The stromal component in rheumatoid arthritis: CD55 expression, cell death and beyond
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CHAPTER 1

INTRODUCTION AND SCOPE OF THE THESIS
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INTRODUCTION

Rheumatoid arthritis and preclinical disease

Rheumatoid arthritis (RA) is a chronic (auto)inflammatory disease of the synovial joints, mainly the small joints of hands and feet, leading to joint destruction and functional disability. RA affects 0.5-1% of the population worldwide with a prevalence of females. The etiology of the disease is not known, however, genetic and environmental factors play a role in RA development1. Phenotypically, development of RA is characterized by a dramatic infiltration of immune cells into the synovial tissue and by changes in the main population of resident stromal cells, the fibroblast-like synoviocytes (FLS)2. Angiogenesis, the development of new blood vessels within the inflamed synovium, further facilitates the chronicity of the disease. Appearance of rheumatoid factor (RF) and/or anti-cyclic citrullinated peptide (ACPA) antibodies in blood years before the onset of disease3 points towards a break of tolerance and activation of an adaptive immune response. This break of tolerance might occur due to environmental factors that trigger the innate immune system, such as obesity or cigarette smoking4. Due to the complexity of the disease, it is currently not possible to predict which individuals with RF or ACPA eventually will develop the disease, even if all risk factors are taken into account. A recent follow-up study comparing synovial tissue biopsies from preclinical RA patients (RF- and/or ACPA-positive) at baseline from individuals who developed RA or did not over time demonstrated no apparent changes in the synovium5, except for a trend towards increased CD3+ T-cell numbers. In line herewith, flow-cytometric analysis of lymph node tissue biopsies from early RA patients and preclinical RA showed increased numbers of T cell expressing IL-17, IL-10 and FoxP36. It is not clear how the imbalance between different subsets of T cells promotes disease development and vice versa, which factors promote T-cell differentiation into different subsets during disease development. Stromal cells are reported to play not only a structural role in secondary lymphoid organs, but are crucial for the promotion of T-cell self-tolerance7. Consequently, changes in the stromal cell compartment might contribute to breaking this tolerance. Therefore, biomarkers related to the stromal cells in synovial tissue or lymph nodes might help to identify disease progression versus self-limitation at an early stage.

The current treatment of RA includes non-steroidal anti-inflammatory drugs (NSAIDs), glucocorticoids and disease-modifying anti-rheumatic drugs (DMARDs). In recent years, treatment options for RA largely improved with the appearance of a novel class of DMARDs, called biologicals and including tumor necrosis factor (TNF) inhibitors, B cell-depleting antibodies and interleukin (IL)-6 receptor antibodies8. Anti-TNF drugs are highly effective, however, some patients do not respond adequately. In this case, therapies with a different mechanism of action are needed, yet availability of such alternatives is limited9. Of note, since the etiology of RA is unclear, current therapies cure the symptoms without eliminating the disease-initiating agent. New insights into the mechanisms underlying the development of RA are therefore of critical importance.

Fibroblast-like synoviocytes are resident stromal cells of the synovial tissue

FLS form the synovial membrane, which encapsulates cartilage surfaces of the joints (Figure 1) and produces constituents of the synovial fluid that lubricate the joints10. In normal synovial tissue, the synovial membrane consists of two layers: a continuous surface layer of cells (the
intimal lining) and the underlying tissue (the sublining). The intimal lining is usually more compact than the sublining and consists of 2-3 layers of FLS. In RA, the intima increases markedly in cellularity and can comprise up to 15 cell layers. In contrast to healthy synovium, macrophages accumulate in the intimal lining in RA. They enter the tissue as circulating monocytes and display a highly activated phenotype, producing various chemokines, cytokines and growth factors. These products in turn promote FLS of the intimal lining to express cytokines, chemokines, matrix-metalloproteinases (MMPs) and other enzymes that cause cartilage destruction in the joint. Thus, FLS actively participate in arthritis development by providing a niche for infiltrating immune cells and by producing inflammatory mediators, finally resulting in chronicity of the disease.

