The stromal component in rheumatoid arthritis: CD55 expression, cell death and beyond
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General discussion and perspectives

Rheumatoid arthritis (RA) is a chronic (auto)inflammatory disease, characterized by infiltration of immune cells into the synovial tissue. Fibroblast-like synoviocytes (FLS) are critically involved in building the structure of the healthy synovial tissue, but are also implicated in the pathogenesis of RA. In RA, FLS provide a niche for incoming immune cells and produce inflammatory mediators that activate these cells. Structurally, synovial tissue can be divided into an intimal lining layer, consisting of activated FLS and macrophages, and a synovial sublining, comprising less compactly packed FLS, various immune cells and blood vessels.

A characteristic marker of intimal lining FLS is CD55, a molecule with at least two functions. First, CD55 prevents the assembling of complement components on the cellular membrane. CD55 binds complement component 3b (C3b) and detaches it from the cell surface (an alternative name is DAF, decay accelerating factor). Activated complement components are found in the synovial fluid of RA patients and CD55, in this context, may protect the tissue against complement-mediated damage. Another activity of CD55 may have an opposite effect in synovial tissue homeostasis. CD55 is a ligand for the Adhesion class G protein-coupled receptor (GPCR) CD97, which is broadly expressed by immune cells. CD55 on synovial lining layer FLS binds CD97+ immune cells and, thereby, may support their retention in the synovium.

In chapter 2, we demonstrated that downregulation of CD97 occurs upon its contact with CD55 in vivo, in the blood stream, and in vitro, under shear-stress conditions. We concluded that this downregulation may restrict CD55–CD97-mediated cell–cell adhesion to solid organs, such as the synovial tissue. In chapter 3, we tested the induction of CD55 expression in FLS using various pro-inflammatory cytokines and danger-associated molecular patterns (DAMPs). We observed that extra- and intracellular application of the double-stranded (ds)RNA mimics poly(I:C) and 3pRNA upregulates CD55 expression and – unexpectedly – found that higher concentrations of intracellular poly(I:C) induce FLS cell death. In chapter 4, we explored the mechanism of FLS cell death after intracellular poly(I:C) application and demonstrated that FLS do not recognize intracellular poly(I:C) through the known cytoplasmic receptor MDA5. We proposed the existence of another – yet to be identified – dsRNA sensor, which senses intracellular poly(I:C) in FLS. Since the function of CD55 in synovial tissue and its importance in health and disease remained elusive, we studied in chapter 5 the role of CD55 in human RA synovial tissue and mouse arthritis models. We unraveled the existence of a collagen network in the intimal lining layer and described the deposition of CD55 on the fibers of this network. Using an immune complex-mediated model of arthritis, we showed that absence of CD55 does not enhance disease activity, but strongly exaggerated arthritis in mice lacking the inhibitory FcgRIIB (CD32). We proposed that CD55 protects synovial tissue from immune-complexes mediated arthritis together with other regulatory molecules. There are numerous studies focusing on the identifications of prognostic biomarkers for early stages of RA. However, these studies fairly ignored stromal cell markers. Stromal cells of synovial tissue, FLS, are hypothesized to play an active role in supporting persistent inflammation. A switch between resolving and persistent disease occurs most likely in early phases of the disease development. An analysis of the expression of the fibroblast markers CD248, podoplanin, CD55 and FAP in two independent early arthritis cohorts is presented in...
chapter 6 of this thesis. In this General discussion, some hypotheses, unresolved issues and future directions are discussed.

A collagen network in the synovial tissue and functional benefits of CD55 deposition

Previous studies demonstrating the existence of an extracellular matrix (ECM) network in the synovial tissue using the Gomori staining technique did not unravel its molecular composition. In chapter 5, we showed the presence of collagen I and III in these fibers. Inflamed synovial tissue is sometimes considered as tertiary lymphoid organ. Reticular fibers in lymph nodes have a highly organized structure, consisting of a collagen core surrounded by fibroblast-like reticular cells (FRCs), which express ER-TR7. Reticular fibers of lymph nodes have a crucial function in antigen transfer and motility of dendritic cells. Considering that collagen fibers in synovial tissue might have a similar function as reticular fibers in lymph nodes, we stained RA synovial tissue with antibodies against ER-TR7. Collagen fibers inside the synovial tissue were not related to lymph node-associated ER-TR7+ fibers.

