Linking fms-like tyrosine kinase 3 and urokinase signalling to survivin expression in experimental arthritis

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Gothenburg 2014

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ISBN 978-91-628-8920-3
http://hdl.handle.net/2077/34820

Illustrations are produced using Servier medical art
Printed by Ale Tryckteam AB, Bohus
Sweden 2014

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ABSTRACT

Rheumatoid arthritis (RA) is a systemic autoimmune joint disease, in which chronic inflammation and hyperplastic synovial tissue mediates destruction of cartilage and bone. Survivin is known as an intracellular inhibitor of apoptosis and a positive regulator of cell division. Previous studies have shown that extracellular survivin can be detected in blood and synovial fluid from patients diagnosed with RA and that survivin in blood can predict destructive course of arthritis and poor response to anti-rheumatic treatment. The role of survivin in arthritis, the cellular source and processes leading to the release of survivin are far from understood. Two proteins, the differentiation factor Fms-like tyrosine kinase 3 ligand (Flt3L) and the Urokinase plasminogen activator (uPA) were positively associated to survivin in rheumatoid arthritis patients. The aim of this thesis was to investigate the role of Flt3 signalling for survivin production for arthritis development using the mBSA arthritis model and the role of survivin and urokinase signalling for the arthritogenic properties of synovial fibroblasts.

Intracellular survivin expression was evaluated in the mBSA arthritis model after Flt3 activation with Flt3L or inhibition using or an Flt3 inhibitor, sunitinib. Changes in the frequencies of immune cell populations and the effect on arthritis development were evaluated after Flt3 inhibition. In addition, RNA silencing was used to directly target survivin in *in vitro* and in a human/SCID chimera model to study the impact of survivin on the arthritogenic properties of fibroblasts.

The results presented in this thesis show that survivin is expressed in bone marrow and DCs in response to activation of the receptor tyrosine kinase Flt3 *in vivo*. Inhibition of Flt3 reduces survivin production, dendritic cell formation and synovial inflammation. uPA release from fibroblasts is survivin dependent and silencing of survivin in human fibroblasts reduced cartilage destruction in the knee joints of SCID mice. In conclusion, survivin may enhance the survival of antigen presenting dendritic cells and the arthritogenic properties of synovial fibroblasts in the RA joint.

Keywords: survivin, rheumatoid arthritis, dendritic cells, synovial fibroblasts, Flt3 ligand, Flt3, uPA, uPAR

ISBN: 978-91-628-8920-3

SAMMANFATTNING PÅ SVENSKA

Ledgångsreumatism (reumatoid artrit) är en sjukdom som drabbar ca 1% av befolkningen. Det är en autoimmun sjukdom där kroppens eget immunförsvar angriper vävnaden i lederna, som om den vore främmande. Den kroniska inflammationen leder till nedbrytning av brosk och benförstörelse, vilket medför kraftigt nedsatt funktion. Möjligheterna att diagnostisera och bromsa förloppet av sjukdomen har förbättrats avsevärt på senare år, men det finns än idag inget botemedel och det behövs bättre markörer för att tidigt identifiera patienter med särskilt snabbt förlopp.

Survivin är ett protein som är viktigt för att celler ska kunna dela sig och för att förhindra programmerad celldöd. I cancer är nivåerna av survivin särskilt höga, vilket leder till överlevnad av cancercellerna. Vi har i tidigare studier visat att höga nivåer av survivin även finns lösligt i blodet och ledvätskan hos vissa patienter med reumatoid artrit och att de oftare drabbas av leddestruktioner än andra patienter. I patientgruppen med högt survivin fanns det två andra proteiner, Flt3-ligand och urokinas, som också förekom i högre nivåer än hos andra patienter. I denna avhandling har vi försökt koppla survivinet till cellulära processer som kan förklara denna förekomst, samt undersöka om de har betydelse för utveckling av artrit.

Flt3-ligand främjar stamcellers utveckling till dendritiska celler, vilka har till uppgift att visa upp främmande proteiner för T cellerna och aktivera dessa, så att den specifika delen av immunförsvaret (den adaptiva) kan rikta sitt svar mot angripare som bakterier, virus och parasiter. Vi provade att behandla möss med artrit, dels med injektioner av Flt3-ligand och dels med en inhibitor (sunitinib), som hämmar den receptor som Flt3-liganden binder till. Vi såg att Flt3-ligand ledde till högre nivåer och sunitinib till lägre nivåer av survivin inuti de dendritiska cellerna. Sunitinib-behandlingen ledde dessutom till mildare artrit, vilket vi tror berodde på att de hade färre dendritiska celler som kunde aktivera immunförvaret att angripa leden.

Urokinas är ett protein som kan bryta ner det extracellulära matrix som omger celler. Vi såg att bindvävsceller som kom ifrån lederna ifrån patienter (synoviala fibroblaster), frisätter urokinas. De hade även höga nivåer survivin och vi kunde hindra urokinas-frisättningen genom med hjälp av RNA interferens, blockera survivin-syntesen i fibroblasterna. Om vi satte in fibroblaster i en mus-led, ledde detta till en kraftig artrit men inte om vi blockerade survivin-syntesen först. Urokinas kan också binda till en receptor,

och om vi stimulerade celler som hade denna receptor kunde vi se att survivin-nivåerna ökade i de cellerna.

Vi har således visat att survivin finns i både dendritiska celler och synoviala fibroblaster och att nivåerna kan öka då nivåerna av Flt3L och urokinas ökar. Survivin kan i sin tur öka frisättning av urokinas och Flt3L. Man kan bromsa denna process genom RNA interferens mot survivin eller genom att blockera Flt3-ligand signaleringen. Denna ökade kunskap om reglering av survivin-produktion och survivinets effekter kan på sikt öppna för nya behandlingsmöjligheter för patienter med reumatoid artrit.

LIST OF PAPERS

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

I. Andersson, SE, Svensson MN, Erlandsson MC, Dehlin M, Andersson KM, Bokarewa MI. Activation of Fms-like tyrosine kinase 3 signaling enhances survivin expression in a mouse model of rheumatoid arthritis. *PlosOne 2012;7(10)*

II. Dehlin, M., Andersson S, Erlandsson M, Brisslert M, and Bokarewa M. Inhibition of fms-like tyrosine kinase 3 alleviates experimental arthritis by reducing formation of dendritic cells and antigen presentation. *J Leukoc Biol 2011; 90:811-817.*

III. Baran, M., L. N. Mollers, S. Andersson, I. M. Jonsson, A. K. Ekwall, J. Bjersing, A. Tarkowski, and M. Bokarewa. Survivin is an essential mediator of arthritis interacting with urokinase signalling. *J Cell Mol Med* 2009; *13:3797-3808.*

OTHER PUBLICATIONS

Other publications not included in this thesis are as follows:

Svensson MN, Andersson SE, Erlandsson MC, Jonsson IM, Ekwall AK, Andersson KM, Nilsson A, Bian L, Brisslert M, Bokarewa MI: Fms-like tyrosine kinase 3 ligand controls formation of regulatory T cells in autoimmune arthritis. *PLoS One* 2013, 8(1):e54884.

Erlandsson MC, Svensson MD, Jonsson IM, Bian L, Ambartsumian N, Andersson S, Peng Z, Vaaraniemi J, Ohlsson C, Andersson KM *et al*: Expression of metastasin S100A4 is essential for bone resorption and regulates osteoclast function. *Biochimica et biophysica acta* 2013, 1833(12):2653-2663.

Isgren A, Forslind K, Erlandsson M, Axelsson C, Andersson S, Lund A, Bokarewa M: High survivin levels predict poor clinical response to infliximab treatment in patients with rheumatoid arthritis. *Seminars in arthritis and rheumatism* 2012, 41(5):652-657.

Erlandsson MC, Forslind K, Andersson SE, Lund A, Bokarewa MI: Metastasin S100A4 is increased in proportion to radiographic damage in patients with RA. *Rheumatology (Oxford)* 2012, 51(5):932-940.

Khan OM, Ibrahim MX, Jonsson IM, Karlsson C, Liu M, Sjogren AK, Olofsson FJ, Brisslert M, Andersson S, Ohlsson C *et al*: Geranylgeranyltransferase type I (GGTase-I) deficiency hyperactivates macrophages and induces erosive arthritis in mice. *The Journal of Clinical investigation* 2011, 121(2):628-639.

Bostrom EA, Svensson M, Andersson S, Jonsson IM, Ekwall AK, Eisler T, Dahlberg LE, Smith U, Bokarewa MI: Resistin and insulin/insulin-like growth factor signaling in rheumatoid arthritis. *Arthritis Rheum* 2011, 63(10):2894-2904.

CONTENT

ABBREVIATIONS

aCCP	Anti-cyclic citrullinated peptide
ACPA	Anti-citrullinated protein antibodies
APC	Antigen presenting cell
BIR	Baculovirus Inhibitor of apoptosis protein Repeat
CCR7	C-C chemokine receptor 7
CD	Cluster of differentiation
CTX-I/II	C-terminal cross-linking telopeptide of type-I/II collagen
DAMPs	Danger associated molecular patterns
DC	Dendritic cell
FcγR	Fc-gamma receptors
Flt3	Fms-like tyrosine kinase 3 (receptor)
Flt3L	Fms-like tyrosine kinase 3 Ligand
FLS	Fibroblast-like synoviocytes
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
IAP	Inhibitor of apoptosis
IFNγ	Interferon gamma
IL	Interleukin
IRF	Interferon regulatory factor
MAPK	Mitogen-activated protein kinase

mBSA	Methylated bovine serum albumin
MCP-1	Monocyte chemotactic protein-1
M-CSF	Macrophage colony-stimulating factor
MHC	Major histocompability complex
MLS	Macrophage-like synoviocytes
MMP	Matrix metalloproteinase
MΦ	Macrophage
PDGF	Platelet-derived growth factor
PGE_2	prostaglandin E2
PI3K	Phosphoinositide 3-kinase
PRR	Pattern recognition receptor
PTPN22	Protein tyrosine phosphatase 22
RA	Rheumatoid Arthritis
RANK(L)	Receptor activator of nuclear factor kappa-B (ligand)
RF	Rheumatoid factor
RTK	Receptor tyrosine kinase
SCF	Stem cell factor
SCID	Severe combined immunodeficiency
shRNA	Short hairpin RNA
siRNA	Short inhibitory RNA
STAT	Signal transducer and activator of transcription

TCR	T-cell receptor
TGFβ	Transforming growth factor beta
Th	CD4+ T helper
TLR	Toll-like receptor
TNFα	Tumour necrosis factor alpha
Treg	Regulatory T lymphocyte
uPA	Urokinase plasminogen activator
uPAR	Urokinase plasminogen activator receptor
VEGF	Vascular endothelial growth factor

1 INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease that leads to inflammation in the synovium in addition to cartilage and bone destruction. The synovial tissue consists mainly of fibroblasts and during RA, these cells become activated and gain tumour-like features such as expansive growth, increased migratory functions [1] and resistance to apoptosis [2]. Further, during the rheumatic inflammation, peripheral leukocytes such as T cells, B cells, dendritic cells (DCs), macrophages and neutrophils invade the synovium. Despite extensive research and development of effective therapeutics, interplay between these different cell types are far from understood [3]. Thus the aim of this thesis was to investigate the importance of Flt3L and uPA signalling on survivin expression in experimental arthritis in order to a) further understand the pathogenesis behind arthritis development and b) investigate new biomarkers and new possible treatments for RA patients.

1.1 Hypothesis

We hypothesised that tyrosine kinase signalling induce survivin, which leads to accumulation of survivin in leukocytes and synovial fibroblasts and subsequent survival of autoreactive cells in RA.

2 RHEUMATOID ARTHRITIS

Rheumatoid arthritis is a systemic autoimmune joint disease, in which chronic inflammation and hyperplastic synovial pannus tissue mediates destruction of cartilage and bone. The disease is one of the most common autoimmune diseases as it affects approximately 1% of the population with some regional variations.

2.1 Autoimmunity and RA

The immune system has evolved to protect us against infections without causing harm to the host, and must therefore be able to distinguish between self and non-self. The ability of the immune system to ignore endogenous structures is called tolerance and is maintained by an intricate network of immunological mechanisms. In autoimmune diseases the tolerance to self-antigens is lost, and the immune system drives a chronic perpetual inflammation that is triggered by endogenous antigens.

Tolerance is maintained by exposure of self-antigens to lymphocytes and subsequent deletion, inactivation or changed function of autoreactive clones. This may occur during development (central tolerance), or in mature lymphocytes in the periphery (peripheral tolerance). Peripheral tolerance is essential to prevent autoreactivity also towards modified antigens and non-self antigens when they do not cause harm. However, tolerance mechanisms can fail if there is a trigger that signals danger.

