Summary and discussion
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This thesis focuses on the consequences of CD97 interaction with its ligands CD55, chondroitin sulfate B (CSB) and very late antigen-5 (VLA-5) in rheumatoid arthritis (RA). Furthermore, the role of the related epidermal growth factor-seven transmembrane (EGF-TM7) family members EMR2 and EMR3 in RA was studied.

BACKGROUND

In RA, the synovial tissue (ST) shows a marked intimal lining layer hyperplasia due to an increase in fibroblast-like synoviocytes (FLS) and intimal macrophages; the latter is thought to be the result of recruitment of bone-marrow derived monocytes from the bloodstream, entering the synovial sublining through the vascular endothelium. These cells might be trapped by FLS as well as by extracellular matrix components. Macrophages and FLS play a key role as effector cells in RA by producing a variety of cytokines, matrix metalloproteinases and other soluble mediators that promote joint inflammation and destruction.

Previous research showed a close association between CD97+ macrophages and CD55+ FLS in the intimal lining layer. This observation suggested a possible role of the CD97–CD55 interaction in macrophage retention and activation at this site. The initial aim of this thesis was to study the consequences of this receptor–ligand pair in RA. The past years, additional ligands for CD97 have been described – CSB and VLA-5 – and novel EGF-TM7 receptors have been identified, necessitating an expansion of the research questions.

Chapter 2 provides a systemic overview of the literature on the EGF-TM7 family of Adhesion type G protein coupled receptors (GPCRs). With the human genome completed, a total of five EGF-TM7 receptors have been identified: CD97 and EMR1 to 4. These five receptors are located on the short arm of chromosome 19. They are composed of an extracellular α chain, containing tandemly arranged epidermal growth factor (EGF)-like domains at the N-terminus, which is non-covalently linked to a seven transmembrane spanning β chain. Due to alternative RNA splicing, isoforms with different numbers of EGF domains exist. These domains can mediate interactions with cellular ligands: CD97 binds CD55 via its first and second EGF domain, CSB via its fourth EGF domain and VLA-5 (α5β1) and possibly also αvβ3 via the stalk region. EMR2 also binds CSB via its fourth EGF domain. Other ligands await identification. With the exception of CD97, expression of the EGF-TM7 receptors is restricted to cells of the myeloid lineage. CD97 is expressed on a broad range of leukocytes as well as on a variety of non-immune cells including (malignant) epithelial and smooth muscle cells.
MAIN FINDINGS

Part I. EGF-TM7 family members and their ligands are expressed in synovial tissue of RA patients.

Chapter 3 describes the expression of EMR2 and the distribution of EMR2 and CD97 ligands within RA ST. EMR2 expression in the synovial sublining was found to be significantly higher in RA compared to disease controls. Most EMR2-positive cells were macrophages and dendritic cells (DCs), expressing co-stimulatory molecules and tumor necrosis factor α (TNFα). CSB was shown to be the ligand of the largest isoform of EMR2 and CD97 in rheumatoid synovium. In addition, the smaller isoforms of CD97, but not EMR2, bound CD55 on FLS. The abundant expression of EMR2 and CD97 on myeloid cells in ST of RA patients in close proximity to their cognate ligands CSB, CD55 and VLA-5 suggests that these interactions may facilitate the retention of activated leukocytes including macrophages in the synovium.

EMR3 is another EGF-TM7 receptor on myeloid cells with highest expression levels on fully maturated granulocytes.\(^\text{13}\) Given the abundant presence of EGF-TM7 family members CD97 and EMR2 in ST of patients with RA, expression of EMR3 was studied in Chapter 4. EMR3 was expressed by granulocytes and DCs in inflamed ST from patients with RA and other arthritides. Synovial fluid (SF) granulocytes expressed significantly more EMR3 – as well as EMR2 and CD97 – than peripheral blood (PB) derived granulocytes. EMR3 expression could be increased on granulocytes by addition of RA SF as well as TNFα or IL-8 (CXCL8). Its expression in the inflamed synovial compartment and its induction by SF and specific pro-inflammatory cytokines suggest that EMR3 plays a role in synovial inflammation.