The ECM is an important component of solid tissues that is located between cells. It provides a barrier between different tissue layers, which facilitates the “navigation” of migratory immune cells and provides a matrix for biologically active compounds, such as growth factors. There are several growth factors including fibroblast growth factor (FGF), tumor growth factor beta (TGFβ) and vascular endothelial growth factor (VEGF) reported to be associated with ECM. In recent years, it became evident that ECM is not only a structural component, but has a broad impact on cell mobility and vascular morphogenesis. As an example, it was suggested that matrix-bound VEGF induces sprouting angiogenesis, while the soluble form of VEGF induces vascular hyperplasia. Angiogenesis, a formation of new blood vessels from pre-existing capillaries, is an essential process in providing inflamed tissue with oxygen and nutrients and conducting inflammatory cells into the tissue. VEGF, hypoxia-inducible factors (HIFs) and angiogenic chemokines are the most potent angiogenic stimuli. Now, we can only speculate that collagen fibers with bound growth factors in the synovial tissue may increase angiogenesis. Future investigations using 3D FLS micromasses co-cultured with endothelial cells might shed light on this subject.

Another important finding of chapter 5 is that CD55 is deposited on collagen fibers in the synovial lining, which explains its local abundance (Figure 1). CD55 is bound to the cell membrane via a glycosylphosphatidylinositol (GPI) anchor and can be cleaved off from the cell surface using phosphatidylinositol-specific phospholipase C (PI-PLC). In general, high expression of CD55 in solid tissues seems to be related to fibrillar deposition. Similar to what we have shown, CD55 is deposited on elastic fibers in enteric plexuses in the gut in its GPI-cleaved form. Furthermore, CD55 deposition on ECM has also been reported for HeLa cancer cells and for human umbilical vein endothelial cells (HUVECs). Studies using primary colorectal cells and gastric cell lines and biopsies extended these results and confirmed the abundant presence of CD55 in the environment of tumors and its deposition on ECM. A mechanism was proposed according to which CD55 deposition on ECM is related to higher expression of VEGF in tumors, which in turn stimulates expression of CD55. VEGF was the only cytokine found to upregulate CD55 on ECM. ECM-bound CD55 was still able to bind the complement component C3b.
Deposition of specifically CD55 on the ECM may require the activity of PI-PLCs. PLCs are able to discriminate between GPI-bound molecules with different molecular weights (MW) and release preferably molecules with a high MW, including CD55\textsuperscript{35}. GPI-bound CD55 has a MW of 70 kDa (for comparison, another GPI-bound complement regulator, CD59, has a MW of 20 kDa) and is shown to be susceptible to PI-PLC cleavage\textsuperscript{36}. An advantage of depositing CD55 on the ECM surrounding synovial tissue could be the resistance of this form of CD55 to further enzymatic cleavage. It might protect synovial tissue from complement attack induced by cartilage-deposited\textsuperscript{37} or soluble\textsuperscript{7,8} immune complexes.

**Stromal markers in the development of RA as predictors for disease outcome**

In chapter 6, we studied the expression of CD55 and other stromal markers (CD248, podoplanin and fibroblast activation protein (FAP)) in two independent cohorts of patients with early arthritis. We did not see differences in CD55 or CD248 expression compared to non-inflamed control tissue. Moreover, expression of CD55 or CD248 did not change between persistent and resolving diseases. These and previous results from chapter 5 about the role of CD55 in the synovial tissue made us see CD55 as a constitutively expressed molecule that protects tissue from complement attack. In the same chapter, we demonstrated a higher expression of the stromal markers podoplanin and FAP in self-limiting or persistent arthritis compared to non-inflammatory controls. Moreover, FAP expression was higher in persistent anti-citrullinated peptide antibody (ACPA) negative RA in comparison to resolving disease. High expression of FAP can have different consequences. Since FAP is a peptidase that can cleave collagen molecules\textsuperscript{38}, FAP might be involved in degrading ECM, thereby promoting immune cell migration into synovial tissue analogous to tumor tissue\textsuperscript{39,40}. As angiogenesis, an important process supporting tissue expansion, is dependent on ECM remodeling, FAP can be indirectly involved in promoting angiogenesis in inflamed tissue\textsuperscript{40}. Therefore, increased expression of FAP in RA may support existing inflammation. However, the precise mechanism of FAP action can be assessed only *in vitro* using 3D models of FLS in combination with endothelial cells.
Interference with CD55 as treatment option for rheumatoid arthritis