Joint inflammation is a hallmark of RA and it is therefore natural to assume that the self-antigen originates from the joint. This could be an intra-articular cartilage component but it could also be an antigen that homes to the joint and binds to joint structures due to charge-mediated binding. Since the antigen remains in the joint and unlike the situation of infection cannot be cleared, chronic inflammation also characterises RA. Today, neither the initiating cause of disease nor the eliciting antigen is known. Similarity between a bacterial component and a self-antigen (molecular mimicry), or posttranslational modifications of self-antigens, which might occur during infections or tissue injury, are mechanisms that are commonly believed to cause break of tolerance and cause autoimmunity.

2.2 Aetiology

Although the exact cause of RA remains elusive, we know that genetic disposition, gender, as well as environmental factors contribute to disease development. There are no strong associations to a single gene, but the concordance rate (around 15%) for monozygotic twins is considerably higher than in the general population [4]. The genetic variability that are most strongly associated to RA are linked to functions of the immune system including the genes for MHC II and PTPN22. Allelic variants of the *HLA-DRB1* in the MHC II locus ("shared epitope") is around 4-5 times higher in RA patients compared to the general population [5]. A single nucleotide polymorphism in the *PTPN22* gene encoding for a tyrosine phosphatase negatively regulating immune responses in T cells is associated with autoimmunity and RA [6]. Females have around three times higher risk of developing RA compared to males.

Environmental triggers associated to development of RA are smoking [7] and silica dust [8], mineral oil [9] and infections [10-13].

2.3 Autoantibodies

2.3.1 Rheumatoid factor antibodies (RF)

Rheumatoid factors (RF) are antibodies of any isotype, with specificity for the Fc-portion of IgG antibodies. RF form immune complexes with IgG and if injected into a joint they cause inflammation [14]. 70% of RA patients are positive for RF. The increase in RF at disease onset is used as a biomarker to distinguish patients with more severe clinical disease that are in need for active treatment. RF antibodies were considered to be the first evidence that the immune response in RA is directed towards self-antigens. However, although characteristic for RA, the specificity and sensitivity of RF for RA are 90% and 78% respectively, which means that these autoantibodies also are also present in other conditions and not an optimal biomarker for the disease.

2.3.2 Anti-Citrullinated Protein Antibodies (ACPA)

The specificity and sensitivity of ACPAs for RA is 95% and 60-70% respectively and therefore constitute a better biomarker for RA than RF. The assay commonly used to detect anti-citrullinated protein antibodies is the anti-cyclic citrullinated peptide (aCCP) assay, which has become a widely used diagnostic tool for RA [15]. Citrullination is the process by which the

amino acid arginine is converted to a citrulline. The reaction is catalysed by peptidyl arginine deaminases (PADs). One possible biological function of citrullination is to degrade intracellular proteins during apoptosis and it seems likely that this process is enhanced during inflammation [16] . Arginine is a positively charged while the citrulline is neutral, which leads to conformational changes of the protein and subsequent exposure of normally hidden antigens. Citrullination of some proteins that are expressed within the joint are of particular interest: α-enolase vimentin, filaggrin, fibrin/fibrinogen and collagen type II [17].

Many parallel findings in patients with RA resulted in the discovery of citrullinated proteins as antigens and the anti-citrullinated protein antibodies (ACPA). In the 1970s the anti-keratin antibodies were found. They were called so because they bound to the squamous epithelium of rat esophagus, even though it was never clear if the keratin was the antigen. This was later shown not to be the case; instead the anti-keratin antibodies recognised epitopes on the citrullinated filaggrin [18] The anti-Sa antibodies was discovered in serum from a patient with rheumatoid arthritis whose name began with Sa, showed specificity for an antigen present in extracts from normal human placenta and spleen and was a poorly soluble protein around 50kD [19] that was later identified as mutated and citrullinated vimentin [20]. The shared epitope predispose RA patients for development of ACPAs [21] and can be explained by the fact that high affinity binding of peptides to the shared epitope MHC II molecule is facilitated by citrullination of the peptide [22]. ACPAs are also present in serum the from healthy individuals, but predict subsequent onset of RA [15,23]

2.4 Disease pathogenesis in RA

In RA the synovial tissue becomes hyperplastic with proliferation and activation of synovial fibroblasts (FLS). There are also inflammatory infiltrates in this tissue that includes cells from the adaptive immune response like DCs, T and B-lymphocytes, which is a sign of chronic inflammation.

DCs are efficient antigen presenters as they take up the autoantigen and present the antigen peptides in lymphoid organs (spleen and lymph nodes) and provide co-stimulation and cytokines that activate T cells and thereby initiate the adaptive immune response.

Ectopic lymphoid structures are often found in the rheumatoid synovium. In these local foci antigens are presented, which result in activation of T and B cells and the production of cytokines and antibodies. T cells function as

enhancers of the innate immune system, by the production of cytokines like IFNγ and IL-17 that activates macrophages and mediate recruitment of inflammatory cells. Activated macrophages show enhanced phagocytosis and are producers of proinflammatory cytokines like TNFα and IL1β. RA-FLS secrete inflammatory mediators like PGE_2, cytokines like IL-6 and IL-18 and chemokines like IL-8, MCP-1 and CXCL10 and proteolytic enzymes (proteases). There are increased levels of RANKL in the inflamed tissue that contributes to osteoclastogenesis. Autoantibodies form immune complexes that activate phagocytes via Fcγ receptors, which enhance antigen presentation and mediate the release of proteases.

Cartilage degradation is performed mainly by soluble proteases such as matrix metalloproteinases (MMPs), serine proteases and cathepsins. The only cell type that can degrade mineralised bone is the osteoclast and they are formed within the joint during arthritis[24]

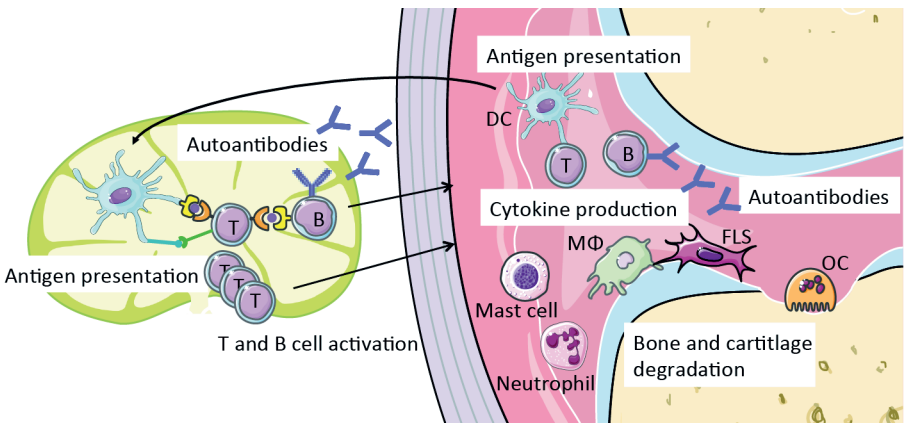


Figure 1. RA pathogenesis. Innate sensing activates DCs that migrate to lymphoid organs to present antigens and activate T cells, which in turn activate B cells. Lymphocytes migrate back to the synovium where adaptive immunity is enhanced by autoantigens. Cytokines, chemokines and antibody immune complexes activate FLS. MΦ, Mast cells and neutrophils to release proteolytic enzymes that destroy cartilage and OC differentiated from MΦ erode bone. FLS=Fibroblast -like synoviocyte, MΦ =macrophage, OC=osteoclast

2.5 The synovial fibroblasts in RA

The lining layer of the synovia in healthy joint is about 1-2 cell layer thick but is increased to about 10-15 layers in RA. It consists of Type A cells, that are macrophage-like synoviocytes (MLS) and type B cells, which are fibroblasts-like synoviocytes (FLS). In humans these cells can be separated by the expression of CD68 on MLS and CD90 and CD29 on FLS [25]. RA-FLS become immunologically active, since they express MHC II which makes them capable of presenting antigens to T cells [26]. RA-FLS express the CCL19 and CCL21 - chemokines that attract the CCR7+ mature DCs and naïve T cells to the lymph node where antigen presentation and T cell activation occur [27]. RA-FLS are transformed in such a way that they retain their phenotype outside the joint, so they can act independently of inflammation and stimuli from inflammatory cells, which has been shown by implants in SCID mice. These implants cause damage to mouse cartilage [28] or human cartilage implants [29] without continuous activation by the immune system. RA-FLS implanted into SCID mouse was even seen to spread between distant implanted pieces of cartilage [30], which is an example of their enhanced migratory ability.

2.5.1 Proliferation and defective apoptosis in RA

Cells of the synovial lining acquire a phenotype resembling epithelial mesenchymal transition, which is a state where the cells loose attachment to the basement membrane and gain enhanced migratory, proliferative and tissue remodelling capacity and enhanced production of components of the extracellular matrix. The state is normal during embryogenesis and wound healing but is also adapted in cancer progression [31]. In RA-synoviocytes this is exemplified by the increased expression of α-smooth muscle actin, cadherin-11 [25], increased expression of vimentin [30], S100A4 [32] [33], and secretion of extracellular matrix degrading proteases (paper III) [30]. RA-FLS can acquire somatic mutations such as in tumour suppressor p53 [34] and a gain of function mutation in a RAF-protein in the MAP kinase pathway [35], and they acquire epigenetic changes such as differentially hypomethylated loci [36] and activation of proto-oncogenes (paper III). RA-FLS themselves secrete growth factor like such as PDGF, TGFβ and GM-CSF in addition to angiogenic factors like VEGF that contribute to continued growth and transition [37].

In line with these changes, RA-FLS do not only display an enhanced proliferation, but also reduced apoptosis, which is retained also when cultured in vitro [38]. Apoptosis, or programmed cell death, is essential for safe removal of damaged or autoreactive cells without provoking an immune response, since danger associated molecular patterns (DAMPs) that are potent activators of innate immunity are hidden in the apoptosome. [39]. Apoptosis can be triggered through the intrinsic pathways by factors such as like cellular stress and DNA damage or the extrinsic pathway through death receptors such as TRAIL receptors or Fas, which contribute to elimination of aberrant immune responses. [40]

2.6 Receptor tyrosine kinases and RA

Many of the growth factors present in high amounts in the rheumatic joint are ligands for receptor tyrosine kinases (RTK) (Figure 2). Tyrosine kinase phosphorylation is a mechanism involved in many cellular processes and signal transduction pathways controlling cell growth, survival, differentiation and angiogenesis [41]. Indeed, tyrosine residues in the RA synovium are heavily phosphorylated [42]. The RTKs are membrane-spanning receptors that receive extracellular signals through binding of a ligand and transmit the signal through tyrosine kinase phosphorylation (Figure 2). Binding of the RTK ligand induces dimerization, which induces a conformational change that makes the tyrosine kinase residues on the intracellular domain able to

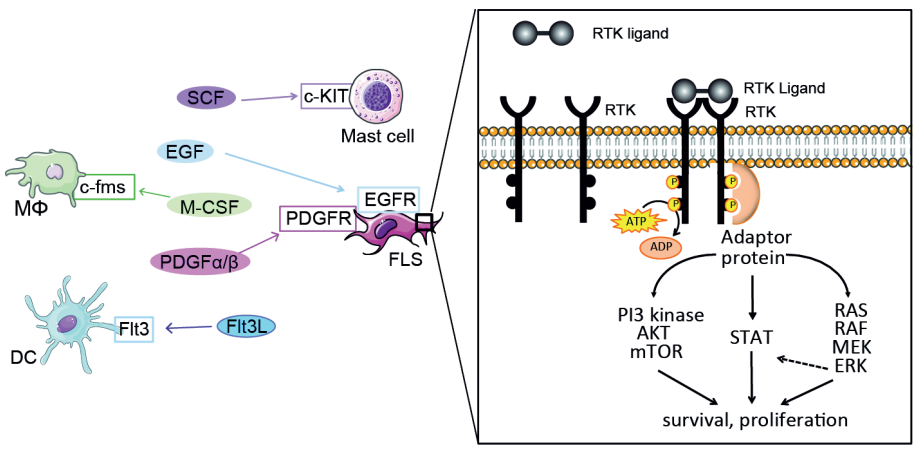


Figure 2. A) RTK and their ligands in the RA joint. B) Dimerization of the receptor induces auto-phosphorylation of the tyrosine residues. An adaptor protein becomes phosphorylated by the tyrosine residues and contains binding sites for downstream signalling proteins.

become auto-phosphorylated by ATP and then able to transfer that group to the adaptor protein. RTK activation leads to several cellular responses depending on the cell type, the proteins expressed and other received signals, as the tyrosine groups of the cytoplasmic tail can phosphorylate several different proteins and initiate various intracellular signalling cascades. Most well studied is the MAP kinase cascade and the PI3 kinase pathway [43]

2.7 Experimental models of RA

There are several models of RA in rodents that are used to study pathogenic mechanisms and evaluate new potential therapeutics.

