA-patients, females, (n=90)</th>
<th>RA-patients, males, (n=40)</th>
<th>Control subjects, serum, (n=70)</th>
<th>Control subjects, synovial fluid (n=37)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (SD)</td>
<td>61 (17)</td>
<td>59 (13)</td>
<td>52 (11)</td>
<td>47 (21)</td>
</tr>
<tr>
<td>Disease duration, years (range)</td>
<td>10 (0-41)</td>
<td>9 (0-39)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>White blood cells, mean (SD)</td>
<td>8.2 (2.6)</td>
<td>7.9 (2.2)</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>RF positive</td>
<td>51 (57%)</td>
<td>27 (68%)</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>Erosive disease</td>
<td>51 (57%)</td>
<td>27 (57%)</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>DMARD treatment</td>
<td>51 (57%)</td>
<td>24 (80%)</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>Methotrexate (Mtx) alone</td>
<td>25</td>
<td>12</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mtx and other DMARD</td>
<td>15</td>
<td>8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mtx and TNFα-IL-1-inhibitor</td>
<td>10</td>
<td>4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Other DMARDs and TNFα-inhibitor</td>
<td>1</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>No DMARD treatment</td>
<td>39</td>
<td>16</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 1. Patient characteristics. SD = standard deviation, n.a. = not assessed, DMARD = Disease Modifying Anti Rheumatic Drug. The table is previously published in paper I and published with permission from PLoS ONE.

3.1.2 pSS, AD and FM

Paired serum and cerebrospinal fluid (CSF) samples were collected from patients with pSS (n=15), fibromyalgia (FM) (n=29). CSF was obtained from 39 patients with AD. pSS was diagnosed according to the Classification criteria for Sjögren’s syndrome proposed by the American-European
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Consensus Group 2002. FM was defined by the ACR 1990 criteria. AD was diagnosed using the Diagnostic and Statistical Manual of Mental Disorders and National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer’s Disease and Related Disorders Association criteria of dementia and probable AD.

<table>
<thead>
<tr>
<th></th>
<th>pSS (n=15)</th>
<th>FM (n=29)</th>
<th>AD (n=39)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years, SD</td>
<td>48 ±12</td>
<td>49±8</td>
<td>69±7</td>
</tr>
<tr>
<td>Gender, f/m</td>
<td>15/0</td>
<td>29/0</td>
<td>22/17</td>
</tr>
<tr>
<td>Salivary gland biopsy,</td>
<td>2.5 (1-5)</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>fold score/4 mm²</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANA, positive</td>
<td>11</td>
<td>0</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

Table 2. Patient characteristics. Six of 15 pSS patients, were treated with immunosuppressive drugs (azatioprin 2, cyclophosphamide 2, hydroxychloroquine 3, methotrexate 1). Oral prednisolone had one patient.

3.2 Mice experiments

3.2.1 Experimental arthritis

Peptidoglycan
The Staphylococcus Aureus cell wall component peptidoglycan (Pep) triggers arthritis in a dose-dependent manner when injected intraarticularly. A single injection in mice knee joint (20 – 100 μg/joint) gives rise to arthritis lasting at least 14 days, occasionally erosive, with an abundance of macrophages and polymorphnuclear cells. Inhibition studies on mice have given evidence for combined impact of acquired and innate immune system for development of peptidoglycan induced arthritis.
Twenty-seven healthy 6-weeks old female NMRI-mice were injected i.a. with Pep, 10 ng/knee, to induce arthritis. In thirteen of these mice we also injected 2 ng/knee of recombinant mouse Flt3-L together with the peptidoglycan. All mice were killed after three days and the knee joints were prepared for histological evaluation.

**Intra-articular transfer of Flt3-L secreting cells**

The mouse hybridoma cell line Sp2.0 (*kindly provided by prof. Robert Rottapel, Dept. of Immunology, University of Toronto, Canada*) transfected with gene for soluble Flt3-L and it’s identical non transfected clone was injected in healthy 6-weeks old female Balb/C mice i.a. in knee joint or intra-peritoneally (Table 3). Mice were killed 3, 7 and 30 days after injection and then the knee joints were prepared for histological evaluation.

<table>
<thead>
<tr>
<th>Number of Sp2.0 cells</th>
<th>Day 3</th>
<th>Day 7</th>
<th>Day 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>1x10^7 i.p.</td>
<td>6</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>1x10^6 i.a.</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>1x10^5 i.a.</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>1x10^4 i.a.</td>
<td>13</td>
<td>13</td>
<td>10</td>
</tr>
<tr>
<td>1x10^3 i.a.</td>
<td>5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Table 3. Injection of Sp2.0 cells. Abbreviations: i.p.=intra peritoneally, i.a.=intra articularly*

**mBSA-induced arthritis treated with sunitinib**

Intraarticular injection of methylated Bovine Serum Albumin (mBSA) in a mice preimmunized with mBSA in a potent adjuvant leads to chronic, T-cell dependent antigen induced erosive arthritis. The histopathological features of mBSA-induced arthritis resemble those of human RA. This model of experimental arthritis has a almost 100 % incidence of arthritis and the time point of induction of arthritis is known enabling start of treatment at known time points in the arthritis process.

**Histological evaluation of joints**

Lower paws of sacrificed mice were removed, fixed, decalcified in Parenzy’s buffer, paraffin embedded and cut into 4µm thin sections. Tissue sections were stained with haematoxylin/eosin. The sections were evaluated by a
Differentiation factor Fms-like tyrosine kinase 3 ligand is a modulator of cell responses in autoimmune disease.

Blinded examiner with respect to inflammatory cell accumulation in synovial tissues (synovitis) and to development of bone/cartilage destruction. Synovitis was defined as a membrane thickness of more than two cell layers, and scored as follows: 1, mild; 2, moderate; and 3, severe synovitis and joint damage (Figure 14). The arthritis index in each group was calculated as a sum of all scores divided by the number of joints. The presence of destructions was registered in each joint and presented as frequency (%) to total number of injected joints.