Expansion of FLS in vivo has been related to a transformed phenotype. Evidence suggests that FLS resemble cancer cells in some respect. For example, mutations in the p53 suppressor gene are regularly found in RA FLS. Moreover, RA FLS lack contact inhibition when growing in vitro and in vivo, in the SCID mouse model, they migrate from one implanted piece of human cartilage to another. Synovial hyperplasia is due not only to enhanced proliferation of FLS but also to resistance against apoptosis. Programmed cell death (apoptosis) can be induced either through the intrinsic mitochondrial pathway or by triggering cell surface TNF receptor family members (extrinsic pathway). FLS have been demonstrated to express a variety of these receptors, however, they are resistant against FasL-induced apoptosis. This can be explained by increased expression of soluble Fas (sFas) in synovial fluid of RA patients. For the intrinsic apoptotic pathway, the balance between pro- and anti-apoptotic proteins is of critical importance. In RA FLS, the anti-apoptotic proteins Bcl-2 and Mcl-1 are upregulated, whereas expression of the pro-apoptotic proteins Puma and Noxa is surprisingly low. Downregulation of Mcl-1 or upregulation of Puma can be used to break FLS resistance to apoptosis.
For experimental usage, primary FLS are obtained by digestion of synovial biopsies with an enzyme mix. The adherent cell fraction consists of two populations: macrophages and FLS. However, after few weeks in culture, only cells of non-hematopoietic origin survive. This FLS population allows conducting experiments in vitro. FLS share common features with other fibroblasts, such as a spin-like shape and the expression of collagens, CD90 and VCAM-1, however, despite of these similarities, FLS are a unique cell type.

As a model for studying the whole spectrum of specific FLS characteristics, 3-dimensional (3D) micromass organ cultures have been established in a Matrigel matrix. Firstly, in contrast to skin fibroblasts, FLS grown in 3D are able to accumulate and compact at the micromass surface, resembling the intimal lining in synovial tissue. Secondly, they express characteristic proteins of synovial lining FLS, such as lubricin, an important component of synovial fluid, and cadherin-11, which facilitates homotypic adhesion between the cells. Thirdly, FLS support cocompaction of primary monocytes into the micromass lining layer. In the absence of FLS or upon replacement of FLS by skin fibroblasts, monocytes do not survive. Finally, FLS in micromass cultures are able to expand in response to inflammatory stimuli, such as TNF, and they express MMPs, cytokines and chemokines.

Modes and consequences of fibroblast-like synoviocyte activation

There are several pathways of FLS activation within the joint. Activation can occur through soluble chemokines or cytokines, such as IL-1β and TNF, via cell-to-cell interactions, such as CD40-CD40 ligand, and, finally, via binding of various danger- and pathogen-associated molecular patterns (DAMPs and PAMPs). In synovial tissue, different molecules can serve as DAMPs, for example, degradation products in the of synovial fluid can activate various extra-
and intracellular sensors\textsuperscript{24}. However, the relative importance of these activation modes \textit{in vivo} is difficult to explore. \textit{In vitro} studies with cultured FLS can help to understand the consequences of FLS stimulation. For example, TLR3, a sensor for dsRNA has been studied extensively. Upon recognition of dsRNA, TLR3 recruits the adaptor molecule TRIF leading to activation of the transcription factors NF-\kappa B and interferon (IFN)-regulatory factors (IRFs), which results in the production of various pro-inflammatory cytokines, type I IFNs and IFN-response genes (IRGs)\textsuperscript{25}. The main result of TLR signaling is cell activation and attraction of other innate immune cells into the site of inflammation. When dsRNA accumulates intracellularly, e.g. during a viral infection, cytosolic dsRNA sensors (helicases) through type I IFNs evoke the production of a wide range of IRGs, which causes dramatic changes in the cell. If this response does not result in clearance of the virus, cells may undergo apoptosis. \textit{In vitro}, to mimic a viral infection, artificial dsRNAs are used. Poly(I:C) can be recognized by TLR3 if delivered extracellularly\textsuperscript{25} or by MDA5 if provided with a transfection reagent\textsuperscript{26}. 3pRNA upon transfection is recognized by RIG-I\textsuperscript{27}. The ability of intracellular dsRNA to induce apoptosis has been used to diminish the growth of cancer cells both \textit{in vivo} and \textit{in vitro}\textsuperscript{28, 29} and to decrease inflammation of the central nervous system in a mouse model of multiple sclerosis\textsuperscript{28}. In these studies, poly(I:C) or 3pRNA was applied systemically, therefore cells in the bloodstream were activated first. \textit{In vitro} studies of non-transformed cells, such as primary tubular epithelial cells of the kidney, showed that intracellular application of poly(I:C) or 3pRNA is not sufficient to induce apoptosis but requires an additional extrinsic stimulus, such as FasL\textsuperscript{31}. These results may indicate therapeutic possibilities for inducing apoptosis through application of dsRNAs. However, it is important to first pinpoint the molecular pathway of recognition and cell death induction.