In induced arthritis models adjuvants are used in combination with an antigen to generate an immune response towards this particular antigen. In the mBSA-induced arthritis, the antigen is introduced directly into the joint after immunisation, which initiate a monoarthritis (paper I, paper II). The mBSA model is discussed in more detail below. As mentioned, humanised models like the transplantational human/SCID chimera mice (paper III) [44] can give information about the arthritogenic behaviour of transformed cells like isolated synovial fibroblast [29] acting in vivo, independently of T cells.

In the collagen-induced arthritis model, adjuvant together with chicken or rat collagen causes a break of tolerance to mouse collagen type II, the major protein of the cartilage, which causes a destructive polyarthritis [45]. Injections with CII antibodies are sufficient to induce arthritis in the collagen-antibody-induced arthritis model [46].

2.7.1 mBSA arthritis

The major principle of the antigen-induced arthritis model is injection of the antigen into a joint of a previously immunised animal. This induces a strong T cell mediated delayed-type hypersensitivity reaction locally. It can be induced in several species like rabbits, rats, guinea pigs and mice [47], the latter being the species used in this thesis (Paper I-II). The mBSA induced arthritis model has been widely used since it was noticed that mBSA has cationic properties that allows it to bind to the anionic surfaces in the joint cavity [48]. This results in chronic arthritis because of antigen retention in the joint [49]. Methylation of BSA will yield a BSA with a pI around 8.5, compared to native BSA that has a pI at 4.5. [47]

The advantages of the model as a model of RA are:

- Simple –fast and with a well defined time of onset
- It does not require any genetic susceptibility and can therefore be induced into any strain including various knockouts and transgenic animals.
- Animal friendly - since it is a monoarthritis and does not severely affect the normal behavioural activities
- Comparison with a normal joint in the same animal
- Control of the severity by the amount of antigen injected locally.

The model is well suited for studies regarding mechanisms of joint destruction following chronic arthritis [47]. It also holds a similarity to RA, since flares can be induced by local or systemic rechallenge with mBSA [50,51], but it is less well suited to study mechanisms of autoimmunity. One thing to consider when using this model is the cross-reaction towards native BSA as many recombinant proteins can contain traces of BSA, which can also cause immune reactions. A DTH reaction in the ear of a mBSA immunised animal could be achieved by native BSA causing a swelling to approximately 1/3 of the swelling caused by mBSA [49]

2.7.2 Immunisation

Mice are immunised at day 0 and boosted at day 7 before arthritis induction at day 21 (Paper I-II). An adjuvant is required to get an immune response towards the mBSA and will enable activation of the pattern recognition receptors (PRRs) so that the DCs will mature and provide co-stimulation to activate T lymphocytes. (Figure 3) The nature of an adaptive immune response is dependent on both the adjuvant and the antigen. The Freunds adjuvant supplemented with mycobacteria (complete Freunds adjuvant, CFA) was used to immunise the mice with mBSA (Paper I-II). Freunds adjuvant is a water-in-oil emulsion containing paraffin oil and a surfactant, which on its own is capable of inducing a Th1/Th2 immune response with antibody production by a mechanism that is not completely understood but may involve PRR stimulation at the injection site due to cellular damage [52]. For further polarisation of T cells into a Th1/ Th17 response, mycobacteria are added. They contain ligands for TLR 2, 4 and 9 [52], peptidoglycans that stimulate NOD receptors [53] and molecules recognised by CARD9 dependent C-type lectin receptors [54]. It also seems to be that the mBSA antigen itself has immunogenic properties and can activate inflammasome-mediated release of IL1-beta [55]. MyD88-dependent IL1-beta signalling is essential for Th17 polarisation [54]. Also, IL-6 has been shown to be crucial for the development of mBSA arthritis [56]

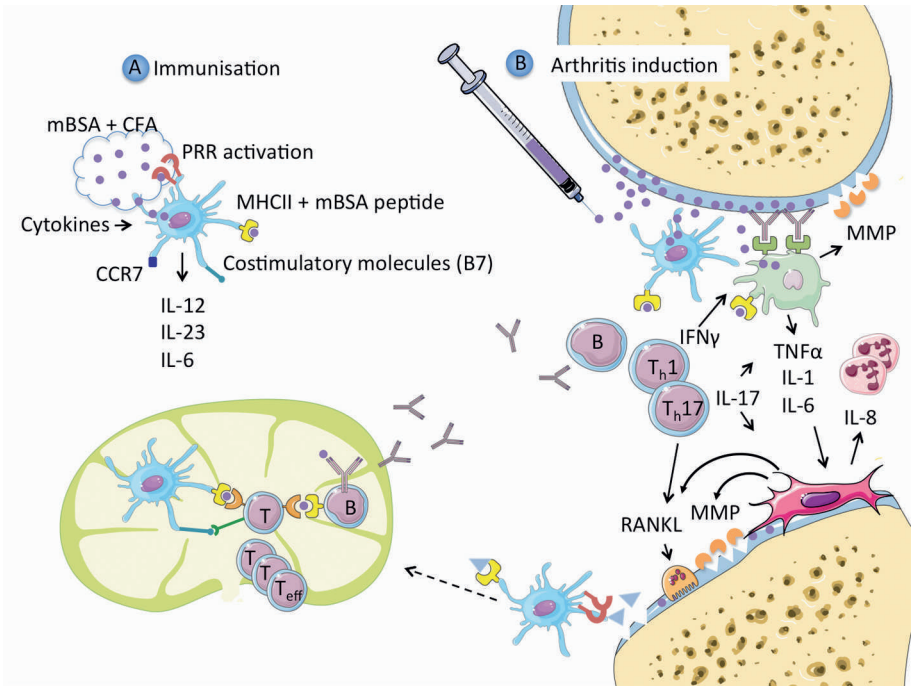


Figure 3. The mBSA arthritis model. A) Immunisation with complete Freunds adjuvant with mBSA activates PRRs on the dendritic cell that takes up the antigen for presentation on MHCII. DCs undergo maturation and increase their expression of MHCII, costimulatory molecules like B7, lymph node homing receptors like CCR7 and cytokines such as IL-12, IL, 23 and IL-6. In the lymph node the dendritic cell present antigen to T lymphocytes and provide costimulation to naive T cells recognizing the MHC with peptide. Activated T cells undergo clonal expansion and differentiate into effector T helper cells. They can then activate B cells to produce antibodies. B) Arthritis is induced by a knee injection with mBSA. Antigen retention is enabled by the positive charge of the mBSA molecule. Antigen presenting cells phagocyte mBSA and present it to effector T lymphocytes, which secrete cytokines like IFNγ and IL-17. Activated macrophages produce TNFα, IL-1 and IL-6. FLS produce IL-8 that recruits neutrophils, enzymes that target cartilage and RANKL that leads to osteoclast

2.7.3 Adaptive immune response with cells infiltrating the synovium

The inflammation caused by the mBSA knee injection, with joint swelling is most prominent after about 3 days. After 7 days the swelling declines but this time-point is more optimal to measure the histopathological changes in cartilage and bone metabolism [47]. The synovitis can then be histologically evaluated after H&E staining of sections according to a 0-3 scale for the degree of synovitis (synovial hypertrophy and accumulation of inflammatory cells) where 1: mild, 2: moderate, 3: severe synovitis (Figure 15, Paper II). The presence of the bone and cartilage damage can also be histologically evaluated using H&E stain (Figure 4).

The synovial infiltrate in the mBSA joint consists of both lymphocytes, neutrophils, plasma cells and mast cells [57]. mBSA arthritis is a T cell driven model and CD4+ T cells are most essential for arthritis development. The RAG-/- mouse lacking B and T cells are completely resistant [58]. Further investigations of the model have shown that it absolutely dependent on CD4+ T cells while the presence of CD8+ T cells are of less importance [58-61]. Adoptive transfer of T cells and DCs from an mBSA immunized mouse 11 days prior to the knee injection was sufficient to induce arthritis in naïve mice, while the transfer of T cells alone was not, which shows that

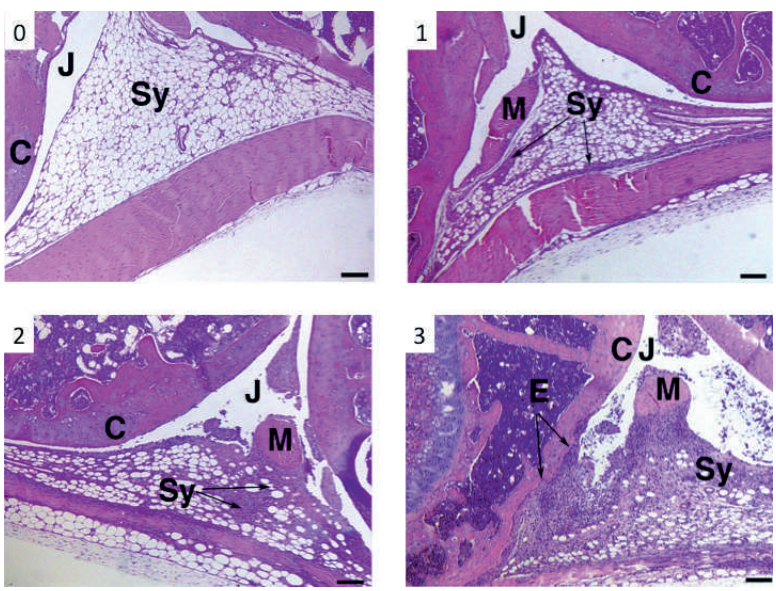


Figure 4. Synovitis and erosions in the knee joint of different severity 7 days after knee injection of mBSA, evaluated by histology. Knees are scored as: 0: no synovitis, 1: mild, 2: moderate, 3: severe synovitis. J= joint cavity. C= cartilage, Sy=synovitis, M=medulla, E=erosions

arthritis induced following injection by antigen specific T cells are further enhanced by supplementary injection of antigen presenting cells [62].

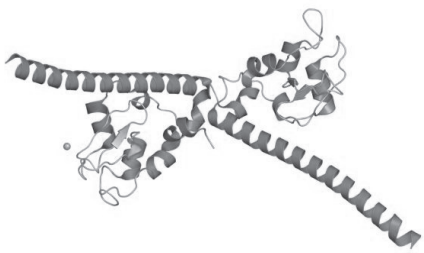
B cells and antibodies seem to be of minor importance for development of synovitis in mBSA arthritis. The μMT/μMT mice lacking B cells and IgM and IgG antibodies developed arthritis without any significant changes compared to the wildtype controls [58,59]. Spleen cell devoided of B cell by passage through a anti-immunoglobulin column could transfer susceptibility to arthritis similarly to transfer of total spleen cells [60]. Serum transfer could predispose mice to arthritis induction with cellular infiltration after knee injected mBSA, but to a very limited extent [60]. Interestingly, activating Fcγ receptors seem to be of importance for cartilage and bone degradation. IgG antibodies binds to FcγRI and FcγRIII on macrophages leading to macrophage activation [63], and to FcγRIV expressed on osteoclast precursors, promoting osteoclastogenesis [64]. Knockout of the activating Fc-gamma receptors reduced cartilage destruction independently of inflammation [65], which suggests that mBSA antibodies are enhancers of damage to cartilage and bone in the mBSA arthritis model. Histological sections of the joint after arthritis induction with radiolabelled mBSA show that some of the mBSA sticks at the cartilage surface together with IgG and complement, indicating that immune complexes are formed. [48].

After mBSA injection activated T_H1 and T_H17 T cells infiltrate the synovium secreting IFNγ and IL-17. IL-17 has been shown to have a pronounced influence on the mBSA mediated response [66] and neutrophil recruitment to the joint [67], which are one of the early effects of arthritis induction [61]. B cells and macrophages will accumulate with time, which indicate that antibodies and activation of Fc gamma receptor on synovial macrophages will occur after some days [61]. The cellular infiltrate is more granulocyte rich in Balb/c mice than in C57Bl/6 [47], which suggests that different mouse strains may respond slightly different.

Osteoclasts mediate bone destruction and target bone through release of enzymes like MMPs and Cathepsin K, while bound to the bone surface by integrin alphaVbeta3. The release of RANKL within the joint will lead to recruitment and osteoclast formation of the cells expressing RANK, which are mainly monocytes stimulated by M-CSF [24]. Arthritis induction by mBSA is followed by increased expression of RANKL in the synovia of the arthritic joint compared to the collateral joint [68]. The C-telopeptide of type I collagen (CTX-1) is a degradation product from Collagen type I (the major protein component in bone) which can be detected in the serum, when bone is degraded by osteoclasts [69](Paper II).