Figure 14. 1-4. Representative images of joint destruction and inflammation in mBSA-induced arthritis. Arthritis index was calculated on the basis of morphological evaluation of the joints with respect to inflammatory cell infiltration in synovial tissues (synovitis) and to development of bone/cartilage destruction. Representative images show arthritis of different severity and scored from 0 (1), 1 (2), 2 (3) and 3 (4). Bone destruction is indicated by arrows (4). Abbreviations: C=cartilage, J=joint space, Sy=Synovium, M=meniscus, P=pannus, E=erosion (destruction). Horizontal bar in the right corner of each image corresponds to 100 micrometres. The figure is previously published in paper II and published with permission from J of Leukocyte Biology.
Sunitinib treatment
Sunitinib (SU11248, Pfizer) was provided by gavage once daily with two different dosages, 10 mg/kg mouse and 40 mg/kg mouse (Fig 15). The treatment regimen used was adopted from mouse models of various established xenografts derived from human or rat tumour cell lines. The drug was provided with starting from vaccination day 7 (n=30) and from the day of intra-articular injection of mBSA, day 21 (n=30), and continued to the end of experiment on day 28. Control mice received citrate buffer by gavage between days 7 and 28. Two independent experiments were performed containing total 75 mice and the results were pooled.

Figure 15. Experimental study design. Balb/c mice were immunized with mBSA on day 0 and 7. The Flt3 inhibitor sunitinib was administered once daily at doses of 10 mg/kg and 40 mg/kg. Treatment was started on day 7 (n=30) and on day 21 (n=30) and continued until day 28. Controls (n=30) received citrate buffer starting from day 7. The figure is previously published in paper II and published with permission from J of Leukocyte Biology.

3.3 Ethical considerations
All studies on patients were conducted in compliance with the Declaration of Helsinki and approved by the Ethics Committee of Sahlgrenska University Hospital (385-04, 220-09). All patients gave written consent to participate in the study. All animal experiments were approved by the Ethics Committee of Göteborg University (28-2007).
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3.4 Methods

Migration assay
The migratory capacity of mouse bone marrow cells was tested using the Transwell system. $5 \times 10^5$ cells/well were placed in the upper chamber and migrated towards Flt3-L containing media (200 ng/ml) or 0.1% BSA-PBS as negative control. Migrated cells were collected from lower chamber and analysed by flow cytometry.

Figure 16. The Transwell system. Cells in the upper chamber migrated towards Flt3-L containing media. Migrated cells were collected from lower chamber and analysed by FACS. Figure adapted with permission from Mikael Brisslert.

Flow cytometry
Flow cytometry was employed to distinguish phenotypes of cells. Bone marrow cells were collected from femur and tibias and splenocytes were mashed through a cell strainer and rinsed with PBS. Erythrocytes were lysed in NH$_4$Cl solution. After incubating with Fc block to eliminate non-specific binding of antibodies to the Fc-receptor, cells were incubated with primary antibodies against different cell surface markers to identify cell phenotypes.

Precursors of DC in bone marrow were defined as Flt3$^+$ cells followed by further analysis in B220$^+$, CD3$^-$, and CD11c$^+$ cell populations. DC in spleen populations were defined as CD11c$^+$ followed by analysis with respect to B220, CD4, CD8, and CD11b phenotype markers. Splenic plasmacytoid DC (pDC) were defined as B220$^-$CD11c$^+$, while conventional DC (cDC) were defined as B220$^+$CD11c$^+$ as proposed. The cDC were further divided into three different subsets, CD4$^+$CD8α$^-$, CD4$^+$CD8α$^+$, CD4$^+$CD8α$^+$.
Measure Flt3-L
Flt3-L levels were determined by ELISA (R&D Systems, Minneapolis, MN, USA). Serum samples were diluted 1:2 respectively 1:10 in PBS containing 1% BSA. Synovial fluid samples were diluted 1:10 and CSF fluid samples undiluted. Minimal detectable level was 10 pg/ml.

3.5 Statistics
Data are presented as median and range or mean and standard deviation (SD). Throughout the thesis non-parametrical statistical methods, the Mann-Whitney U-test was used to analyse differences in continuous variables between groups with two exceptions. $\chi^2$-test was performed to evaluate differences on the frequency of arthritis in the peptidoglycan induced arthritis and in the mice that received the cell line overexpressing Flt3-L. Relationships between variables were examined by Spearman’s correlation coefficient. All statistical evaluation of data was done using the statistic programmes Microsoft Excel 2004, Prism 4 and SPSS 11.
4 RESULTS

To investigate the role of the growth factor Flt3-L in rheumatic disease we induced Pep induced arthritis in mice with addition of Flt3-L. Suboptimal doses of Pep were chosen not to overshadow the possible effects of Flt3-L.

4.1 Adjuvant properties of Flt3-L

The histological findings of arthritis in the Pep-injected NMRI-mice were of moderate severity. Microscopic examination of the knee joints showed significantly higher (p<0.05) frequency of arthritis of the mice co-injected with Pep and Flt3-L. Indeed 12 of 13 (92%) mice showed signs of mild to moderate arthritis compared to 8 of 14 (57%) in the group that only received Pep. Notably, a single i.a. injection of Flt3-L (0.02 ng, 0.2 ng, 2 ng/joint) did not cause joint inflammation.

Thus, Flt3-L injected at one time point worsens arthritis. To further investigate the effects of increased levels of Flt3-L in the joint compartment we analysed the possible chemotactic properties of Flt3-L.

4.2 Flt3-L display chemotactic properties

Bone marrow cells from healthy mice were subjected to migration for 12 h towards a Flt3-L rich medium and a control, Flt3-L deficient medium. The cells migrated in a significantly higher number towards the Flt3-L containing chamber (%: 2.9 ± 1.2 vs. 1.4 ± 0.5 in Flt3- controls, p=0.0007). Flow cytometry of the migrated cells showed no differences in number of DC progenitors (CD11c\(^{-}\)Flt3\(^{+}\)) in the migrated cells. However, percentages of CD3\(^{+}\) cells (%: 15 ± 3 vs. 62 ±17 in Flt3L\(^{-}\) controls, p=0.0007) and CD11c\(^{+}\) cells (%: 30 ± 5 versus 60 ± 16 in Flt3L\(^{-}\) controls, p=0.0007) were higher in the controls, suggesting that the migrating cells were mostly composed of less differentiated bone marrow cells. Thus, we show that Flt3-L is a potent chemoattractant facilitating mobilization of Flt3\(^{+}\) cells from the bone marrow.