\textbf{CD55 and other markers for fibroblast-like synoviocytes}

The abundant expression of CD55 in RA synovial tissue is appreciated for some time now. First studies by Stevens et al., Edwards & Wilkinson and our laboratory showed highly specific staining of synovial lining layer fibroblasts with monoclonal antibodies directed against CD55\textsuperscript{32-34}. Due to the unusually high expression in the intimal lining of synovial tissue, CD55 is now widely used as FLS marker\textsuperscript{19}. However, the mechanism and possible functional implications of its expression have remained elusive. CD55 is a well-established complement regulator. It binds to C3b (and C5b) and prevents accumulation of complement on the cell surface by facilitating their decay (an alternative name of CD55 is DAF, decay accelerating factor)\textsuperscript{35}. Moreover, our group showed that CD55 is a binding partner of CD97, a non-classical G protein-coupled receptor (GPCR)\textsuperscript{36, 37}. CD97 is present on synovial macrophages, which are in close contact with lining layer FLS\textsuperscript{34}. Affinity of CD55 interaction with CD97 is rather low and CD55 is likely to bind C3b and CD97 through different protein domains\textsuperscript{38, 39}. Interestingly, \textit{Cd55}\textsuperscript{-/-} mice are more susceptible to various autoimmune diseases\textsuperscript{40, 41}; however, lack of CD55 rather had a mild protective effect in experimental models of RA\textsuperscript{42}. The dual function of CD55 as complement regulator and binding partner for CD97 may result in different and potentially opposing roles in the pathogenesis of RA (Figure 3).

There are several other fibroblast markers, which are present on FLS in synovial tissue. CD248 (endosialin) is a molecule expressed by perivascular cells and fibroblasts in the intimal lining layer of synovial tissue\textsuperscript{43}. CD248 is a highly sialylated cell surface antigen that comprises an N-terminal
C-type lectin-like domain, a Sushi domain, 3 epidermal growth factor (EGF)-like repeats, a mucin-like region, a single transmembrane segment and a short cytoplasmic tail. Due to the complex structure of CD248, different domains may be involved in regulating inflammation. CD248 on lymph node stromal cells participates in lymph nodes expansion after immunization via increasing the proliferative and migratory capacity of stromal cells. Podoplanin (gp38) is a small mucin-type transmembrane glycoprotein expressed on human lymphatic endothelia, follicular dendritic cells and fibroblastic reticular cells of lymphoid organs. The physiological function of podoplanin during embryonic development is to separate blood and lymph vessels. Mice lacking podoplanin die shortly after birth as a result of respiratory failure and generalized lymphoedema. After embryonic development, podoplanin expressed by fibroblastic reticular cells in lymph nodes allows dendritic cells to enter the lymphatics and migrate within lymph nodes. Expression of podoplanin was also reported in human cancers and has been associated with tumor cell invasion. In RA synovial tissue, podoplanin is expressed in the intimal lining layer where its presence coincides with the expression of α-smooth muscle actin (α-sma).
and might be involved in epithelial-mesenchymal transition of RA-FLS into myofibroblasts\textsuperscript{50}. Fibroblast activation protein (FAP) is a peptidase with two enzymatic activities, a dipeptidyl- and endopeptidase, that allows it to cleave after a proline residue two amino acids or more (respectively) off the N-terminus of a protein\textsuperscript{51}. One of known substrates for FAP is collagen I, an important component of the extracellular matrix. FAP is hardly present in healthy adult tissues, however it is greatly upregulated in sites of tissue remodeling, such as liver fibrosis\textsuperscript{52}, arthritis\textsuperscript{53}, tumors\textsuperscript{54} and embryonic tissues\textsuperscript{55}.