CD55 is a complement regulator with a wide cellular distribution. As explained above, CD55 is overexpressed in the ECM surrounding certain tumors. That is why most of the available data regarding the therapeutic approaches targeting CD55 are available from cancer studies. A role for high CD55 expression in worsening tumor development and outcome has been demonstrated in several independent studies, but contradictory data has been reported as well. As an example, CD55 overexpression in breast cancer was associated with a more favorable outcome, whereas an increased expression of CD55 in nasopharyngeal carcinoma correlated with tumor progression and poor prognosis. Blockade of CD55 with monoclonal antibodies inhibited the ability of CD55 to protect tumor cells against complement activation making them more susceptible to complement-mediated killing by membrane attack complex (MAC) formation or immune cell recruitment and activation. Since many tumors overexpress CD55, blocking of the molecule might be a useful approach to decrease tumor size and prevent metastasis. For gastric cancer, a prospective study using a single injection of monoclonal antibodies against CD55 prior to tumor surgery resulted in tumor size regression and a higher survival rate over 10 years of follow-up.

There are no clinical trials reported using anti-CD55 monoclonal antibodies for the treatment of arthritis. Results of animal experiments from chapter 5 of this thesis suggest that CD55 plays a protective role in the development of RA both in immune and stromal cells when an immune-susceptible model (Fcgr2b−/− mice) is used. In this case, an interplay between two key receptors, FcγRIIB (an inhibitory Fcγ receptor) and CD55 (a complement regulator), dramatically worsens experimental arthritis in compound knockout mice. However, previous results from our group and an experiment from chapter 5 with Cd55−/− mice showed that lack of CD55 did not worsen K/BxN serum induced arthritis and even ameliorated collagen-induced arthritis (CIA). Here, other complement regulators, such as CD46 and CD59, might compensate the complement-inhibiting function of CD55, or CD55 may have an alternative function, namely the binding and retention of CD97+ immune cells. Therefore, an approach to block the function of CD55 with monoclonal antibodies in human arthritis could be ineffective. A naturally occurring condition resulting in loss of all GPI-linked proteins, including the complement regulators CD55 and CD59, is paroxysmal nocturnal hemoglobinuria (PNH). PNH is caused by the clonal expansion of hematopoietic stem cell with a defect in one of the enzymes required for GPI-anchor biosynthesis. The most evident clinical manifestations of PNH arise from dysregulated complement activation on blood cells, which leads to hemolytic anemia due to lysis of red blood cells. There are no reports about an association between PNH and arthritis development. Lack of those associations can be explained by the partial loss of CD55 on blood cells and the still complete presence of CD55 on stromal cells.

Intracellular poly(I:C) causes FLS apoptosis independent of MDA5

In chapter 3, we showed that CD55 can be upregulated by extracellular poly(I:C), a known TLR3 ligand. However, when we tested intracellular poly(I:C), which in many cells is recognized by the dsRNA sensor MDA5, FLS underwent cell death. In chapter 4, we investigated in detail the mechanism of cell death and the involvement of MDA5 in the recognition of intracellular poly(I:C) in FLS. We showed that cells undergo intrinsic apoptosis, however, we did not completely exclude other mechanisms of cell death, since we saw that knockdown of TRIF reduced cell death at
least partially. Figure 2 illustrates the different processes of death that cells can undergo and that have been studied in chapter 4.

In vitro, FLS are difficult to transfect with plasmids of any size; the cells shrink and detach from the culture plastic. However, the precise link between recognition of dsRNA (in case of intracellular delivery of poly(I:C)) and dsDNA (in case of transfection with a plasmid) and induction