In order to resemble in vivo role of continuous Flt3-L exposure in the joint we transplanted Flt3-L secreting cells into healthy mouse joints.
4.3 Intra-articular transfer of Flt3-L secreting cells

Balb/C mice that received i.a. 1x10⁴ Balb/C derived B cell clone transfected with the gene for murine Flt3-L developed after three days histopathological signs of arthritis. Indeed, there was a significantly higher frequency of arthritis since 8/13 (62%) mice developed joint inflammation compared to 3/13 (23%)(p<0.05) (Table 4) in the control group. After seven days most of the arthritic process has disappeared since only 2/13 mice in each group showed signs of arthritis (Table 4). The mice that were killed thirty days following injection showed no difference between the groups in frequency of arthritis but there were signs of bone erosions only in the joints that received Flt3-L expressing cells. Five out of ten of the knee joints injected with Flt3-L-expressing cells showed severe signs of arthritis, and all these joints also showed great tumour masses intra- and extraarticularly. Three out of five joints in this group also showed severe destruction of bone. In the control group 4/10 showed signs of arthritis but great tumour masses were only seen in one of the controls and no signs of bone erosions were visible (Table 4, Figure 17).

<table>
<thead>
<tr>
<th>Number of Sp 2.0 Filt3-L+/- cells</th>
<th>Day 3</th>
<th>Day 7</th>
<th>Day 30</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Arthritis</td>
<td>Arthritis</td>
<td>Arthritis</td>
</tr>
<tr>
<td>1x10⁴ i.a. Flt3-L+</td>
<td>8/13</td>
<td>2/13</td>
<td>5/10</td>
</tr>
<tr>
<td>1x10⁴ i.a. Flt3-L-</td>
<td>3/10 p&lt;0.05</td>
<td>2/13</td>
<td>4/10</td>
</tr>
</tbody>
</table>

Table 4. Frequency of arthritis and erosions 3, 7 and 30 days following i.a. transfer of 1x10⁴ Balb/C derived B cell clone (Sp 2.0) transfected with the gene for murine Flt3-L compared to control group injected with the same B cell clone not transfected with the gene.

There were no signs of arthritis when the number of hybridoma cells exceeded 1x10⁵/knee or if the cells were provided i.p.
Differentiation factor Fms-like tyrosine kinase 3 ligand is a modulator of cell responses in autoimmune disease

Figure 17. Joint histology. A Balb/C mouse knee joint 30 days following intra-articular transfer of cells overexpressing Flt3-L. Arrows indicate bone erosions. B Balb/C mouse knee joint 30 days following intra-articular transfer of cells not overexpressing Flt3-L. Arrows indicate bone erosions. Abbreviations: C=cartilage, M=meniscus, S=synovitis. The histology is previously published in paper I and published with permission from PLoS ONE.

Thus, continuous exposure in vivo to Flt3-L induces arthritis in mice with significant articular erosivity.

To verify the effects of Flt3-L on arthritis and erosivity inhibition of Flt3, the receptor for Flt3-L, was performed with sunitinib in a model of antigen-induced arthritis in mice.

4.4 mBSA-induced arthritis treated with sunitinib

Balb-c mice with mBSA arthritis and sunitinib treatment were evaluated for degree of synovitis and erosivity. In addition, effects of Flt3 inhibition with sunitinib upon effector cells, antibody production and bone metabolism were evaluated.

4.4.1 Inhibition of Flt3 reduces the frequency and severity of mBSA-induced arthritis

Arthritis index was constructed for each group and showed a significant reduction in the severity of arthritis in the sunitinib-treated mice as compared with controls (Fig. 18). Indeed, the arthritis index was reduced in direct proportion to the sunitinib dose and to the duration of treatment. The arthritis
index was reduced significantly in mice treated with a sunitinib dose of 40 mg/kg between days 7 and 28, compared with citrate buffer-treated controls (0.8±1.0 vs. 1.5±0.75 in the control group; \( p=0.02; \) Fig. 18). Treatment with sunitinib between days 21 and 28 had no significant effect on the arthritis index (Fig. 18). Sunitinib treatment led to a dose-dependent reduction in bone-destruction score in mice treated days 7-28 (Fig. 18) and from day 21 (Fig. 18).

**Figure 18. Arthritis index and destruction.** Balb/c mice treated with sunitinib between days 7-28 (10 mg/kg, S10, n=15; 40 mg/kg, S40, n=15) showed a dose-dependent reduction in the arthritis index as compared to control mice treated with citrate buffer (n=30) (1) and in the incidence of bone and cartilage destruction (2). Balb/c mice treated between days 21-28 (10 mg/kg, S10, n=15; 40 mg/kg, S40, n=15) had no reduction of arthritis index (3), while the reduction of bone and cartilage destruction was obvious (4). The figure is previously published in paper II (ref nr). Published with permission from J of Leukocyte Biology.

### 4.4.2 Inhibition of Flt3 reduces formation of DCs

DC precursors were evaluated by FACS analysis performed on bone marrow cells from 24 mBSA immunized mice, 12 of which had been treated with sunitinib. The total number and per cent of Flt3+ cells in the bone marrow of sunitinib treated mice was similar to that of control mice (Table 5). However, the number of Flt3+ cells was significantly reduced in sunitinib treated mice within CD3+ population (Table 5), while CD11c+Flt3+ population (Table 5) and B220+Flt3+ population were similar to controls (Table 5).

**Table 5. Sunitinib effects on bone marrow cells.** Sunitinib treatment (40 mg/kg, days 7-28) reduced Flt3 expression on bone marrow cells. Sunitinib reduced the number of Flt3+ cells in the subpopulation of CD3+, while the population of CD11c+ as well as the population of B220+ in sunitinib treated mice was similar to citrate buffer treated controls.
Differentiation factor Fms-like tyrosine kinase 3 ligand is a modulator of cell responses in autoimmune disease

CD11c^+ precursors of DC were analysed in spleens of sunitinib treated mice (n=9) and citrate buffer treated controls (n=9). Sunitinib treated mice displayed a 36% reduction in the total CD11c^- population when compared to controls (Table 6). Consequently, sunitinib treated mice had a significantly smaller population of plasmacytoid (p)DCs (Table 6) and of conventional (c)DCs (Table 6). Further analysis showed that sunitinib treated mice had a significant reduction of all cDC populations in comparison to controls (Table 6). Sunitinib treatment had no effect on spleen B and CD4^+ T cell populations while the population of CD8^+ T cells was increased in sunitinib treated mice (Table 6).