Part II. Antibodies to the EGF domains of CD97 do not affect T cell proliferation and granulocyte migration \textit{in vitro}.

Chapter 5. To gain more insight into the functional consequences of the CD97–ligand interaction, the effects of CD97 ligation were studied in mixed lymphocyte cultures. Antibodies directed at the CD97 stalk region, marginally but significantly inhibited alloantigen-induced T cell proliferation. Since no effect on T cell apoptosis was observed, decreased proliferation was presumably caused by augmentation of the threshold for T cell activation. CD97 stalk region-directed antibodies significantly decreased the release of the T cell effector molecule granzyme B. Finally, a tendency towards inhibited expression of the adhesion molecules CD11a and CD49d was observed. Antibody binding to EGF domain 1 (CD55 binding site) or 4 (CSB binding site) of CD97 did not inhibit any of these key parameters of T cell activation. Furthermore, we studied expression of CD97 on different lymphocyte subsets and found that naive and memory T cells express more CD97 than effector cells and that CD97 is upregulated upon T cell activation.
**Chapter 6.** Since antibodies against CD97 have been implicated to hinder leukocyte trafficking in mouse models\(^{14-19}\), we studied the effects of CD97 antibodies on human neutrophil migration in a flow chamber model. Ligation of CD97 at the stalk region impeded neutrophil rolling and increased firm adhesion to activated human umbilical vein endothelial cells (HUVEC) under flow conditions, possibly by altering the activation state of β2 integrins while not affecting expression of CD62L, CD18 or CD11b. An antibody binding to EGF domain 1 (CD55 binding site) did not affect rolling, adhesion or clustering. We concluded that ligand interactions mediated by the EGF domains of CD97 are likely not involved in the recruitment of granulocytes to sites of inflammation.

**Part III. CD97 antibody interference and gene targeting ameliorates arthritis in different in vivo models of RA.**

**Chapter 7.** We evaluated the consequences of CD97 blockade in collagen induced arthritis (CIA), a mouse model of RA. Mice receiving CD97 monoclonal antibody 1B2, blocking the CD55 binding site \textit{in vitro}, at the onset disease developed significantly less disease activity and hind paw swelling compared to control mice. Furthermore, joint damage and inflammation were reduced and granulocyte infiltration was decreased. The effects of CD97 antibody treatment on established disease were similar, albeit less pronounced. The results supported the notion that CD97 contributes to synovial inflammation and joint destruction in arthritis, possibly by interfering with migration and/or retention of the immune cells.

**Chapter 8.** To further investigate the role of CD97–CD55 interaction in the development of arthritis, we studied mice that lack expression of these molecules. The onset of CIA was delayed in both CD97 and CD55 null mice. CD55 knockout mice also showed less disease activity and hind paw swelling. In addition, passively induced K/BxN serum transfer arthritis was diminished in both knockout mice. CD55 protects cells from complement attack. Since CIA and K/BxN serum-transfer arthritis both partially depend on complement activity, one could have predicted an aggravated course of arthritis in CD55 null mice. However, the pathogenic aspect of CD97–CD55 interaction seems to outweigh the beneficial effect of complement inactivation during the course of arthritis development, providing further support for the detrimental role of the CD97–CD55 interaction in RA.
CONCLUDING REMARKS AND DISCUSSION