Figure 2. Schematic overview of different mechanisms of controlled cell death: intrinsic/extrinsic apoptosis and necroptosis. a. Intrinsic apoptosis can be induced by several factors such as DNA damage, metabolic stress and nutrient deprivation, which induce a shift in the balance between anti-apoptotic (eg, Bcl2, Bcl-xI) and pro-apoptotic (eg, Noxa, Puma) proteins. This shift results in association of Bak and Bax, which form mitochondrial outer membrane pores (MOMPs), release of cytochrome C from mitochondria, binding of cytochrome C to APAF-1 and finally formation of an apoptosome. The apoptosome complex converts pro-caspase 9 into caspase 9, which in turn converts pro-caspase 3 into caspase 3. Caspase 3 is known as an effector caspase, which induces several cellular processes and as a final step nuclear and cytoplasmic condensation. b. Extrinsic apoptosis is initiated after ligation of death receptors, such as FASL binding to FAS. Formation of FASL-FAS-FADD involves binding of pro-caspase 8, which is turned to an active caspase 8. The downstream pathways are similar to the intrinsic apoptotic pathway. c. Necroptosis is executed by a ripoptosome complex: RIP1-RIP3-FADD-pro-caspase 8 that assembles after extracellular poly(I:C) induces dimerization of TLR3 and binding of an adaptor molecule TRIF. Depending on which isoform of caspase inhibitor cFLIP is bound to the complex cell death is executed in two different manners. A large isoform of cFLIP (cFLIPL) inhibits ripoptosome formation and cells undergo apoptosis via cleavage of pro-caspase 8 into an active caspase 8. A short isoform of cFLIP (cFLIPS) stabilizes ripoptosomes and executes cell death in another manner – with intact nucleus, but release of the internal compartment of the cell – necroptosis.
of apoptosis in FLS is not known. A mechanism of cell death proposed for melanoma cells\textsuperscript{53} is that recognition of intracellular poly(I:C) by MDA5 through downstream signaling induces expression of the pro-apoptotic protein Noxa, which eventually induces cleavage of pro-caspase 9 and execution of apoptosis with caspase-3 (Figure 2). In our study, we showed that intracellular poly(I:C) is recognized neither by MDA5 nor involves engagement of the adaptor proteins IPS and STING. The mechanism of recognition can be the following: an unknown dsRNA receptor recognizes intracellular poly(I:C), which induces phosphorylation of IRF transcription factors. IRF1 has been shown to induce expression of pro-apoptotic Puma\textsuperscript{54}, and IRF3 can either translocate the apoptotic MOMP-forming protein Bax into mitochondria\textsuperscript{55} (Figure 3, ①) or induce expression of IFNα/β. When IFNα/β binds to its receptor (IFNAR), phosphorylation of JAK kinases occur, which phosphorylate STAT1/2 transcription factors. STAT proteins migrate into the nucleus and induce expression of more than a hundred IFN-regulated genes (IRGs)\textsuperscript{56}, including RNA-dependent protein kinase R (PKR) and 2', 5'-oligoadenylatesynthetase (OAS), which inhibit translation and induce RNA degradation. This, in turn, might activate intrinsic apoptotic pathway (Figure 3, ③). We detected significantly less apoptosis after knockdown of IFNAR (see chapter 4). Alternatively, since we also saw reduction (not significant) of apoptosis after knockdown of TRIF, we could not exclude another mechanism of cell death involving ripoptosome formation and stabilization (Figure 3, ②).

Decreasing FLS proliferation as a therapeutic option

Apoptosis induction of FLS after administration of intracellular poly(I:C) could be considered as a therapeutic approach to decrease stromal hyperplasia and, in combination with other

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**Figure 3. A model showing a connection between dsRNA/dsDNA sensors and controlled cell death.** See text for a detailed description.
interventions, to decrease the number of immune cells in the synovium. Immune cells are transiently present in the synovium with a relatively short half-life; therefore, drugs need to be applied with short intervals. In contrast, FLS are resident cells of the synovial tissue with a rather long half-life, and even single administrations of intracellular poly(I:C) might have a long-term effect. Administration of intracellular poly(I:C) has been successful in mice to treat melanoma and experimental autoimmune encephalomyelitis (EAE), a model for multiple sclerosis (MS). In both cases, symptoms of the disease were diminished due to apoptosis of effector cells – tumor cells in melanoma and dendritic cells (resulting in diminished Th1 and Th17 cell differentiation) in EAE, respectively. Importantly, intracellular poly(I:C) was administered systemically, so the first cells that encountered poly(I:C) complexed with a transfection reagent were dendritic cells and macrophages in the blood stream. However, depending on the mechanism of the disease, different cells need to be targeted. Tumor growth and metastasis are dependent on proliferation activity and epithelial-mesenchymal transition EMT of tumor cells. Tumor cells are more susceptible to apoptosis after administration of intracellular poly(I:C) than cells in the blood stream. This makes tumor cells excellent targets for this therapy. The development of multiple sclerosis is highly dependent on the expansion of antigen-specific T cells, which got primed after the contact with professional antigen-presenting cells, mainly dendritic cells (DCs). Interferon receptor (IFNAR)-mediated cell death of DCs, induced after a systemic administration of poly(I:C) complexed with a transfection reagent, prevented the expansion of antigen-specific T cells and thereafter diminished symptoms and pathology of EAE.