<table>
<thead>
<tr>
<th>Spleen cells</th>
<th>Sunitinib, n=9</th>
<th>Control, n=9</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11c+, %</td>
<td>3.5 ± 0.4</td>
<td>5.5 ± 0.5</td>
</tr>
<tr>
<td>pDC, CD11c+B220+, %</td>
<td>2.1 ± 0.5</td>
<td>3.4 ± 0.5</td>
</tr>
<tr>
<td>cDC, CD11c+B220-, %</td>
<td>2.0 ± 0.5</td>
<td>3.2 ± 0.8</td>
</tr>
<tr>
<td>cDC, CD11c+B220-CD4+, %</td>
<td>0.6 ± 0.2</td>
<td>1 ± 0.3</td>
</tr>
<tr>
<td>cDC, CD11c+B220-CD8+, %</td>
<td>0.7 ± 0.1</td>
<td>1 ± 0.3</td>
</tr>
<tr>
<td>cDC, CD11c+B220-CD4-CD8-, %</td>
<td>0.7 ± 0.1</td>
<td>1 ± 0.2</td>
</tr>
<tr>
<td>B-cell, B220+, %</td>
<td>61 ± 11</td>
<td>59 ± 9</td>
</tr>
<tr>
<td>T-cell, CD4+, %</td>
<td>31 ± 4</td>
<td>26 ± 1</td>
</tr>
<tr>
<td>T-cell, CD8+, %</td>
<td>13.7 ± 1.7</td>
<td>10.7 ± 1.4</td>
</tr>
</tbody>
</table>

Table 6. **Sunitinib effects on spleen cells.** Sunitinib treatment (40 mg/kg, days 7-28) reduced B220+CD11c+ population of plasmacytoid DCs and all sub-populations of B220-CD11c+ conventional DCs in the spleen. CD8^+ T-cell population was increased in spleen upon sunitinib treatment.
4.4.3 Inhibition of Flt3 decreases antibody production

Sunitinib treatment resulted in a significant reduction in the number of splenic CD11c^+ cDCs as well as pDCs, while no reduction in mature B and T cell populations was observed. To assess if this reduction in the DC population was functionally significant for T cell-dependent antigen presentation, titres of antibodies against mBSA, as well as autoantibodies to cyclic citrullinated peptide (aCCP) and to the Fc-gamma part of Ig (known as rheumatoid factor, RF) were measured in sunitinib treated mice and citrate buffer treated controls.

Sunitinib treatment on days 7-28 significantly reduced the levels of mBSA-specific antibodies as compared to controls (Table 7). Analogously, sunitinib caused a significant reduction in the levels of anti-CCP antibodies (Table 7), while the levels of RF were not changed (Table 7).

<table>
<thead>
<tr>
<th>Antibody, absorbance</th>
<th>Sunitinib, n=15</th>
<th>Control, n=7</th>
</tr>
</thead>
<tbody>
<tr>
<td>mBSA, IgG</td>
<td>0.8 ± 0.3</td>
<td>1.5 ± 0.3  p&lt;0.0006</td>
</tr>
<tr>
<td>Anti-CCP, IgG</td>
<td>0.8 ± 0.4</td>
<td>1.2 ± 0.4  p&lt;0.02</td>
</tr>
<tr>
<td>Anti-Fc, IgG</td>
<td>0.9 ± 0.5</td>
<td>1 ± 0.4</td>
</tr>
</tbody>
</table>

*Table 7. Sunitinib effects on antibody production. Sunitinib treatment (40 mg/kg, days 7-28) reduced levels of antibodies to mBSA and cyclic citrullinated peptides (anti-CCP) while the levels of antibodies to Fc-IgG (RF) were not changed.*

4.4.4 Inhibition of Flt3 increases Flt3-L

Sunitinib treatment was accompanied by a pronounced, significant increase in circulating Flt3L as compared to citrate buffer treated controls. This effect was observed irrespectively of dose or duration of treatment (Fig 19A). Levels of VEGF did not show this pattern and were only significantly increased with high dose of sunitinib initiated at day 21 (Fig 19B).
Differentiation factor Fms-like tyrosine kinase 3 ligand is a modulator of cell responses in autoimmune disease

4.4.5 Inhibition of Flt3 decreases bone metabolism

In agreement with the morphological findings, mice treated with sunitinib on days 7-28 displayed significantly reduced serum levels of the bone degradation marker CTX-I when compared to citrate buffer treated controls (ng/ml: 18±6.5 versus 24±3.8, p=0.018. Figure 20). Serum levels of the cartilage degradation marker, CTX-II, were not changed significantly by sunitinib treatment (ng/ml: 7.3±7.8 versus 6.8±5.4).

Levels of osteoclast regulators OPG and RANKL also showed correlation to the morphological findings. Treatment of low dose sunitinib, 10 mg/kg, between days 21 and 28 led to a significant reduction of the osteoclast inducing factor RANKL (Table 8). Levels of the osteoclast maturation...
inhibitor OPG were not changed significantly; however, the OPG/RANKL ratio was increased in the sunitinib treated mice supporting bone formation (Table 8).

| Table 8. Sunitinib effects on bone metabolism. Treatment of low dose sunitinib, 10 mg/kg, between days 21 and 28 led to a significant reduction of the osteoclast inducing factor RANKL. The OPG/RANKL ratio was increased in the sunitinib treated mice supporting bone formation. Abbreviations: osteoproteogrin = OPG, receptor activator of nuclear factor kappa-B ligand = RANKL.

Thus, inhibition of Flt3 in mBSA arthritis decreases antigen-induced immune responses, synovial inflammation and bone resorption. The inhibitory effects are dose-dependent and potentially mediated through inhibition of DC formation and antigen-presentation and effects on bone metabolism.

To investigate the role of growth factor Flt3-L in human rheumatic disease levels of Flt3-L were measured in matched serum and synovial fluid samples from 130 RA patients and 107 controls.

### 4.5 Flt3-L in RA SF and serum

We found significantly higher levels of Flt3-L in the SF (mean 218 pg/ml, SEM 19) as compared to serum levels (mean 141 pg/ml, SEM 35) in RA patients (p=0.0001)(Figure 21). In addition, RA synovial fluid levels of Flt-3-L were significantly higher than these obtained from synovial fluids originating from non-inflammatory joint diseases (mean 132 pg/ml, SEM 12, p=0.022). There was no significant difference in the circulating Flt3-L levels between patients and controls (mean 74 pg/ml, SEM 4). Interestingly,
controls with degenerative/traumatic joint diseases also showed significantly elevated levels of Flt3-L in SF compared to serum (p=0.0001)(Figure 21).

**Figure 21. Flt3-L levels in synovial fluids and sera of RA patients and control subjects.** Median displayed in the horizontal line. RA serum (n=130, median 80, min-max (0–3320), RA SF (n=130, median 160 pg/ml, min-max (20–1980), control subjects serum (n=70, median 70.5 pg/ml, min-max (0–160), and control subjects SF (n=37, median 120 pg/ml, min-max (0–360). The figure is previously published in paper I and published with permission from PLoS ONE.