3 FUNCTIONS OF SURVIVIN IN CANCER AND AUTOIMMUNITY

Survivin, (*API4,* TIAP) is the smallest inhibitor of apoptosis protein (IAP) with its 142 amino acids. It is a multifunctional protein, most extensively studied in the cancer field, where it has been implicated in cell proliferation, inhibition of apoptosis and angiogenesis [70]. Survivin is encoded by the *Birc5* gene at chromosome 17q25 in the human, has a basal isoelectric point at 5.66, and exhibit important intracellular functions [71]. Furthermore, survivin has also been found in the extracellular space, however the source and function is at present not well known [72-74]. The function of survivin in different biological processes will be described below.



3.1.1 Is survivin a cancer gene?

Ambrosini et al first described survivin as a gene essential during fetal development and absent in terminally differentiated tissues, but re-expressed in cancers [75]. Survivin was then identified as 1 out of 40 differentially expressed genes in a large study using serial analysis of gene expression, in which around 3.5-million transcripts from different human cancer tissue (colon cancer, brain cancer, breast cancer, lung cancer, melanoma) was compared to terminally differentiated healthy tissue [76]. Expression of survivin in malignancies is associated with unfavourable outcome [77,78] and resistance to cytostatic treatment [79-81] Survivin is used as a tumor biomarker [70] and is also considered to be a promising target for cancer therapeutics [82].

On the other hand, survivin is essential during normal embryogenesis, which is clearly demonstrated by lethality of the homozygous knockout mice. [83]. Later on, several studies have shown the requirement of survivin for normal proliferation of several different cell types also in adults. Conditional knockouts show that survivin is essential for adult haematopoiesis [84]. We (paper I), and others [85] have shown that survivin is expressed in terminally

differentiated DCs, and is linked to their maintenance (paper I). Further, survivin is expressed in terminally differentiated neutrophils [86] and crucial for T cell development [87,88]. Survivin transcription seems to be active in constantly renewing tissues like the testis, spleen, bone marrow and thymus, but almost absent in fully differentiated tissue that rarely divide such as brain, heart, kidney, ovary and skeletal muscle [89,90]

In conclusion, although overexpressed in most cancers, survivin also is important for normal adult tissue homeostasis of constantly renewing tissues.

3.1.2 Survivin in cell division and apoptosis

The knockout of the other anti-apoptotic IAPs is not lethal, but the survivin knockout is [91]. The severe defects at the embryonic stage with reduced number of nuclei and polyploidy in animals lacking survivin, demonstrate that the protein is necessary during cell division [83] shRNA knockdown studies in a primary cell line and in in normal differentiated cells showed that survivin is essential for proliferation and proper chromosome segregation and cells lacking survivin will suffer severe defects [92]. In the HeLa human cervical cancer cell line - survivin is expressed in a cell-cycle dependent manner with a peak at G2/M [93]. Survivin is a part of the chromosomal

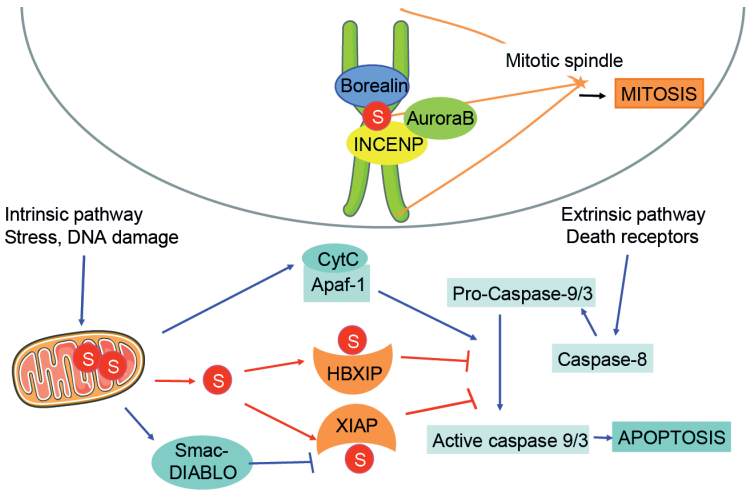


Figure 5. Survivin in cell division and apoptosis. Survivin in complex with Borealin, inner centromere protein (INCENP) and Aurora B kinase forms the chromosomal passenger complex that binds to the centromere and segregates the chromosomes between daughter cells at mitosis. Survivin also takes part as an inhibitor in apoptosis in complex with caspase inhibitors HBXIP and XIAP. Caspases are activated by death receptors through caspase-8 (extrinsic pathways), or cellular stress leading to release of Cytochrome c and the IAP inhibitor SMAC from the mitochondria (intrinsic pathways).

passenger complex, which during cells division facilitates the segregation of chromosomes [94] (Figure 5). The chromosome segregating function of survivin is evolutionary conserved and the essentiality of survivin homologs during mitosis has been shown in yeast [95] and *Caenorhabditis elegans* [96]

Also, cell-cycle independent expression of survivin has been described. It can be observed after stimulation with cytokines or growth factors [97,98] and studies in transgenic mice suggest that survivin also may be up-regulated during interphase *in vivo* [99] and that the expression overlaps with stem cell markers [100]. At the N-terminus survivin has a BIR domain, which is a 73 amino acid (15-87) domain containing a zinc-binding motif, and that characterises the members of the inhibitors of apoptosis proteins. In contrast to other IAPs with known anti-apoptotic function survivin has only one BIR domain, [101] and lacks also other domains present in IAPs, like a C-terminus RING domain and a CARD domain. Transgenic expression of survivin has been shown to counteract apoptosis in several experimental settings like in ultra violet B (UVB) light induced apoptosis in kerationcytes [102]. Attempts to show that survivin can inhibit apoptosis on its own have not been fruitful, instead it has been suggested that it does promote survival through modulation of other proteins. [103]. Survivin is released from mitochondria in response to death stimuli through intrinsic or extrinsic pathways and inhibit apoptosis in complex with hepatitis B X interacting protein [104] or in complex with another inhibitor of apoptosis; XIAP [105] perhaps by separating Smac/Diablo from XIAP, thus enabling caspase degradation [106]. (figure 5).

3.2 What regulates the intracellular functions of survivin?

It is not clear how survivin exerts its different actions, but there are many hypothesis. Cellular localisation might be one determinant [107]. It has been shown that cytoplasmic survivin inhibits cell death [108], promote tumour progression [109] and accumulates in cell culture [110], mitochondrial survivin play a role in apoptosis [71], whereas nuclear survivin in some studies is associated with the chromosomal passenger complex that regulates cell division [111]. It has been proposed that function is depending on whether survivin acts as a monomer or a dimer [112]. Also, posttranslational modifications, such as phosphorylation, seem to be important for its functions in cell cycle and apoptosis [113]

The survivin gene generates several alternatively spliced transcripts that are translated into several isoforms and these isoforms might be the key determinant of the many functions of survivin [114]. It has been suggested that the splice variants can have different properties and that that splice variants can interact with and modulate the functions of full-length of survivin [115,116]. In the human there are at least five different transcripts; survivin, survivin-2B, Survivin-ΔEx3, Survivin-3B, survivin-2α and (Figure 6A). There are somewhat conflicting reports about their exact biological function. The splice variants all share homology in the N-terminus, but they differ at the C-terminus. Almost all of them, are associated with unfavourable disease outcome of cancer [114], but the Survivin 2B variant might be a natural antagonist of the anti-apoptotic survivin and has been seen to promote apoptosis experimentally [117] and is in some cases associated with a better disease outcome in some cancer types [114]. In mice there are three known transcripts of the survivin gene: survivin140, survivin121 and survivin40 [89] (Figure 6B). The survivin 40 variant is expressed in mouse embryos [89] and bone marrow (paper I). But, we (paper I), and others [89] could not detect any protein of that size (paper I)

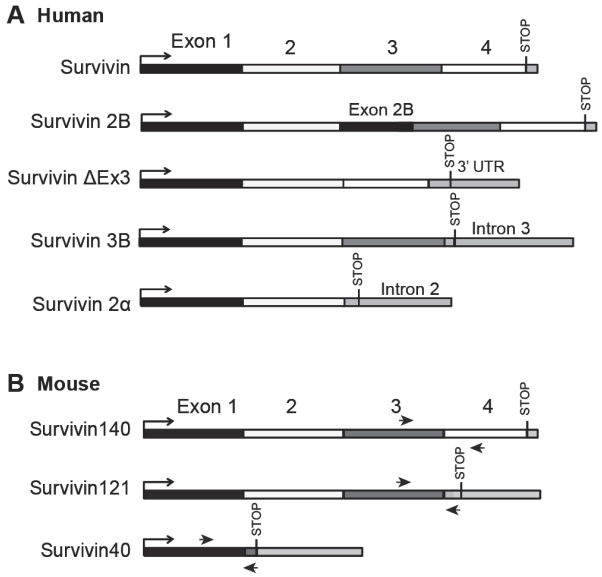


Figure 6. Splice variants of (A) human and (B) mouse survivin. Sites of the primer binding sequences for gene expression analysis of the mouse survivin splice variants are marked (paper I).

3.3 Survivin in RA

In 2005 Bokarewa et al [73] discovered that extracellular survivin was present in the blood and synovial fluid, in around 21% of RA patients (n=131), with levels exceeding those in healthy controls. Furthermore, survivin levels was associated with the degree of bone and cartilage erosivity [73]. Analysis of survivin in serum in 651 patients from the BARFOT cohort of showed that 60% of the patients with early RA were positive for survivin. The cohort was followed for 5 years and the survivin positive group developed erosions faster than the survivin negative group [118]. New studies have shown that extracellular serum survivin could even precede the onset of RA and predict development of disease [119].

Several studies have confirmed the presence of extracellular survivin in RA patients and the link to erosivity (paper I, paper III) [73,74,118], disease activity [74,120], presence of aCCP [118,120], RF (paper I)[118] and smoking [121]. No significant correlations to neither age [118,122] nor to inflammation – measured as CRP – were found (paper I) [73,74] The levels of circulating survivin in RA patients are similar to the levels in the joints (paper III) [73] but there is a strong correlation between the blood survivin and synovial fluid [73,122] survivin suggesting the joint to be the source of survivin secretion.

The survivin levels in synovial fluid in RA patient were significantly higher compared those obtained osteoarthritic patients [74]. Survivin is expressed in the lining layer of synovial tissue from RA patients (paper III) [2,74] and has also been confirmed by gene expression analysis of RA synovial tissue [2] In murine histology sections from mice with collagen-induced arthritis, survivin is expressed in the tissue surrounding ectopic lymphoid structures. In mBSA induced arthritis survivin is found in the synovial lining layer, in the chondrocytes and in bone marrow (Figure 7).

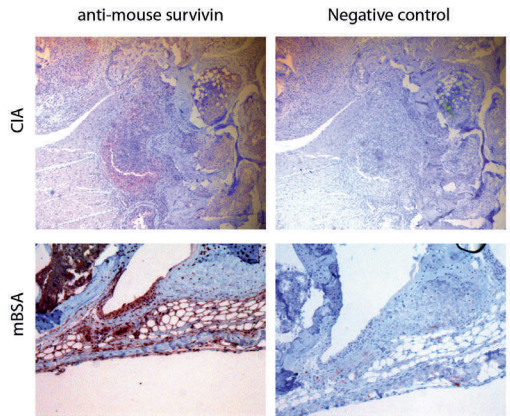


Figure 7. Survivin expression in the joints of murine collagen induced arthritis and mBSA induced arthritis

3.3.1 Autoantibodies against survivin

Autoantibodies against survivin of both IgG and IgM isotypes are found in RA patients, and are increased in patients without erosions. There are no correlations between the serum levels of survivin and the anti-survivin autoantibodies [73]. Anti-survivin autoantibodies are found in a substantial portion (41%) of patients with systemic sclerosis, rarely in patients with SLE [123,124] and were not detected in patients with Sjögren's syndrome [124]. In a study of women with endometriosis and healthy controls anti-survivin autoantibodies were negatively associated with smoking [125], thus smoking might be a confounding factor for survivin autoantibody levels.

3.4 Survivin in other autoimmune diseases

Survivin is detected in blood plasma and synovial fluid of and patients with juvenile arthritis and the levels of survivin was associated to a more severe course of disease [126]. Survivin is expressed in psoriatic skin and decrease upon infliximab treatment [127]. It is also expressed in oral lichen planus lesions [128] and is increased in T lymphocytes from patients with multiple sclerosis [129].

3.5 A link between Flt3 ligand and survivin in RA?

Flt3L is a hematopoietic differentiation factor stimulating growth of hematopoietic progenitor and DCs [130,131]. Analysis of Flt3L in paired serum and synovial fluid samples in a cohort of RA patients with established disease showed that Flt3L is strongly expressed at the site of inflammation [132]. In addition, in an experimental setting Flt3L exerts both pro-inflammatory and tissue destructive properties once in the joint cavity [132]. Also, Flt3 has been shown to be a marker with predictive value for development of RA [133].