If we consider specific targeting of FLS using intracellular poly(I:C) as treatment for RA, then the therapeutic agent should be administered locally into the joints. In this case, FLS will be the first cells to encounter intracellular poly(I:C) and undergo cell death. Before apoptosis occurs, the first response of FLS to intracellular poly(I:C) is the expression of IFNβ. IFNβ has an ability to reduce expression of pro-inflammatory mediators, such as interleukin (IL)-6, tumor necrosis factor (TNF) and matrix-metalloproteinases (MMPs), that are key players in RA. IFNβ acts through its receptor (IFNAR) and induces expression of numerous IRGs, including the chemokine CXCL10. In chapter 4, we showed that FLS apoptosis is IFNAR-dependent and occurs 24 h after the treatment, whereas upregulation of CXCL10 on mRNA level was detected 16 h after the treatment. CXCL10 plays an important role in the homing of leukocytes into synovial tissue, and its cross-talk with receptor activator of NF-κB ligand (RANKL) may induce bone destruction in RA. The expression of CXCL10 in FLS after administration of intracellular poly(I:C) and its functional consequences require further investigation.

Concluding remarks and perspectives
This thesis addressed the expression and function as well as the prognostic and therapeutic value of CD55. In synovial tissue, CD55 is produced by FLS and found at large amounts in the intimal lining layer. We hypothesized that due to the abnormally high expression, and based on previous results from our group, CD55-mediated binding of CD97-expressing immune cells may play an even more important role than the control of complement activity by CD55 in the synovial tissue. To study the function of CD55 in the development of arthritis in vivo, we used the passive K/BxN serum transfer model of arthritis in mice that lack both CD55 and the inhibitory FcγRII. We found that lack of CD55 both on immune and stromal cells worsens disease activity,
proving that CD55 also contributes to the control of immune-complex mediated inflammation in the joints. Importantly, expression of CD55 stays at the same high level in synovial tissue of non-inflammatory controls and patients who develop arthritis overtime (our study from chapter 6). These results suggest that CD55 is constantly needed for the protection of synovial tissue.

Another aspect, discovered in this thesis, is the recognition and apoptosis-inducing capacity of intracellular poly(I:C) in FLS. In the current discussion we propose to use intracellular poly(I:C) as a theurapeutical agent to decrease the amount of proliferating FLS. However, therapies that combine the targeting of stromal cells with the targeting of immune cells might be more powerful. It is crucial to understand the mechanism of action of different interventions, which in turn might help to explain why some patients respond to a certain therapy and others do not.

Since transfection of some primary cells results in apoptosis, gene manipulation of these cells is possible only using viruses. This is a time-consuming technique especially when combinations of manipulated genes are required. Therefore, the identification of receptors, involved in the recognition of dsRNA (including poly(I:C)) or plasmid DNA is crucial. Blockage of these receptors may prevent cells from undergoing apoptosis without influencing the expression of genes encoded by a transfected plasmid. Identification of the exact molecular pathway connecting dsRNA or dsDNA receptors to apoptosis will help to understand complications related to cell transfection.

The role of the stromal component in the development of RA remains an interesting subject. FLS have unique capabilities when isolated and used for in vitro analysis. They are able to express pro-inflammatory cytokines and actively respond to the given stimuli. Clearly, it is difficult to detect soluble cytokines and MMPs in stromal cells locally in the tissue. The exact role of stromal cells in RA is not well understood, a situation that will improve with novel approaches, such as the 3D micromass FLS model. In this model, FLS can be cultured alone in Matrigel matrix in 3D mode or together with other cells, such as immune or endothelial cells. This approach may help to understand the impact of FLS on immune cells or the vasculature and thereby help to develop novel therapeutic approaches targeting the stromal compartment of the synovial tissue.

REFERENCES


GENERAL DISCUSSION AND PERSPECTIVES