Further statistical analysis showed that Flt3-L had a positive correlation to age, both regarding serum and synovial fluid levels. This was not seen in the controls. The RF+ patients displayed significantly increased (p<0.0001) levels of Flt3-L in serum but not in SF compared to RF- patients. The older cohort of RA-patients (>53 years, n=97) had significantly higher levels of Flt3-L both in serum (p=0.0155) and SF (p=0.0232) compared to the younger cohort (<53 years, n=33).

Thus, Flt3-L accumulates in the synovial fluid of RA patients. The highest levels are seen in two groups of RA patients with bad prognostic factors, namely RF+ patients and patients of high age.

Flt3-L is linked to immunoglobulin production through it’s effects on development of B-cells and DCs following antigen presentation. A rheumatic disease characterized by B cells and a huge production of various antibodies is pSS with its variety of autoantibodies, polyclonal IgG and cryoglobulins. Levels of Flt3-L were measured in serum and CSF from patients with pSS and related to markers of inflammation and degeneration. For comparison we chose two non-inflammatory conditions with CNS involvement, AD and FM.
4.6 Flt3-L in pSS

4.6.1 Clinical characteristics of the patient material

All the patients in pSS and FM groups were females, while 17 patients with AD were men (Table 9). pSS and FM patients were younger than AD patients. Three of 15 pSS patients reported CNS symptoms (depression, cognitive impairment and change in personality). All patients in the AD group had severe cognitive deficiency. No cognitive impairments were reported for FM patients, while depression was registered in 38% (11 of 29) FM patients. Fatigue was observed as a major problem for the pSS and FM patients, and was similar between these groups. pSS patients experienced significantly less pain than FM patients (p=0.002) (Table 9).

<table>
<thead>
<tr>
<th></th>
<th>pSS n=15</th>
<th>FM n=29</th>
<th>AD n=39</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>48±12</td>
<td>49±8</td>
<td>69±7</td>
</tr>
<tr>
<td>Gender, f/m</td>
<td>15/0</td>
<td>29/0</td>
<td>22/17</td>
</tr>
<tr>
<td>Salivary gland biopsy, foci score/4 mm²</td>
<td>2.5 (1-5)</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>ANA, positive</td>
<td>11</td>
<td>0</td>
<td>n.a.</td>
</tr>
<tr>
<td>Fatigue, VAS mm</td>
<td>90 (10-100)</td>
<td>77 (11-99)</td>
<td>n.a.</td>
</tr>
<tr>
<td>Pain, VAS mm</td>
<td>21 (0-99)</td>
<td>72 p=0.002 (7-97)</td>
<td>n.a.</td>
</tr>
<tr>
<td>IgG serum, g/L</td>
<td>18 (9-29)</td>
<td>12 p=0.0006 (6-17)</td>
<td>n.a.</td>
</tr>
<tr>
<td>IgG CSF, mg/L</td>
<td>59 (14-143)</td>
<td>26 p=0.003 (12-81)</td>
<td>n.a.</td>
</tr>
<tr>
<td>Flt3-ligand, pg/ml serum</td>
<td>109 (78-693)</td>
<td>127 (74-837)</td>
<td>n.a.</td>
</tr>
<tr>
<td>Flt3-ligand, pg/ml CSF</td>
<td>53 p=0.004 (28-76)</td>
<td>65 (46-95)</td>
<td>45 p=0.0003 (21-111)</td>
</tr>
</tbody>
</table>

Table 9. Patient characteristics. Six of 15 pSS patients, were treated with immunosuppressive drugs (azathioprin 2, cyclophosphamide 2, hydroxychloroquine 3, methotrexate 1). Oral prednisolone had one patient.
Differentiation factor Fms-like tyrosine kinase 3 ligand is a modulator of cell responses in autoimmune disease

### 4.6.2 Flt3-L in serum and CSF

![Graph A: Flt3-L serum](image)

![Graph B: Flt3-L CSF](image)

**Figure 22. Flt3-L in serum and CSF.** Levels of Flt3-L in serum showed no difference between pSS and FM patient groups. Levels of Flt3-L were significantly higher in FM patients compared to pSS and AD.

Levels of Flt3L in CSF were significantly higher in FM patients compared to pSS and AD (*Fig 22B*). Serum levels of Flt3L were significantly higher compared to CSF (*p*<0.0001) and showed no difference between the pSS and FM patient groups (*Fig 22A*). Serum levels of Flt3L in patients with pSS were neither related to the inflammation score in salivary gland biopsies nor to the presence of ANA. Levels of Flt3L in CSF showed no correlation to age and had no gender difference. Neither fatigue nor pain showed correlation to CSF levels of Flt3L in pSS and FM patients. Five of 15 pSS patients displayed oligoclonal bands of IgG in CSF but there were no differences in Flt3L when pSS patients were stratified accordingly.
4.6.3 Flt3-L and microglia regulators

CSF levels of IL-6, IL-8, MMP-3 and MCP-1 (Fig 23A-D) were somewhat higher in pSS patients compared to the FM and AD groups but the difference did not reach statistical significance.

![Figure 23. Levels of IL-6, IL-8, MMP3 and MCP-1 in CSF. CSF levels of IL-6 (A), IL-8 (B), MMP3 (C) and MCP-1 (D) in pSS, FM and AD showed no significant differences.](image)

CSF levels of Flt3L correlated with MCP-1 both in patients with pSS (r=0.714, p=0.03) (Fig 24A), and with FM (r=0.667, p<0.001) (Fig 24B).

![Figure 24. Spearman correlation between CSF Flt3-L and MCP-1. CSF levels of Flt3-L and MCP-1 correlate in pSS (A) and FM (B).](image)
Differentiation factor Fms-like tyrosine kinase 3 ligand is a modulator of cell responses in autoimmune disease

### 4.6.4 Flt3-L and Tau / pTau

CSF levels of Flt3L correlated to T-tau levels in all patient groups (*Table 10*). The strongest correlation was seen in pSS patients, followed by FM and AD. Furthermore, Flt3-L correlated to P-tau in pSS and FM (*Table 10*).