Mutations in Flt3 are found in 30% of patients with acute myeloid leukaemia [134] and cells expressing Flt3 with internal tandem duplication mutations, which keep Flt3 signalling constantly active, have an increased survivin expression [135]. We found that in the survivin positive RA patients, the levels of Flt3L in are also elevated. Thus, there is a positive association between the levels of survivin and Flt3L in blood ($p=0.003$) (Paper I).

3.6 A link between urokinase plasminogen activator and survivin in erosive RA?

uPA is a serine proteinase responsible for activation of plasminogen. This leads directly to lysis of fibrin clots, but also to the breakdown of the extracellular matrix and invasive migration of cells. This plays an important role in wound healing and inflammation, but also in cancer development and metastasis.[136]. The link between survivin and uPA was noted in breast cancer [137], but there are reasons to suspect that those two could also be linked in RA. The synovial tissue in RA patients has an increased uPA activity in the proliferating lining layer [138] (paper III). uPA has been shown to induce arthritis after intraarticular injection in healthy mice [139]. Since erosivity in RA seems to be linked to high serum levels of survivin we postulated that survivin could be involved in cell transformation leading to increased migration of synovial cells and breakdown of the extracellular matrix- that is in processes typically linked to the activity of uPA. Indeed, survivin and urokinase are both elevated in the patients with erosive RA and there is a correlation ($r = 0.46$, $P = 0.0015$) between the two proteins (Paper III).

4 FLT3L AND DENDRITIC CELLS

4.1 The expression of Flt3L in RA

Although, the exact cellular source of Flt3L in RA patients has not yet been determined, Dehlin et al. observed that the levels of soluble Flt3L are significantly higher in synovial fluid from patients with RA compared to synovial fluid from patients with joint damage caused by osteoarthritis or traumatic joint injury, while the serum levels of Flt3L did not differ between the patients and the controls [132]. These findings suggest the synovial cells could be a major source of Flt3L in RA. Flt3L is expressed both as an intracellulary stored soluble protein (sFlt3L) and as a membrane bound protein (mFlt3L) and the balance between them are mediated through alternative splicing [140,141]. The Flt3L is released *in vivo* in response to intracellular infections such as malaria [142] or viral infections, in response to stimulation with TLR ligands [143] and after bone marrow ablation [144]. Human synovial fibroblasts express mFlt3L on their surface in culture (previously unpublished, Figure 8), but release barely detectable amounts spontaneously. Also bone marrow stromal cells [145,146] and thymic stromal cells [147] express mFlt3L and sFlt3L. Both human [148] and mouse (unpublished data) T lymphocytes express mFlt3L and have intracellularly stored sFlt3L. It is not known whether soluble Flt3L in RA patients is generated through alternative splicing or through shedding of the membrane bound form. In T cells sFlt3L can be released when the cells are stimulated with cytokines that signal through the common cytokine receptor γ-chain (IL-2, IL-7, IL-4 and IL-15) [148] and uric acid was recently shown to mediate Flt3L release from mast cells [142]. Soluble Flt3L can also be generated through shedding of the membrane bound protein by proteases like TNFα converting enzyme (TACE/ ADAM17) [149].

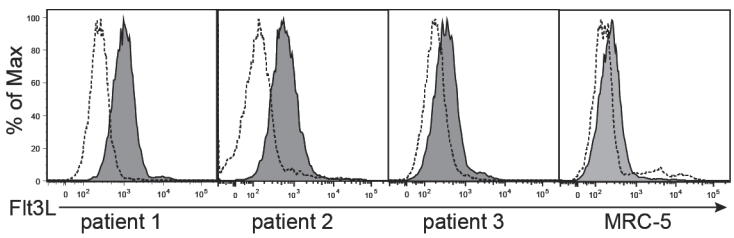


Figure 8. Surface staining of Flt3L (tinted) and control IgG (dashed) on in vitro cultured synovial fibroblasts and the primary human cell line MRC-5.

4.2 What cells does Flt3L affect?

The only known receptor for Flt3L is Fms-like tyrosine kinase 3 (Flt3, also known as FLK-2, STK1 or CD135), that is a receptor tyrosine kinase class III. These include c-KIT, FLT3, PDGFRα/β and c-fms receptors. Binding of Flt3L causes auto-phosphorylation of the tyrosine residues of the intracellular domain of Flt3 and both mFlt3L and sFlt3L exhibit this activity [150]. Flt3 was originally cloned by two independent groups, found to be expressed by hematopoietic progenitors [151] and named by the similarity to c-fms [152]. Multipotent progenitors that are generated through haematopoietic stem cell division express Flt3 among other growth factor receptors and the signal that they receive from their ligands determine the fate of cell [153]. Flt3 is the key receptor for the differentiation of the professional antigen presenting DCs from bone marrow progenitors [153] (Figure 9). Mice lacking Flt3 show a marked reduction in the number of DC in peripheral lymphoid organs [154] and ectopic expression of Flt3 restore DC potential in haematopoetic progenitors [155] The expression of Flt3 also remain on fully differentiated

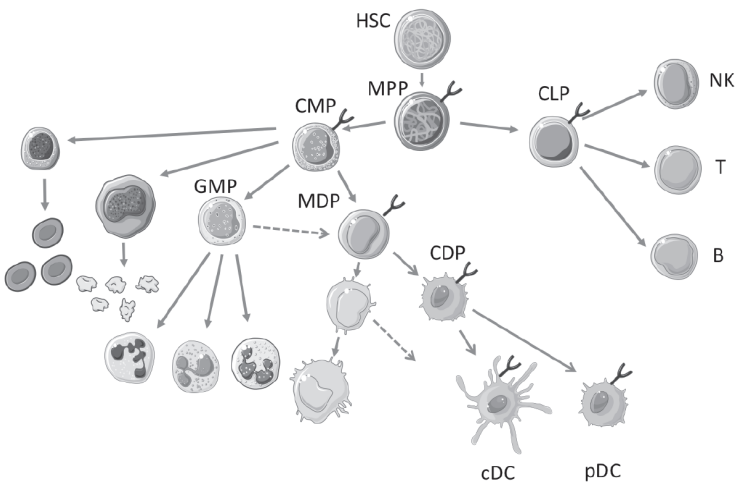


Figure 9. Flt3, together with other growth factor receptors, are expressed on haematopoietic precursors in the bone marrow guiding their development into different cell types. Flt3 is essential for the differentiation of DCs and is also expressed on peripheral cDCs and pDCs. MPP = multipotent precursors, CMP=common myeloid progenitors, CLP= common lymphoid progenitors, MDP= macrophage-dendritic cell precursors, CDP=common dendritic cells precursor.

DCs [153,156] and is present on the surface of the CD11c^{hi} DCs in lymphoid organs [62]. In addition, a small fraction of splenic B cells express Flt3 and they up-regulate surface expression after *in vitro* stimulation with LPS or CD40 and IL-4 [157]. Mice lacking Flt3 have a reduction in B cell progenitors but normal numbers of mature B cells [158]. Flt3L treatment promote the differentiation of DCs *in vivo* in mice [130] and humans [131]. Mice that lack Flt3L have reduced cellularity in the bone marrow, peripheral blood, lymph nodes and spleen, and a particularly large reduction in the number of DCs, but also in NK cells [159]. The slight differences in the phenotypes of the Flt3 and Flt3L knockouts suggest that Flt3L can affect other receptors. DCs can be generated *in vitro* by 8-10 days culture in Flt3L supplemented medium. This will generate DCs that represent steady state splenic DCs with respect to TLR expression, transcription factors and function [160].

4.3 DC development and subtypes

The DC is the most important antigen-presenting cell in the immune system and a bridge between the innate immune sensing and the adaptive immune system triggering either immunity or tolerance. Most information regarding different DC subtypes come from mouse experiments and when it comes to human DC subsets the blood derived DCs are the most studied. Mouse lymphoid-tissue DCs can broadly be divided into plasmacytoid DCs (pDCs) and conventional DCs (cDCs). pDCs express low to intermediate CD11c, MHC II, B220 and PDCA1 (Figure 10A). The cDCs has a high expression CD11c and are MHC II positive (Figure 10A) and can be further divided in to CD8α+CD4^{-}CD11b^{-} (CD8+ DC), CD8α–CD4+CD11b+ and CD8α–CD4–CD11b+ cDCs [161]. The two latter CD8- cDC populations are sometimes grouped and called the CD11b+ cDCs, or alternatively the CD4+ cDCs and the CD8-CD4- cDCs (Figure 10B) [162]. The CD8+ cDC are the most well

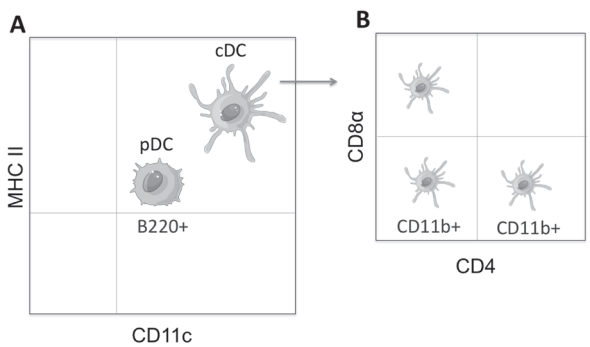


Figure 10. Flow cytometry gating of DC subsets in mouse spleen. A) pDCs are MHCII and CD11c^{int}, cDCs are MHCII+ and CD11c^{hi} B) cDCs can be further subdivided into CD8+, CD4+ and CD8-CD4-

studied subset, which have the same origin and a similar phenotype as the CD103+CD11b- in tissues. They are dependent on the transcription factor IRF8 and are highly sensitive to Flt3L stimulation [62] and are almost absent in Flt3L knockout mice [159]. The CD4+CD8- and CD8-CD4- cDCs are a more heterogeneous population that also dependent on Flt3L possibly in combination with GM-CSF [153]. These DC they express the IRF4 transcription factor and they correspond to the CD103+CD11b+ DCs found in tissues [163].

Differentiation of the skin epidermal Langerhans cell is independent of Flt3L-Flt3 signalling, since they are unaffected in Flt3 knockout mice [163]. Langerhans cells are also present in the human syndrome of DC, monocyte, B and NK lymphoid deficiency (DCML), a condition, which in many ways resemble Flt3L/Flt3 knockout phenotypes [164]. Monocytes that enter lymph nodes can differentiate into a DC phenotype under inflammatory conditions (mo-DCs) [165] DCs that are generated from bone marrow *in vitro* cultures supplemented with GM-CSF with or without IL-4, resemble the monocyte derived DCs [166] and the DCs derived from GM-CSF supplemented PBMC cultures are distinct from the Flt3L derived DCs and they loose the expression of Flt3 [167]. Also *in vivo,* Flt3L and GM-CSF results in different DC populations, with different cytokines profiles [168], they promote different antibody subclasses [169], and Flt3L-induced DCs migrated more efficiently to lymph nodes [168] . Since the GM-CSF knockout mice only has a minor reduction in splenic DC frequencies in the spleen the Flt3L is probably the most important cytokine for maintaining DC number in lymphoid organs [170]

4.4 Flt3L as a chemoattractant

Flt3L is highly released/expressed at the site of inflammation [132] and Flt3+ cells are present in synovial tissue from RA patients [167]. Injection with Flt3L directly into the knee joint enhance inflammatory cell infiltrate in mice [132] and injection of Flt3L into human subjects enlarges lymph nodes and gives a reaction at the injection site [131]. We observed an enhanced migration of freshly isolated mouse bone marrow cells towards a higher Flt3L gradient in a transwell system (paper II). This means that Flt3L could possibly function as a chemoattractant for Flt3 positive cells from the blood or from the bone marrow into the joint space. Other researchers have also observed enhanced migration towards Flt3L of human CD34+ cells [171], but also without gradient, so it cannot be excluded that Flt3L besides chemotactic properties also can display chemokinetic properties.

4.5 DC activation and response

In response to growth factors DCs leave the bone marrow and travel via the blood to nearby tissues and lymphoid organs [172], where they attain their typical morphology and reside and wait for a pathogen encounter. Before any pathogen encounter the DCs are immature, meaning that they express MHCI and MHCII, without providing co-stimulatory molecules. Stimulation of DC through PRR, either attached to the cell surface or present inside the cell, leads to DC maturation. In addition to presentation of the retrieved antigen on MHC II, the mature DCs express co-stimulatory molecules such as B7-1 (CD80) and B7-2 (CD86) and CD40. DCs also express chemokine receptors and e.g. CCR7 makes them home to a nearby lymph node. In the lymph node the activated DC can encounter naive T cells with a cognate TCR that can receive the activating signals through CD28 that interacts with B7 molecules on the DC. Activated T cells can stimulate DCs to produce cytokines through the CD40L interacting with CD40 on the DC. The different DC subsets are described in more detail in the mouse [163,173] and below follows some characteristics of the major DC subsets in mice:

- pDCs produce type I interferons in response to viral and bacterial infections, caused by activation of the nucleic acid-sensing TLRs; TLR7 and TLR9 [174].
- CD8+ cDCs are good at cross-presenting antigens from intracellular microbes to CD8+ T cells through a phagosome-to-cytosole pathway [175], they express TLR3, TLR9, TLR11 and TLR13 and produce IL-12 and are therefore important for the T_H1 response.
- The CD4+ and CD8-DC4- cDCs present peptides on MHC II to CD4+ T cells, express various TLRs, and inflammasome activating PRRs, and a tissue equivalent IRF4 dependent DC express IL-6 and is important for the activation of the T_H17 response [176].