Mice

During the research described in this thesis, monoclonal antibodies against mouse CD97 were found to block the recruitment of granulocytes to sites of inflammation in vivo. Impaired recruitment of neutrophils resulted in defective anti-bacterial host defense and stem cell mobilization from bone marrow. In line with these findings, we showed that monoclonal antibody 1B2, directed against the first EGF domain of CD97 (CD55 binding site), was protective in the CIA model of RA (Chapter 7). This finding was confirmed and extended by an independent study using the same antibody as well as an antibody to the third EGF domain of CD97 (the presumed CSB binding site). Both antibodies efficiently prevented granulocyte infiltration, inflammation and bone destruction in DBA/J1 mice during early and late CIA. More recently, a mouse with a defective CD97 gene was generated in our laboratory. We showed that this mouse as well as a mouse lacking CD55 developed reduced arthritis in actively (CIA) and passively (K/BxN serum transfer) induced models of RA (Chapter 8). We concluded that abrogation of the CD97–CD55 interaction by means of antibody interference and antibody treatment protects against experimental arthritis.

The findings summarized here let us to hypothesize that the interaction between CD97 and its ligands likely is involved in the trafficking of immune cells to sites of inflammation. Recently, this concept was challenged. Firstly, granulocyte infiltration in thioglycollate induced peritonitis – a model strongly affected by CD97 antibody treatment – was not disturbed in two independently developed CD97 knockout mice. Secondly, studies on the working mechanism of CD97 antibodies raised questions as to whether these antibodies indeed interfere with cell trafficking: (1) Despite the rather ubiquitous presence of CD97 on immune cells, targeting of CD97 with antibodies selectively affected immune responses depending on granulocytes. (2) Antibodies that prevented recruitment of mouse granulocytes in vivo did not impact on the migration of these cells in vitro. (3) Antibodies that block CD55 interaction of human CD97 did not inhibit interaction of granulocytes with activated endothelium in the flow chamber model (Chapter 6). Collectively, these data suggested that CD97 could be dispensable for leukocyte migration and CD97 antibodies may actively disturb extravasation of granulocytes in a different way. Recent work from our laboratory has proven this idea. The CD97 antibody 1B2 (also used in this thesis) was shown to prevent neutrophil recruitment by inducing neutropenia under specific conditions of acute inflammation, which was solely dependent on Fc receptor activation.

The unexpected mechanism of action of CD97 antibodies in vivo not only explained the different consequences of antibody treatment versus gene targeting with respect to cell recruitment. It also raised novel questions about the physiological function of CD97. These questions directly relate to the role of the ligand interactions of CD97.
The role of ligands for the functioning of nonclassical GPCRs of the Adhesion type is currently a matter of intensive debate (5th Adhesion-GPCR Workshop, Leipzig 2010). It is not clear whether molecules that bind Adhesion-GPCRs act as agonists that activate the receptors or whether their activity is restricted to the generation of adhesive contacts between cells. So far, none of the known Adhesion-GPCR binding partners has been demonstrated to initiate signaling. In contrast, a number of studies have shown involvement of Adhesion-GPCRs in the positioning of cells within tissues and in the formation of cell-cell contact. It seems possible that the interaction between CD97 and CD55 facilitates contacts between cells expressing these molecules like FLS, including the surrounding extracellular matrix, and immune cells in the synovium. Whether the amelioration of RA in CD55 and CD97 null mice (Chapter 8) could be explained by lack of establishing cell-cell contact or interference with cell positioning in ST is still the subject of research.