**CD97 is an Adhesion class G protein-coupled receptor regulated by CD55**

Seven-span transmembrane (7TM) are the largest superfamily of receptors regulating crucial cellular processes such as perception (vision, odor and taste) as well as the responses to hormones, chemokines, peptides and ions. Binding of a ligand induces changes in the conformation of 7TM receptor, which leads to the dissociation of receptor-associated G-proteins into subunits that initiate intracellular signal cascades\textsuperscript{56}. Accordingly, 7TM receptors are often called GPCRs (G protein-coupled receptors). According to the current nomenclature, GPCRs are divided into five major classes: Glutamate, Rhodopsin, Adhesion, Frizzled/Taste and Secretin\textsuperscript{57}. Adhesion-GPCRs have unique characteristics that set them apart from all other GPCRs\textsuperscript{58}. Firstly, Adhesion-GPCRs are present on the cell surface as non-covalently bound heterodimers, consisting of an extracellular N-terminal fragment and a 7TM/cytoplasmic C-terminal fragment, that arise from intramolecular processing at a GPCR-proteolytic site (GPS) (Figure 3). Recently it became clear that the GPS motif is a part of a much larger (about 320 residues) GPCR autoproteolysis-inducing (GAIN) domain\textsuperscript{59}. Secondly, most Adhesion-GPCRs have an extended extracellular region containing various domains, which might be involved in cell-cell or cell-matrix interactions. Thirdly, unlike in classical GPCRs, G-protein activation upon ligand binding has been reported so far only for two Adhesion-GPCR, namely latrophilin\textsuperscript{60} and GPR56\textsuperscript{61}. Fourthly, most Adhesion-GPCRs are still orphan receptors\textsuperscript{57}, neither functions nor ligands are known. CD97 is an archetypical Adhesion-GPCR; it’s ability to interact with CD55 has been demonstrated by our group\textsuperscript{36}. Later, other binding partners, such as dermatan sulfate\textsuperscript{62}, α5β1 integrin\textsuperscript{63} and CD90\textsuperscript{64} have been identified. CD97 is broadly expressed by hematopoietic and non-hematopoietic cells\textsuperscript{65}; however, its function is not fully defined. Cd97\textsuperscript{−/−} mice are overall healthy\textsuperscript{66} but, similar to Cd55\textsuperscript{−/−} mice, develop a mild granulocytosis\textsuperscript{67}. Whether phenocopies found in CD55 and CD97 knockout mice are indeed due to a functional link between the molecules requires further investigation. Moreover, studying consequences of the interaction between the Adhesion-GPCR CD97 and its interacting partner CD55 might help to understand the biology of Adhesion-GPCRs in general.

The research described in this thesis aimed to explore the role CD55 in the biology of the synovial tissue as well as in the pathogenesis of RA, thereby considering its ability to interact with both, C3/C5 convertases and the Adhesion-GPCR CD97. Applying in vitro and in vivo approaches, including cell culture systems and gene-deficient mice, we formally confirmed the interaction of CD55 with CD97 in vivo, unraveled the molecular basis of its abundant presence in the synovial lining and described a protective role related to immune complex-meditated arthritis.
Scope of this thesis

In chapter 2, we showed how expression of CD97 is regulated by its interacting partner CD55. *Cd55<sup>-/-</sup>* mice have a higher expression of CD97 on all blood cells, and expression levels are reversibly regulated by CD55 on stromal and hematopoietic cells. Regulation *in vivo* occurred within minutes after transfer of *Cd55<sup>-/-</sup>* cells into wild-type mice. We found that CD97 regulation *in vitro* and *in vivo* occurred only when shear stress, mimicking the situation in the circulation, was applied. Our data suggest that continuous downregulation of CD97 in the blood stream might prevent leukocyte clustering via CD55-CD97 interaction.

In chapter 3, we tested the induction of CD55 expression in FLS using various pro-inflammatory cytokines and DAMPs, which might become available during the course of chronic inflammation in the synovial tissue. We found that TLR3 triggering and, to a lesser extent, ligation cytosolic helicases induces upregulation of CD55. Unexpectedly, we observed that higher concentrations of intracellular poly(I:C), but not 3pRNA, induced FLS cell death.

In chapter 4, we explored the mechanism of FLS cell death after intracellular poly(I:C) application and demonstrate using gene silencing and pharmacological inhibitors that FLS do not recognize intracellular poly(I:C) through the helicase MDAS, the adaptor proteins IPS, TRIF or STING and the kinase TBK1. We propose the existence of another – yet to be identified – dsRNA sensor, which senses intracellular poly(I:C) in FLS.

In chapter 5, we explained the discrepancy between high CD55 expression locally in the intimal lining of synovial tissue and moderate CD55 expression in cultured FLS. We demonstrate that in RA synovial tissue, CD55 is deposited on collagen fibers. In a 3D FLS organ culture model, expression of CD55 appeared together with an extracellular collagenous network. Using K/BxN serum transfer, an immune complex-mediated model of arthritis, we showed that absence of CD55 did not enhance disease activity, but further exaggerated arthritis in *Fcgr2b<sup>-/-</sup>* mice. Using bone marrow chimeras, we confirmed a protective role of stromal CD55.

In chapter 6, we quantified expression of the stromal cell markers CD248, podoplanin, CD55 and fibroblast activation protein (FAP) on synovial tissue biopsies of patients included in two independent early arthritis cohorts. In both cohorts, we observed higher expression of podoplanin and FAP when comparing self-limiting or persistent arthritis with non-inflammatory controls. Significantly greater expression of FAP was found in ACPA-negative RA patients compared to patients developing resolving disease. This suggests synovial fibroblast activation in the early phase of RA and implies that these markers (in combination with the ACPA status) may allow to distinguish between persistent and resolving courses of arthritis.

In chapter 7, we discuss the findings of this thesis in relation to Adhesion-GPCR biology and RA pathology.
REFERENCES