When antigens are presented to a naïve helper T cells in the peripheral lymph node the T lymphocytes differentiate into different subclasses of T helper lymphocytes depending on the cytokines released by the DC, the co-stimulatory molecules they express and the strength binding in the MHC-TCR complex. The naïve CD4+ T lymphocytes differentiate into different subclasses. The different T helper subsets have different cytokine profiles and transcription factors governing lineage commitment of these subsets have been identified (Figure 11). The lineage commitment of effector T lymphocytes is important for initiation of an appropriate programme of defence against the intruder.

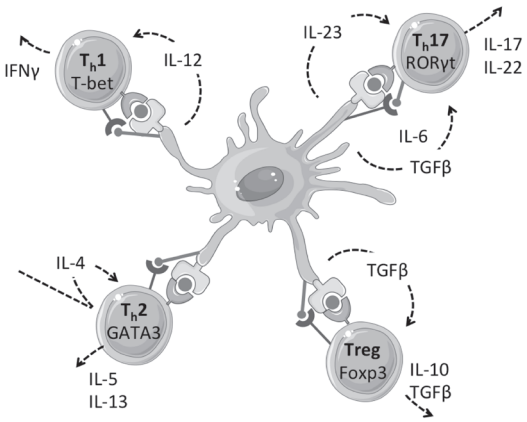


Figure 11. Differentiation of naïve CD4+ T cells into helper T subsets. Lineage commitment depends on the cytokine signals given by the DCs.

4.6 Tolerance induction by DC

Besides their function as initiators of adaptive immune responses, DCs are important for the maintenance of central and peripheral tolerance and e.g. absence of CD11c+ cells in mice causes autoimmunity [177]. Flt3L treatment *in vivo* increases the numbers of regulatory T lymphocytes (Tregs) in mice [62,178] and in humans [179]. This is believed to be mediated by the expanded DC population and in particular the pDCs [178,179] as Tregs can proliferate *in vitro* by addition of antigen loaded DCs [180].

Tregs are T helper cells that are commonly characterized by high expression CD25 and the intracellular transcription factor Foxp3. [181] Tregs suppress immunity through several different mechanisms. They secrete suppressive cytokines like TGFβ and IL-10 and they consume IL-2 [182]. They also constitutively express the receptor CTLA-4 of the CD28 family. CTLA-4 is a receptor with high affinity for the B7 molecules and binding will prevent co-stimulation of naïve T cells via CD28, and thus inhibit their activation.[183] The Treg has also been shown to reduce the APC function by removing B7 from its surface [184]

Although the exact mechanism is not known, DCs are most likely involved both in Treg differentiation [185] and clonal deletion in the thymus [186]. DCs are important also for the induction of Tregs in the periphery [187] together with the presence of TGFβ [188]. Interaction of PD-1 with the PD-L1 on the DC is essential for the induction and sustained function of peripheral Tregs [189]. The immature DC that has not been activated by any

pathogen-associated signals are important to maintain tolerance since their low levels of co-stimulatory molecules induce anergy in T cells [190].

4.7 Inhibition of Flt3 signalling reduces the frequency of DCs and the degree of synovitis in mBSA arthritis

Flt3 expression can be upregulated by inflammatory mediators like PGE_2 [85] which is released by synoviocytes in response to proinflammatory cytokines in RA [191] and is produced during mBSA arthritis [67]. It is known that Flt3 is essential for the development of DCs [154]. Sunitinib, a synthetic inhibitor of Flt3, reduces the frequency of DCs (Figure 12) in

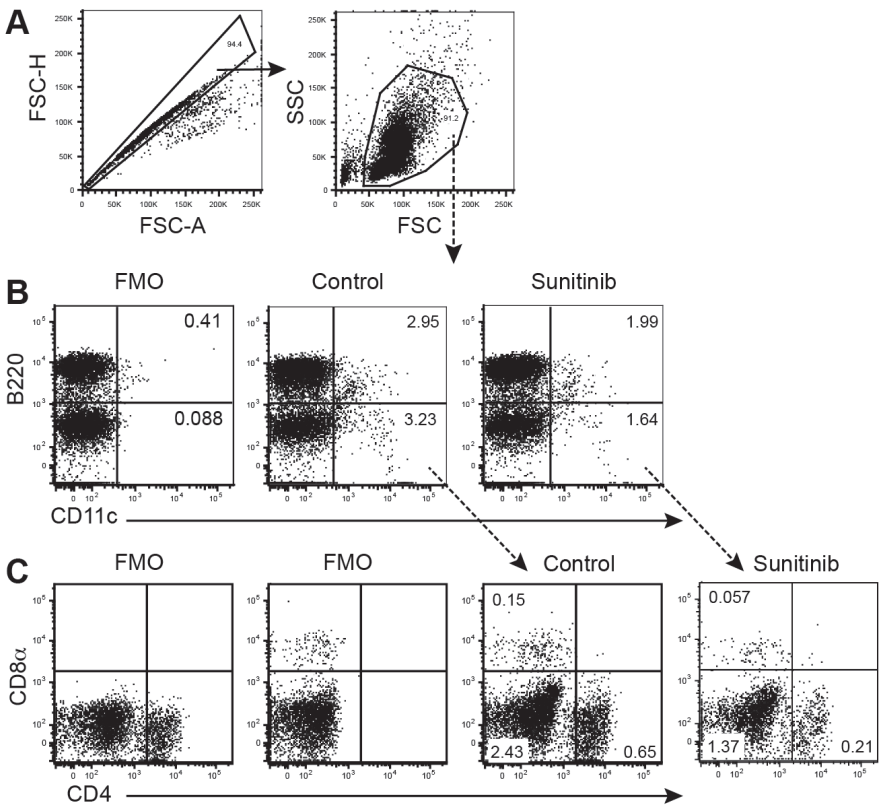


Figure 12. Representational illustration of Flow cytometry gating strategy for the analysis of pDC and cDC frequencies in the spleen after sunitinib treatment.A) Cells were fist gated on singlets, then gated by FSC and SSC to remove dead cells and debris. B) A gate was made for the B220+CD11c+ (pDCs) and the B220-CD11c+ (cDC). C) cDCs where further gated after the expression of CD8α and CD4 into CD8α+$CD4^-CD11b^-$ (CD8+ DC), CD8α–CD4+CD11b+ and CD8α–CD4–CD11b+ cDCs . The frequencies in the gate are relative to the SSC/FSC gate in A. Statistics are shown in paper II

mouse spleen while there are no major differences in the frequency of B cells, T cells or granulocytes (Paper I). Flt3 inhibition by sunitinib results in major decrease in all of the DC subsets (Figure 12 and paper II) that could be explained by the dependency of all subpopulations on Flt3L stimuli [153] and the rapid turnover of DCs [192].

Sunitinib treatment initiated at day 7, the 2nd immunisation, reduces the synovial inflammation in a dose dependent manner and the levels of antigen specific antibodies. However, starting sunitinib treatment at the time of arthritis induction does not reduce synovitis, but instead tended to increase the inflammatory cell infiltration (paper II).

One of the primary features of the human DC deficiency syndromes, like DCML syndrome and the IRF8 mutation, is susceptibility to mycobacterial infections, probably caused by a lack of DCs and subsequent defects of the IL-12/T_h1/IFNγ axis [193]. The DCML syndrome is most likely caused by a defect in a progenitor common to DC, monocytes, B cell and NK cell lineages and can be treated by hematopoietic stem cell transplantation [164]. Flt3L knockout mice have similar cellular defects as DCML patients and they both have reduced number of regulatory T cells [164,194]. Mice with a loss of function mutation in Flt3 show a susceptibility to mouse cytomegalovirus infections [143] and Flt3L treatment increased survival after challenge with *Listera monocytogenes* and herpes simplex virus type 1 in newborn mice [195]. Ectopic expression of Flt3L generated a stronger T_h1 respons to *Mycobacterium tuberculosis* antigen in adult mice [196]

It is possible that the DC deficiency induced by sunitinib in the mBSA model reduces the response to Freunds adjuvant, which is important for proper immunisation, which is crucial for arthritis development after mBSA injection. When sunitinib was given at day 7, from the 2nd immunisation, the inflammatory reaction following intraarticular injection of mBSA was impaired and serum levels of anti-mBSA antibodies were lower, which is a clear sign that sunitinib reduced the response to mBSA. In contrast, when sunitinib treatment was initiated at the time of arthritis induction, when the adaptive immune response was already established, there was no effect on synovitis (paper II).

Taken together, this suggests that the inhibition of Flt3 signalling limit development of arthritis when administered during the immunisation phase by interfering with DC development. However, the effects of Flt3 inhibition by sunitinib on already an established immune response are limited.

4.8 Sunitinib could potentially reduce bone destruction

The frequency of bone destructions in sunitinib treated mice seems to be uncoupled from the degree of synovitis (paper II). Sunitinib is known inhibit all RTKs type III to a different extent, which include the receptors for M-CSF and VEGF [197]. M-CSF and RANKL are potent inducers of osteoclastogenesis in mouse bone marrow cultured *in vitro*, which result in the generation of bone resorptive osteoclasts [198]. The serum levels of RANKL in mice receiving the higher sunitinib dose do not differ from serum levels in control mice, so that does not explain any influence on bone destructions. Sunitinib mediated inhibition of M-CSF could potentially have an effect on the osteoclastogenesis during arthritis. Indeed, it has been shown that sunitinib can reduce the number of osteoclasts mice [199,200]. VEGF, also inhibited by sunitinib, has been shown to enhance the bone resorption by mature osteoclasts [201]. Taken together, it is possible that other actions of sunitinib besides Flt3 inhibition that could have impact on bone destruction.

4.9 Sunitinib the increase the serum levels of Flt3L

The serum levels of Flt3L increased after treating mice with the Flt3 inhibitor sunitinib, (Figure 13 and paper II), which can be interpreted as a negative feedback loop where tissues in lack of DCs release more Flt3L or alternatively a lack of Flt3 positive DCs that consume Flt3L [202]. It is e.g. known that both after DC ablation in mice [177,203] and in human DC deficiencies [164,204] the serum levels of Flt3L are also elevated. The exact mechanism of this feedback loop is not known. But there seem to be no direct effect since there are no differences in release of Flt3L from synovial fibroblasts after adding sunitinib in vitro (unpublished data).

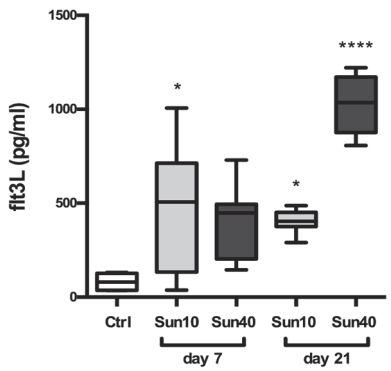


Figure 13. Serum levels of Flt3L in mBSA arthritis mice treated with Flt3 inhibitor sunitinib (sun) at 10 mg/kg/day or 40 mg/kg/day starting from the second immunisation at day 7 or at the arthritis induction at day 21. A kruskal-Wallis test was used to calculate P-values

4.10 Survivin is up-regulated in bone marrow and splenic DC following Flt3 activation

STAT3 is phosphorylated by Flt3 signalling [155] and is essential for development of both pDCs and cDCs in lymphoid tissues. Mice lacking STAT3 cannot produce sufficient amounts of DCs, even after Flt3L injections [205] while expression of STAT3 induced by viral transduction could provoke DC development in Flt3 negative bone marrow progenitors [155]. Phosphorylated STAT3 can bind to the STAT response element in the survivin promoter and induce expression of survivin in haematopoietic progenitor cells [206] Daily systemic Flt3L injections upregulates survivin expression in mouse bone marrow (Figure 14). The MHC II+CD11c^{hi} cDCs in the spleen express high levels of survivin compared to total splenocytes in the mononuclear gate, at baseline (paper I). And the levels of intracellular survivin in the cDCs are significantly upregulated by daily systemic flt3L injection to Balb/c mBSA mice. (Figure 15 and paper II).