<table>
<thead>
<tr>
<th>Correlations</th>
<th>pSS, n=15</th>
<th>FM, n=29</th>
<th>AD, n=39</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF Flt3-L – T-tau</td>
<td>r=0.679, p=0.005</td>
<td>r=0.49, p=0.008</td>
<td>r=0.45, p=0.006</td>
</tr>
<tr>
<td>CSF Flt3-L – P-tau</td>
<td>r=0.646, p=0.009</td>
<td>r=0.41, p=0.028</td>
<td>r=0.27, p=n.s.</td>
</tr>
</tbody>
</table>

*Table 10. Spearman correlation between CSF Flt3-L and MCP-1. CSF levels of Flt3-L correlates positively to T-tau in pSS, FM and AD. P-tau correlates positively to P-tau in pSS and FM. Spearmans rho(r) and two-tailed significance (p) are indicated in the table.*

As expected, the fraction of phosphorylated-tau (P-tau) was highest in AD patients (Fig 25 B).

![Figure 25. Levels of T-tau and P-tau. CSF levels of T-tau (A) and P-tau (B) in pSS, FM and AD. *AD patients were analysed by xMAP technology that gives different absolute concentrations than the corresponding ELISA measurements of T-tau and P-tau performed on pSS and FM.*](image-url)
4.6.5 Flt3-L and amyloid biomarkers APPα/β and Aβ 38, 40, 42

The strong correlation between CSF Flt3L and tau proteins in pSS and FM encouraged us to evaluate levels of sAPPα and sAPPβ and their degradation products β-amyloid (Aβ) 38, 40 and 42, the latter of which reflects brain amyloid build-up in AD by correlating inversely to senile plaque counts. Comparison of APP in CSF of pSS and FM patients showed that patients with pSS had lower levels of APPβ (Table 11) and proportionally low levels of Aβ40 and Aβ42 (Table 11). As expected, AD levels of Aβ42 are very low.

<table>
<thead>
<tr>
<th>Amyloid biomarkers</th>
<th>pSS, n=15</th>
<th>FM, n=29</th>
<th>AD, n=39</th>
</tr>
</thead>
<tbody>
<tr>
<td>APPα, ng/mL</td>
<td>671 (191-1264)</td>
<td>744 (396–1188)</td>
<td></td>
</tr>
<tr>
<td>APPβ, ng/mL</td>
<td>263 (62-556)</td>
<td>383(138-648)$^{p=0.02}$</td>
<td></td>
</tr>
<tr>
<td>Aβ38, ng/mL</td>
<td>1974 (625-3208)</td>
<td>2204 (688-5689)</td>
<td></td>
</tr>
<tr>
<td>Aβ40, ng/mL</td>
<td>8549(3957-12028)</td>
<td>10357(4560-19197)$^{p=0.03}$</td>
<td></td>
</tr>
<tr>
<td>Aβ42, ng/mL</td>
<td>874(331-1292)</td>
<td>1050 (451-1672)$^{p=0.01}$</td>
<td>173(113-333)</td>
</tr>
</tbody>
</table>

Table 11. Levels of CSF amyloid biomarkers. CSF levels of APPβ, Aβ 40 and Aβ42 are significantly decreased in pSS compared to FM.

Flt3-L level below those of 97% of the FM group were considered low = 47 pg/ml. Low levels of Flt3L were found in 33% of pSS and 61% of AD. Patients with low Flt3L were similar to the rest of the group with respect to age, symptoms of fatigue and pain, and CSF levels of IgG. All 3 pSS patients with CNS symptoms had normal levels of Flt3L.
Differentiation factor Fms-like tyrosine kinase 3 ligand is a modulator of cell responses in autoimmune disease

pSS patients with low levels of Flt3L had also low levels of T-tau (Fig 26 A) and P-tau (Fig 26 B). pSS patients with low CSF Flt3L had also lower levels of GFAP (Fig 26 C)(ng/L: 555[298-860] vs. 350[170-440], p=0.04) while there was no difference in levels of NFL (Fig 26 D).

Figure 26. Levels of T-tau, P-tau, GFAP and NFL in pSS with low CSF levels of Flt3-L. pSS patients with low levels of Flt3L had also low levels of T-tau (A), P-tau (B) and GFAP (C) while there was no difference in levels of NFL (D).
pSS patients with low levels of Flt3L displayed a further decrease of Aβ42 levels compared to pSS patients with normal levels of Flt3-L (Fig 27 A). Additionally, pSS patients with low levels of Flt3L had lower levels of MCP-1 (p=0.008) (Fig 27 B).

![Figure 27](image)

**Figure 27.** Levels of CSF Aβ42 and MCP-1 in pSS patients with low levels of CSF Flt3-L. pSS patients with low levels of CSF Flt3L displayed significantly decreased levels of Aβ42 (A) and MCP-1 (B) in CSF compared to pSS patients with normal levels of CSF Flt3-L.

Thus, Flt3-L in CSF in pSS is strongly correlated to Tau/pTau implying Flt3-L mediated effects on neuroaxonal structures. These effects are also reflected in the supporting glia system. Flt3-L in CSF is linked to activation of microglia. Furthermore, patients with pSS display decreased levels of amyloid biomarker APPβ and Ab42. This relation is most pronounced in the pSS patients with low levels of CSF-Flt3-L.
5 DISCUSSION

There is life without Flt3-ligand, at least in mice. Flt3-L KO mice develop normally but do exhibit reductions in lymphoid and myeloid progenitors in bone marrow and also reduced levels of lymphocytes, NK-cells and dendritic cells in the periphery. In this thesis, I have focused on the role for Flt3-L in autoimmune rheumatic disease.

5.1 Flt3-L, an adjuvant

Flt3-L in rheumatic disease could be considered an adjuvant, an agent that enhances other agents. In part I of my study, Flt3-L when injected in the joint does not induce arthritis on its own but worsens the immune response to peptidoglycan, mBSA or mouse B cells. Flt3-L does not apparently exhibit pro-inflammatory properties on its own, merely helps effector cells to trigger the immunological response. Clonal expansion of dendritic cells by Flt3-L is crucial for antigen presentation and amplified activation of B- and T-cells. Flt3-L stimulates formation of both lymphoid and myeloid progenitors providing effector cells. Flt3-L is expressed locally in the joint recruiting cells through chemotaxis but also exerting direct effects on bone metabolism, probably through induction of osteoclasts. Flt3-L induces formation of microglia and is linked to their activation. Thus, Flt3-L has adjuvant properties increasing effects independent of origin of initial response.