Humans
At the onset of the work described in this thesis, effects of CD97 blockade on human cells had been studied sparsely. Moreover, although murine CD97 has an expression profile similar to human CD97, CD55, and its EGF domains presumably recognize the same ligands (CD55 and CSB), it lacks an RGD motif in the stalk region that facilitates integrin binding in humans (Figure 3 in Introduction). Effects of CD97 ligation at the stalk region can therefore not be investigated in mice. In our experiments with human cells (neutrophil migration and mixed lymphocyte culture), we did not find consequences of blocking CD97–CD55 interaction but observed effects when applying monoclonal antibody CLB-CD97/3 directed against the CD97 stalk region. The leukocyte adhesion cascade comprises of eight steps: capture or tethering, rolling, activation, slow rolling, arrest, adhesion strengthening, intraluminal crawling, and finally paracellular or transcellular migration (and references therein). In the flow chamber model we used, the first six steps of the neutrophil adhesion cascade can be studied. We can therefore conclude that in humans, these steps do not depend on CD55–CD97 interaction (Chapter 6). In contrast, ligation of CD97 at the stalk region partially impeded neutrophil rolling and increased firm adhesion to TNFα-activated human umbilical vein endothelial cells (HUVEC) likely by increasing the activation state of β2 integrins. Integrins on circulating neutrophils are in a fairly inactive state under physiological conditions. Upon exposure to an appropriate external stimulus, functional activity of integrins is rapidly enhanced, resulting in increased adhesion that does not require changes in levels of integrin expression on the cell surface, but instead, affects the activation state of these integrins, a process called inside-out signaling. While blockade of CD97–CD55 interaction did not affect T cell proliferation, ligation of the stalk region of CD97 in mixed lymphocyte cultures resulted in a decreased frequency of alloreactive T cells (Chapter 5). Of note, expression of CD11a, the α
chain of LFA-1, was decreased. On neutrophils LFA-1 mediates firm adhesion and neutrophil arrest by binding ICAM-1. Possibly ligation of CD97 at the stalk region exerts it effects (decreased T cell proliferation, decreased rolling percentage neutrophils) by affecting CD11a. The group of Spendlove found that blockade of the CD97–CD55 interaction resulted in a proliferation block of CD4+ and CD8+ T-cell clones, co-cultured with peptide-pulsed autologous monocytes.29 However, we could not reproduce these data in mixed lymphocyte cultures, and an explanation for this incongruence is still not found.

Together, antibody binding to the stalk region of CD97 affected neutrophil migration and T cell responsiveness. Yet, it needs to be stressed that these effects were limited and that they are not properly understood. Up to now, signaling in response to ligation by physiological binding partners or antibodies has not been demonstrated for CD97 or any of the other EGF-TM7 family members.

**CD97–CD55 blockade: a potential target in RA?**

RA is characterized by the influx of inflammatory immune cells including macrophages and neutrophils.30 In chronic RA, inflammation of the joints is sustained by retention and reduced apoptosis of these cells.1,31,32 A therapy, which reduces migration or retention of immune cells to/in the joint or induces their death might therefore be successful in preventing and treating disease. CD97–ligand interactions could be interesting novel targets for the treatment of RA. Mainly the finding that lack of CD97 and CD55 in mice has a protective effect in the CIA and K/BxN serum transfer models of RA is encouraging. In vitro and in vivo studies have excluded a role for CD97–CD55 in leukocyte migration (and this thesis).21 However, it seems likely that CD97–ligand interactions contribute to the retention of immune cells in inflamed tissue. Due to the very abundant presence of the CD97 ligand CD55 on FLS, synovial tissue might be an interesting site for therapeutic intervention. It seems possible that biologicals that target CD97–ligand interactions have a protective effect in RA. For the development of potential therapeutics, several points need to be considered. Firstly, antibodies need to be developed in such a way that they do not deplete or activate leukocytes. Secondly, interfering with the first line of immune defense, it will be of crucial importance to exclude uncontrolled suppression or activation of the immune system. Thirdly, given the abundance of CD97 expression on all immune cells, unwanted therapeutic effects need be controlled properly. Here, EMR2 and EMR3 could be alternative targets, as expression of both EGF-TM7 receptors is restricted to myeloid cells. EMR2 has been implicated in potentiating the inflammatory response of neutrophils by augmenting the effects of proinflammatory mediators. Furthermore, ligation of EMR2 increased neutrophil adhesion to endothelium and migration.33 However, EMR2 and EMR3 have no ortholog in mice, which limits experimental approaches.

In conclusion, CD97, EMR2 and EMR3 are interesting novel targets for the treatment
of RA. Future research needs to address the feasibility of therapeutic approaches directed at these EGF-TM7 receptors and to unravel the role of these molecules in the pathophysiology of RA.
References