Mice treated with sunitinib have a lower expression of survivin in the bone marrow and in the cDCs (paper II), which shows that the upregulation of survivin is mediated through the receptor. The frequency of DCs in the spleen are reduced by sunitinib treatment (Paper II) and increased in the spleen and lymph nodes by daily injections with recombinant Flt3L [62]. Thus survivin expression by Flt3 signalling is strongly linked to the number of DCs in lymphoid organs (paper I, II)[62], but is that due to proliferation or differentiation of DCs?

DC depletion experiments have shown that, except for the Langerhans cells, DC homeostasis and DC numbers are maintained by continuous differentiation of DC from bone marrow precursors rather than proliferation of differentiated DCs [202]. The mature DCs also have the ability to divide in lymphoid organs (spleen and lymph nodes) which was shown by injecting mice with BrdU [172]. However, several studies imply that survivin expression in Flt3+ progenitors mainly promote survival during differentiation. For instance, in Flt3L knockout animals BrdU incorporation in Flt3+ bone marrow progentitors was similar to the incorporation in the wildtype animals, but apoptosis markers were elevated [207]. Singh et al showed that Flt3L induced DC development *in vitro* from haematopoietic progenitors could be enhanced by addition of prostaglandin E_2 that enhanced survivin expression, and was associated with reduced apoptosis but not with proliferation [85].

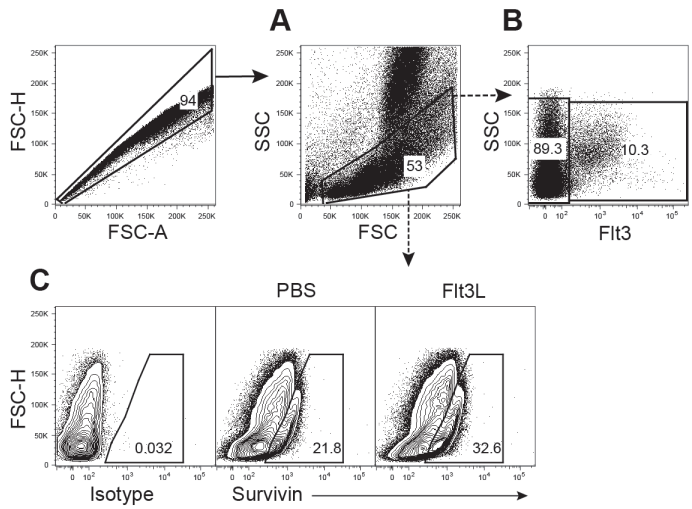


Figure 14. Flow cytometry analysis of bone marrow from mBSA arthritic Balb/c mice that daily were injected with PBS or Flt3L. A) SSC/FSC gate of bone marrow mononuclear cells. B) Bone marrow with positive and negative gate for surface Flt3. C) Intracellular survivin in bone marrow mononuclear cells

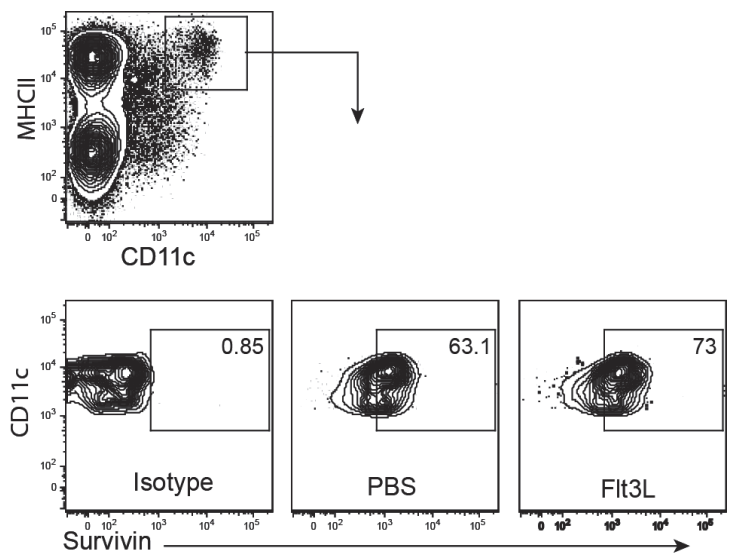


Figure 15. Flow cytometry analysis of intracellular survivin in the MHCII+CD11c^{hi} population in spleen from mice treated with PBS or Flt3L.

Survivin in DCs might also be linked to maturation and enhanced antigen-presenting capacity. Splenocytes stimulated with TLR ligands up-regulated intracellular survivin after stimulation with agonists for TLR9, TLR4 and TLR2 (Sofia Andersson, unpublished data). In human DCs differentiated *in vitro* with GM-CSF/IL-4 an increase of survivin was observed after stimulation with TNFα [208]. The survivin expression in DC is associated with an increased frequency of splenic DCs (paper I, paper II) [62] and increased surface expression of the integrin CD11c, as well as increased expression of MHC II [62]. In RA, local Flt3L might lead to survival of autoantigen presenting cells and the modulation of DC turnover. In addition, inflammatory mediators like PGE_2 enhance the expression of Flt3 and render the DCs more susceptible to Flt3L induced resistance to apoptosis.

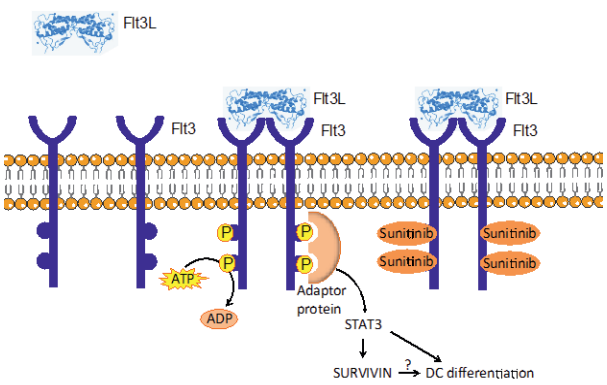


Figure 16. Flt3 signalling and inhibition by sunitinib. Binding of Flt3 induced dimerization of the receptor and autophosphorylation of the intracellular tyrosine residues. A phosphorylation cascade will lead to phosphorylation of STAT3 and survivin expression. Sunitinib prevents autophosphrylation.

4.11 Survivin mediates the effect by tyrosine kinase activation

The specific up-regulation of survivin by Flt3L in cell populations sensitive to Flt3L shows that tyrosine kinase signalling is a powerful inducer of survivin production. Survivin has also been observed to mediate the effects of other RTKs besides Flt3. RNAi targeting survivin remove the cytoprotetive effect of VEGF on human endothelial cells [209] GM-CSF signalling induces survivin expression via STAT3 and STAT3 siRNA induces and apoptosis

[206] SCF, GM-CSF and G-CSF causes upregulation of survivin in bone marrow cells from patients with AML [98].

4.12 Immunity or tolerance by Flt3L?

The effects of Flt3 signalling in mBSA arthritis are contradictory: On the one hand, as was discussed above, Flt3 inhibition by sunitinib significantly reduces the mBSA arthritis synovitis-score in a dose dependent manner when treatment is initiated at immunisation, accompanied by lower levels of mBSA antibodies (paper II). On the other hand, mice injected with recombinant Flt3L show a tendency to reduced joint damage in the mBSA arthritis model, and they also display significantly lower levels of mBSA antibodies [62]. The fact that the seemingly opposite treatments, resulting in reversed DC number and survivin expression, they nevertheless show similar anti-arthritogenic effects, requires an additional explanation. DCs can in contact with naïve T cells give rise to many different responses and transcriptional programmes in the T cell, depending on the activation state and the subtype of the DC, as discussed earlier. Flt3L treatment in mice is accompanied with an increased number of regulatory T cells [210], which can explain the attenuated response to mBSA immunization [62]. The Tregs effect on mBSA arthritis is probably due to the fact that Flt3L treatment is starting before the immune response to mBSA is established. However, Tregs injected at the arthritis induction was n ot sufficient to cure established mBSA arthritis [211].

5 UROKINASE SIGNALLING AND SURVIVIN

5.1 Survivin and the uPA/uPAR system in fibroblasts

Urokinase plasminogen activator (uPA) is a serine protease and it cleaves the zymogen plasminogen to plasmin. Plasmin in turn, can degrade fibrin clots, degrade extracellular matrix components and cleave pro-MMPs into active MMPs that can cleave collagen (figure 18). Besides its proteolytic actions, uPA can bind to the uPA receptor (uPAR, CD87), a glycoprotein lacking an intracellular domain. The binding of uPA to uPAR has two functions for the cell: 1) it localizes uPA activity to the cell surface, which can be used by the cell as a way to degrade extracellular matrix at the leading edge during migration [212]; 2) it can induce intracellular signalling via integrins as co-receptors, leading to cell adhesion, migration and proliferation (figure 17). The system is utilized by cancer cells to enhance invasive properties during metastasis, and is also expressed by synoviocytes in RA *in vivo* [138] and by cultured RA-FLS [213,214]. It has been shown that uPA is essential for the development of collagen-induced arthritis [215] and that uPA injected into a mouse joint can trigger arthritis [139] (paper III).

Survivin is essential for the uPA secretion from fibroblasts (paper III). When injected into a SCID mouse joint, human MRC-5 fibroblasts caused arthritis with synovitis and cartilage destruction after 3 days, but fibroblasts subjected to the survivin targeting siRNA before injection caused a significantly milder arthritis (paper III). It is possible that the observed effect of survivin RNA interference on arthritis is due to decreased uPA secretion by the injected cells.

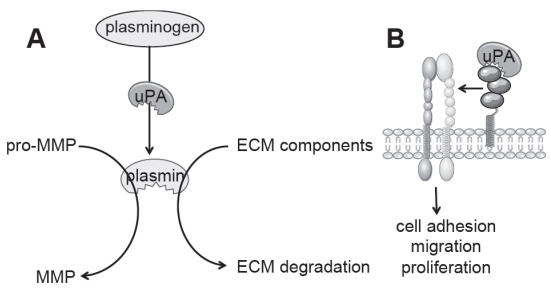


Figure 17. A) uPA cleaves the zymogen plasminogen to plasmin. Plasmin cleaves pro-MMPs into active MMPs and degrades extracellular matrix (ECM) components. B) uPA bind the uPAR at the cell surface and promote intracellular signalling

5.2 uPAR signalling and survivin

The expression of uPAR is complex. It has been shown that Hypoxia-inducible factor 1 (HIF-1) that can bind to the survivin promoter and enhance transcription [216] and it has also been shown to enhance expression of uPAR [217]. We could show that uPAR expression is dependent on survivin (paper III) and survivin is up-regulated by stimulation of uPAR through PI3 kinase signalling (paper III). Indeed, these findings are interesting as local hypoxia characterizes the hyperplastic synovial tissue and might contribute to survival of synoviocytes through survivin and uPAR.

The uPA expressed by synovial fibroblasts can contribute to the arthriogenic behaviour of synoviocytes in at least three different ways 1) uPA promotes the breakdown of the extracellular matrix 2) uPA can induce survivin after binding to its receptor uPAR (paper III), which is expressed by synoviocytes in RA tissue, and thus enhance survival and promote increased uPA synthesis 3) uPA promotes the release of pro-inflammatory cytokines [139], and the chemokine IL8 (paper III), which is a major neutrophil chemoattractant in humans. The uPA induced IL8 release is independent of uPAR (paper III), which means that there might be another not yet discovered mechanism of uPA signalling.

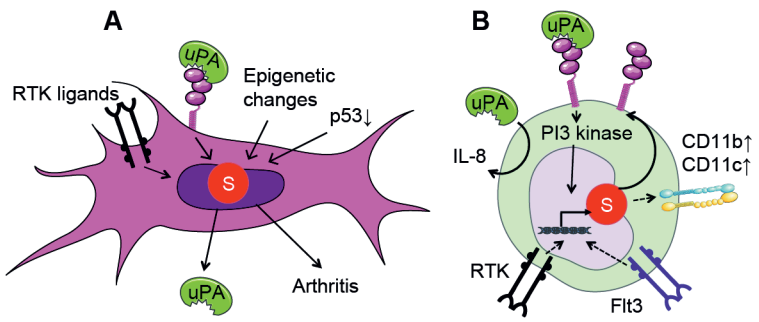


Figure 18. Survivin as a regulator of uPA/uPAR signalling in the inflamed joint. A) Survivin expression in RA-FLS is induced by several possible mechanisms, such as the presence of RTK ligands, uPAR expression, epigenetic changes or modulation of tumor suppressor genes e.g. p53. Survivin expression enhances uPA release from RA-FLS. B) uPA/uPAR signalling enhances survivin expression in monocytes via PI3 kinase, which leads to the up-regulation of uPAR and also to the expression of integrins such as CD11c+ and CD11b+. uPA up-regulates IL-8 in monocytes independently of uPAR signaling. RTK = receptor tyrosine kinase

6 GENERAL DISCUSSION

6.1 Have we found a possible cellular source for the extracellular survivin seen in RA patients?

We have shown that survivin is induced in DCs following Flt3L administration (paper I), in monocytes following uPA stimulation (paper III) and expressed by synovial fibroblasts (paper III). Intracellular survivin may potentially protect inflammatory fibroblasts and autoimmune cells that infiltrate the synovia from apoptosis and impair immune regulation. These findings suggest that the intracellular functions of survivin are important in RA, but what is the source of the extracellular pool seen in the RA patients?