5.2 Why is Flt3-L increased in RA SF?

The DC and the macrophage have been highlighted for their role in the early innate immunological response in arthritis and play a crucial role in the rheumatoid synovium. The healthy synovium is sparsely cellular and is made up of macrophage-like synovial cells (MLS) and fibroblast like synoviocytes (FLS) in a two-cellular layer. The macrophage-like synovial cells express markers of hematopoietic origin most consistent with the monocyte–macrophage lineage (CD11b, CD68, MHC class II and FcR) and are derived from the bone marrow. The FLS are mesenchymal cells that display many characteristics of fibroblasts but do also display unique properties such as expression of the adhesion molecule cadherin-11.

The rheumatoid synovium is a hyper cellular structure where the healthy two-cellular layer is manifold increased. The sublining layer, facing the joint cavity, is infiltrated by T- and B-cells, DCs and NK-cells.
How do these cells find their way to the synovium? Potent chemoattractants, such as MCP-1 and IL-8, are produced in the joint and recruit inflammatory cells. Furthermore, adhesion molecules, such as ICAM-1 and VCAM-1, are expressed on the synovial blood vessels binding and recruiting cells to the synovium. In part II of my study, we showed that Flt3-L display chemotactic properties attracting immature bone marrow cells. Thus, Flt3-L could attract cells in surrounding blood vessels but also in adjacent bone marrow.

Approximately 20% of RA patients display synovial ectopic germinal centres and local autoantibody production takes place. This may contribute to the improper regulation of emerging self-reactive B cells. The intimal synovial layer, facing the joint cartilage and bone, consists of MLS secreting pro-inflammatory cytokines (TNF-α, IL-1β) activating FLS which in turn secrete IL-6, MCP-1 and MMPs and a paracrine/autocrine network is formed perpetuating synovitis. A neovascularized synovial front, pannus, develops and produce villous projections that grow invasively and destruct cartilage and bone. Cartilage is broken down by FLS producing huge amount of proteases. Bone erosion is handled by osteoclasts differentiated in the joint from monocytic progenitors under the influence of RANKL and M-CSF/Flt3-L.

There is a clear correlation between synovial macrophage infiltration and subsequent radiographic joint destruction as well as degree of clinical improvement this further emphasizing the crucial role of the macrophage in RA. Thus, there is a great demand of macrophages in the RA joint. It is not clear whether they arrive differentiated or undergo last stages of maturation in the joint.

Thus, Flt3-L in the joint has a role providing myeloid progenitors from adjacent bone marrow by chemotaxis and stimulate them to proliferate and differentiate. Furthermore, Flt3-L in the joint supports differentiation of osteoclasts.

### 5.3 Why is Flt3-L decreased in pSS and AD CSF?

Part 3 of my study shows that a large proportion of the pSS and AD patients display a pronounced decrease of levels of CSF Flt3-L compared to FM. Low levels of Flt3L were found in 33% of pSS and 61% of AD. Patients with low levels of CSF Flt3L were similar to the rest of the group with respect to age, symptoms of fatigue and pain and CSF IgG-levels. All 3 pSS patients with
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CNS symptoms had normal levels of CSF Flt3-L. The low Flt3-L levels in pSS were accompanied by low levels of T-tau, P-tau and GFAP. This may represent a picture of CNS decline with decreased homeostatic activity presenting itself with diminished axonal and microglial activity. Indeed, Flt3-L has been proposed a diagnostic biomarker in degenerative CNS conditions, for instance separating Parkinson disease from an overlapping condition, multiple systemic atrophy.

The APP metabolite Aβ42 has been highlighted as an early predictor for cognitive decline in healthy individuals. In part 3 of my study, the pSS patients displayed significantly decreased Aβ42 levels compared to FM and this difference was even more pronounced in the pSS patients with low CSF Flt3-L. Interestingly, the area of the brain affected early in AD with deposition of APP metabolites and abnormally hyperphosphorylated Tau is also the CNS region with the highest expression of Flt3 and its ligand, namely the hippocampus. Thus, our findings could be interpreted as deposition of APP metabolites resulting in hippocampal degeneration and loss of Flt3-L expression in pSS.

On the other hand, the combination of decreased levels of amyloid precursor proteins, APP α and β, and their degradation products, Aβ 38-42, have been shown before in multiple sclerosis, SLE and HIV and there considered an inflammatory down-regulation of synthesis or secretion of APP. However, in these earlier studies levels of both amyloid precursors, APP α and β, were decreased. In our study only APP β was affected maybe suggesting a different mechanism.

5.4 Flt3-L – a marker?

5.4.1 Flt3-L in pSS CNS

Microglia, the primary inflammatory effector cell in CNS, has a common origin with macrophages and dendritic cells. They are differentiated by Flt3-L. The chemokine MCP-1 attracts microglia but also induces their proliferation. Microgliosis is considered one of the hallmarks of neuroinflammation. Tau is generally considered a marker for axonal degeneration reflecting degree of damage to CNS. In our study, CSF Flt3-L correlates to CSF MCP-1 and Tau/pTau implying a role for Flt3-L in microglial activation and Tau/pTau turnover. Hence, Flt3-L is correlated to activation of microglia and axonal degeneration, two central aspects of neuroinflammation. However, these correlations are present also in the non-inflammatory conditions FM and AD implying general effects of Flt3-L in
CNS homeostasis. This does not necessarily pose a problem, it is the same mechanisms in different contexts. In the pSS patient with symptoms of CNS-involvement, who displays CSF findings with signs of inflammation and no signs of infection, increased levels of Flt3-L would strengthen the suspicion of ongoing neuroinflammation with microgliosis and axonal degeneration. Thus, CSF Flt3-L may prove to be a future marker for neuroinflammation.

The majority of rheumatic diseases are chronic and exhibit great variation in degree of severity and involvement of organs. This, in combination with greatly improved pharmacological treatment possibilities and the observation that prognosis of rheumatoid arthritis is improved if treatment is provided early, have created an urge for improved prognostic factors allowing individualisation of treatment. In rheumatoid arthritis, erosive course of disease is especially important to pinpoint early since it has the potential of inflicting great disability.

5.4.2 Flt3-L, a marker for erosive arthritis?
Is Flt3-L a prognostic marker for erosive disease?

In part 1 of my study, serum Flt3-L in rheumatoid arthritis correlates to negative prognostic markers age and RF+ but not erosivity. Flt3-ligand is outlined as a prognostic factor for RA development in healthy military volunteers. However, our study patients had established RA with a mean disease duration of 10 years. Thus, a substantial part of the erosive course of disease already having taken place and the current levels of Flt3-L not necessarily reflecting erosive events. Inhibition of Flt3 in experimental arthritis lead to reduced levels of RANKL and increase in OPG/RANKL ratio indicating bone formation, thus decreased bone erosivity. There is clearly a need to investigate early arthritis and correlate erosivity to Flt3-L with the expectation to find a good prognostic marker for erosive course of rheumatoid arthritis.