6.1.1 Flt3L induced survivin in DCs?

Previous studies have shown that survivin is not released by human PBMCs isolated from RA patients or healthy controls after stimulation by TNFa, the TLR4 ligand LPS or mitogens such as ConA and PHA [73] The survivin positive RA patients have higher frequency of circulating CD11c+ cells and higher intracellular expression of survivin in the CD11c+ subset [218], which indicate that survivin in DCs can be associated with the extracellular pool of survivin. We found that splenocytes from Flt3L treated mice released low but detectable levels of survivin (paper I). However, we did not observe any changes in serum levels of survivin in Flt3L treated mice or sunitinib treated mice (paper I). In a study of pre-symptomatic individuals sampled before the onset of RA, serum survivin levels are increased compared to controls, while Flt3L levels are not [119]. Thus, there is some evidence suggesting that Flt3L stimulated DCs might be associated with increased levels of extracellular survivin, but it needs to be further investigated.

6.1.2 Synovial fibroblasts?

As there is a correlation between levels of survivin in the blood and synovial fluid [73,122] the joint is a plausible source of survivin secretion. We and others have noticed the local expression of survivin in the lining layer of the RA synovium (paper III) and that synovial fibroblasts cultured *in vitro* express intracellular survivin (paper III). We also found that survivin expression could be enhanced by uPA signalling through uPAR (paper III). Furthermore, although we did not observe any detectable release of survivin into the supernatant *in vitro* (Sofia Andersson, unpublished data) extracellular survivin in RA patients show a correlation to markers of epithelial

mesenchymal transition in synoviocytes, such as S100A4 [219] and uPA (paper III). Thus, synovial fibroblasts expressing survivin is a potential source of extracellular survivin but the explanatory mechanism leading to its release is still lacking.

6.1.3 Cell death?

Necrotic cell death or cellular damage results in the release of intracellular components. In RA there is increased cell death e.g. of chondrocytes due to breakdown of cartilage. It is possible that these cells could release their intracellular storage of survivin. Other intracellular proteins that are not normally secreted as Hsp70 have also been found in synovial fluid from RA patients [220]. An increased general cell-death as a consequence of the anti-rheumatic treatment is a less likely cause since the levels as patients have high levels of extracellular survivin before the initiation of treatment [118]. Thus, it is likely that cellular damage caused by disease progression can lead to the release of survivin.

6.1.4 Possible functions of extracellular survivin

There is a possibility that the release of survivin and its different splice variants have extracellular functions. Previous studies of PBL pulsed with recombinant survivin showed an increased expression of CD11c and CD11b in the monocyte- and granulocyte population [218]. The extracellular survivin may exhibit properties of an endogenous adjuvant i.e. a danger-associated molecular pattern. Several intracellular molecules, such as HMGB1 and heat shock protein, can trigger arthritis when they are localised extracellularly [221]. A receptor or PRR molecule recognising survivin is however not yet identified and survivin could possibly also be taken up passively. It has been shown that extracellular survivin can be taken up by cancer cells and promote cell growth [72]. There are also examples of intracellular proteins acting as co-receptors when present outside the cell [222].

A more detailed dissection is needed to understand the cellular processes that lead to the release of the extracellular pool of survivin. Measurement of specific survivin isoforms in the serum and synovial fluid from RA patients could give more information about the origin and maybe also the function, since it is known that the isoforms of survivin are unable to take part in mitosis [116] and might instead have extracellular functions. Active secretion might be possible under certain circumstances Cervical carcinoma cells (HeLa cells) can secrete survivin via exosomes [223] and extracellular survivin can be taken up by other cells and enhance their proliferation [72].

In conclusion, our studies suggest that there elevated levels of extracellular survivin in a subgroup of RA patients. The cellular source of survivin in this condition is not known, but our data indicate that bone marrow cells, dendritic cells, and fibroblasts are possible candidates. We have shown that down-regulation of survivin signalling by various methods of intracellular targeting as RNA interference and tyrosine kinase inhibitors can ameliorate arthritis. However, we still do not know whether extracellular survivin actually provides additional arthritogenic danger signals. This may be further evaluated by targeting of intracellular and extracellular survivin in models of arthritis.

6.2 Can Flt3 inhibition by sunitinib be a way to target survivin production in RA?

One clinical question that arises from this thesis is whether the targeting of Flt3 with sunitinib is a potential therapy for the treatment of RA patients, since it is already approved and widely used for the treatment of renal carcinoma [197]. However, there are several reasons for not using it:

First of all, we did not see a change in mice when treatment started at the arthritis induction, when the immune response was already established. Secondly, even if inflammatory DCs presenting autoantigens are potential enhancers of an already established autoimmunity, reducing DC number is by far a too crude method of treatment. DCs are essential also for maintaining tolerance; it is known that DC deficiency in humans [164,224] or experimental depletion in mice [177] will lead to autoimmunity. Sunitinib treatment reduces the number of Tregs cells in mice [194] and in renal cell carcinoma patients [225]. In fact, reducing the frequency of Tregs could be an additional mechanism by which sunitinib exerts its effect on renal cell carcinoma [225], although it is generally believed to be mediated by inhibition of VEGFR and PDGFR [226]. In RA, there is risk that by disturbing the balance of haematopoiesis, even more inflammatory DC subsets, less dependent on Flt3 will expand when blocking the Flt3 signalling in the bone marrow progenitors.

Several severe side effects after sunitinib usage, like (nausea and vomiting, anemia, neutropenia, thrombocytopenia etc) that are acceptable for the treatment of metastatic renal cell carcinoma [227] would not be acceptable in the situation of prolonged treatment of RA.

One possibility that remains an option for further studies is instead to focus on the Flt3L. Flt3L was in previous studies shown to enhance peptidoglycan induced inflammation by local injection. However, Flt3L alone could not cause joint inflammation [132], which might suggest that PRR ligands are needed to induce inflammation and that Flt3L can prolong inflammation by recruiting mediators of adaptive immunity. Perhaps by recruiting (paper II) and enhance survival of antigen presenting DCs (paper I).

In these studies we have focused on sFlt3L and the effect on survivin in DCs. We do not know whether the mFlt3L has functions in the communication between DCs and for example the synovial fibroblasts and T cells in RA, and if these cells locally can support the survival of Flt3 positive cells. Ectopic expression of mFlt3L as a vaccine adjuvant has been evaluated in mice with good results. As we observed that Flt3L is expressed on the surface of synovial fibroblasts, there could be a possible interplay between synovial fibroblasts and DCs in RA, where mFLt3L on the synovial fibroblasts enhance survivin expression in the DCs. FLS also express CCL19 and CCL21, ligands for the lymph node homing molecule CCR7, which is expressed on DCs and naïve T cells, making local antigen presentation and lineage commitment possible. Maybe local injections of targeting antibodies or a decoy receptor, interfering with the interaction between receptor and ligand, could function to reduce local expansion of DCs presenting self-peptides. It is not known whether the Flt3 can transmit signals to cells expressing membrane bound Flt3L, but its an intriguing idea that the mode of inhibition could play a role.

7 CONCLUSIONS

- Survivin is expressed in bone marrow and DCs in response to activation of the receptor tyrosine kinase Flt3 *in vivo*.
- Increased survivin levels in DCs coincide with accumulation of DCs in mBSA arthritis, but expression of survivin in DC do not determine whether the T cell response to mBSA is tolerogenic or immunogenic.
- Flt3 inhibition with sunitinib decreases the frequency of DCs and the priming against mBSA when administered at immunisation.
- Intracellular survivin boost the arthritogenic properties of fibroblasts.

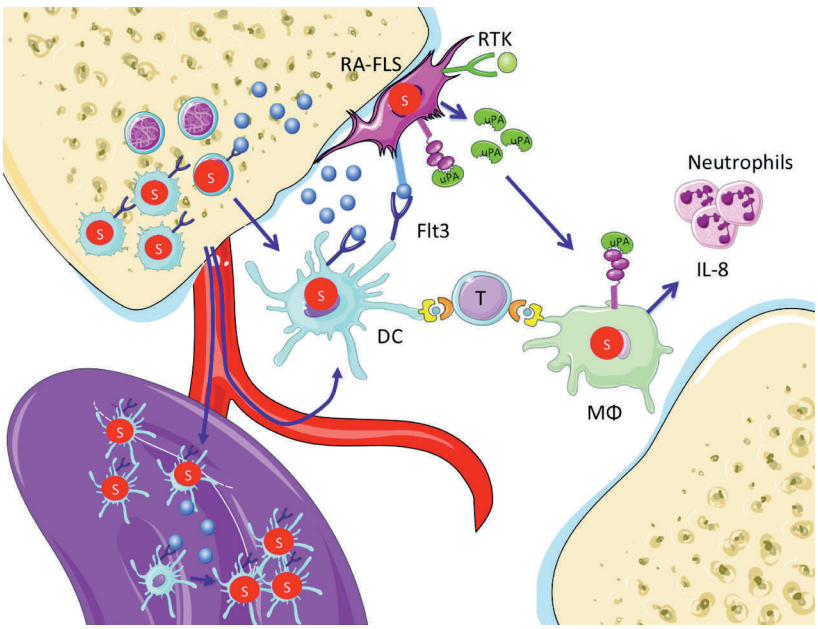


Figure 19. This figure summaries the main findings in the thesis. Flt3L is expressed within the arthritic joint. Flt3 signalling in hematopoietic progenitors will lead to the up-regulation of survivin and differentiation into DCs. DCs will accumulate in secondary lymphoid organs, due to differentiation or clonal expansion. Flt3L also enhances DC migration into the joint, where survivin expression is further enhanced by contact with the RA-FLS expressing membrane bound Flt3L. This will potentially enhance local presentation of autoantigens to T cells. Activated RA-FLS express survivin and release uPA. uPAR is expressed by both RA-FLS and and monocytes in the RA joint. Expression of uPAR that can bind uPA will lead to further up-regulation of survivin. Monocytes activated by uPA express IL-8 that will lead to neutrophil recruitment.

ACKNOWLEDGEMENT

Först och främst vill jag rikta ett stort tack till min handledare **Maria** för att jag har fått möjligheten att arbeta i din grupp. Det har varit en mycket lärorik och utvecklande tid.

Karin – tack för att du har varit en utmärkt bihandledare. Ditt engagemang och stöd har varit mycket värdefullt.

Tack till alla medförfattare och nya och gamla medarbetare i labbet: **Elisabeth AB** och **Linda** – som guidade mig under min första tid, **Malin** - för ditt erfarna lugn, och alltid lika trevliga sällskap, **Ing-Marie** – du har lärt mig allt jag kan om immunohistokemi, **Anna-Karin** – för goda råd och för att du introducerade mig till fibroblasternas värld, **Mats** – för samarbete med sunitinibmössen, **Mattias** – för många sena nätter med marathon-FACSande, för samtal och vänskap, **Annelie** – för pepp och snabba svar, **Sofia** – för goda råd och utflykter, **Mia, Li**, **Mikael**, **Kuba**, **Elisabet J**, **Tao**, **Ali**, **Helene**, **Janne**, och **Rille** – förutom stor excellens i arbetet har ni bidragit med god stämning och gemenskap.

Tack till **Esbjörn** och **Inger -** för många kloka råd och korrekturläsning av ramen.

Tack **Linda B**, **Ola**, **Olof, Katrin, Berglind,** och **Pernilla** för glatt sällskap under skrivandet. Tack till **Lois** för hjälp med presentationen, pepp och härliga skratt.

Tack till nuvarande och före detta doktorander vid avdelningen för reumatologi: **Alexandra**, **Annica**, **Vanja**, **Halla**, **Anna S**, **Maria**, **Gabriel**, **Lucija**, **Hardis**, **Martina**, **Karin**, **Emil**, **Andrey**, **Angelina** och **Jessica.**

Tack till **Hans** och **Kristina**, till **Eva**, **Harriet** och **Cathrine**, samt till all övrig personal på avdelningen för reumatologi och inflammationsforskning.

Ett stort tack vänner, till syskon och föräldrar, i Tidaholm, Härjevad och i Fritsla och sist men inte minst ett särskilt tack till **Alexander**, **Leo** och **Tora**. Utan ert stöd vore det inte möjligt!

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