5.4.3 Flt3-L, a disease activity marker in arthritis?
Is Flt3-L a marker for disease activity?

There is a great need for disease activity markers in most rheumatic diseases. The ideal marker is only influenced by the rheumatic disease in question and
makes it possible to rule out infections or malignant disease as alternative aetiology to the increase in symptoms.

Part 1 of my study shows increased levels of Flt3-L in SF of RA patients. Levels of Flt3-L increases rapidly upon inoculation/induction of mouse cytomegalo virus infection in mouse \(^{103}\). Increased levels are seen in leukopenia, Fanconi anaemia, in human \(^{104}\). Lack of DCs, irrespective of aetiology, increases Flt3-ligand. A study of paediatric SLE patients showed increased levels of Flt3-L in serum although no significant correlation with disease activity defined by SLEDAI was seen \(^{105}\). On the contrary, in a study with pSS patients serum levels of Flt3-L correlated to disease activity defined by ESSDAI score \(^{24}\). Hence, one may conclude that serum levels of Flt3-L is not thoroughly examined in regards of responses to infection and inflammation. In our study, serum levels of Flt3-L were not increased in RA or pSS in comparison to controls with non-inflammatory diseases. Thus, Flt3-L in serum does not obviously offer specific advantages as a disease activity marker compared to already existing.

### 5.5 Inhibition of Flt3 in rheumatic disease?

In the present study, we treated mice with the Flt3-inhibitor sunitinib. It offers obvious advantages with high bioavailability and per oral administration. It’s side effects, such as anorexia, skin toxicity, thyroid dysfunction, myelotoxicity, and hypertension \(^{106}\), are deemed tolerable in treatment of cancer but not obviously in rheumatic diseases.

Side effects are often dose related and that was the case with sunitinib in treatment of AML. It was noted that a cytotoxic effect was only seen in FLT3-dependent leukaemia cells when FLT3 phosphorylation was reduced to below 10 – 15% of its baseline level.

Hence, the poor patient tolerability to sunitinib doses required to continuously inhibit FLT3 may ultimately limit the further development of sunitinib in AML \(^{107}\). Other cancer forms are though treated but they lack activating mutations of Flt3 and probably benefit to a larger extent from the multikinase inhibitory properties of sunitinib.

What shall we treat – the receptor or the ligand? There is no evidence for other ligands activating the Flt3 receptor or Flt3-L binding to any other protein besides Flt3. Thus, it would be expected that mice carrying a targeted disruption in the gene for either the receptor or the ligand would have an
identical phenotype which they don’t. Thus, inhibition of the Flt3 receptor doesn’t necessarily give the same effect as removing the ligand.
6 CONCLUSION

- Flt3-L worsens experimental arthritis, in reference to both frequency of synovitis and degree of erosivity

- Inhibition of Flt3 alleviates antigen induced arthritis in mice by reducing number of DCs, decrease antibody production and bone metabolism

- Flt3-L accumulates in the synovial fluid of RA patients, the highest levels seen in patients with the poor prognostic factors RF+ and high age

- Flt3-L in CSF in pSS correlates to T-tau/P-tau and to activation of microglia
En dag hände det sig, att konungen i landet beordrade en fest med dans och alla kvinnor bjöds in. Festen anordnades för att kungens son skulle få välja sig en hustru. Alla var glada och förväntansfulla.


En annan kvinna, Snödrottningen, i grannbyn Spetsbergen har också fått ledvärk men därtill uttalad torrhet i ögon och mun och kan i all sin bedrövelse knappt gråta. Hon har också blivit mycket sömnig och man misstänker att hennes huvud är drabbat. Även detta öde finns omvittnat sedan tidigare. De ursprungliga rapporterna kom från vår druid Henrik Sjögren (1899-1986) och benämns därför Sjögrens syndrom.

Förvirrade, självdstruktiva ynglingar i byn Immunsystemet, som ligger i benmärg och mjälte, är sedan lång tid tillbaka misstänkta för att vara inblandade i dessa nesligheter. Vår högt skattade och sanningssökande druid, Mats Dehlin, tror att de bland mycket annat använder sig av ett elixir, en dekokt på fms-lik tyrosinkinas 3 ligand, för att utföra sina tarvligheter. Dock kan ingen minnas det namnet så vi kallar det Flt3-ligand (Flt3-L). Byborna i immunsystemet dricker Flt3-L så det står härliga till. Det får dem att utvecklas, att mogna och föröka sig, kort sagt ett tillväxelixir. Herr B, fru T och局长armade morbror DC är speciellt begivna i Flt3-L.

Upprörd över Askunges och Snödrottningens belägenhet och fast beslutet att ordna danskvällen för dem har vår sanningssökande druid Dehlin experimenterat i sin grotta. Hans efterforskningar beskriver elixiret Flt3-ligands roll vid ledgångsreumatism och Sjögrens syndrom.

Människor med ledgångsreumatism uppvisar förhöjda nivåer av Flt3-L i sin ledvätska. Tillförsel av Flt3-L vid experimentell ledgångsreumatism hos mus förvärrar förstörelse och inflammation i leden. Druiden Dehlin har givit
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mössen ett motgift mot Flt3-L, sunitinib, vilket givit god lindring av inflammation och förstörelse av leden vid experimentell ledgångsreumatism.

Vid Sjögrens syndrom är Flt3-L i hjärnan hos människa länkat till nervcellernas ombyggnation. Vidare påvisas en nära koppling mellan Flt3-L och ett annat elixir, MCP-1. Det aktiverar byn Hjärnans dominerande immunokompetenta bybo, farbror mikroglia, för övrigt en nära släkting till långarmade morbror DC.

Sålunda, druiden Dehlin efterforskningar ger stöd för att Flt3-L är inblandad i inflammation och ledförstörelse vid artritsjukdom. Dehlin har också visat att Flt3-L är nära sammankopplat med nervcellernas ombyggnation och aktivering av mikroglia.

Askungen har nu fått modern anti-reumatisk behandling och mår mycket bättre. Snödrottningen har fått behandling mot sin ledvärk men har fortfarande ont om tårar. De båda kommer kunna bevisa den kungliga danskvällen. Och vem vet, i en ej så avlägsen framtid kommer kanske motgift mot Flt3-L vara en kur för ledgångsreumatism.
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