EGF-TM7 receptors in rheumatoid arthritis
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General introduction
Pathogenesis and treatment of rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory disease that mainly targets the synovial membrane in the joints. The disease typically manifests itself as a symmetrical peripheral inflammatory polyarthritis that leads to joint destruction and may be associated with extra-articular features.\(^1\)

RA affects 1% of the population (varying across racial and ethnic groups, reflecting the prevalence of predisposing genes such as HLA-DR4) and is associated with significant morbidity and increased mortality.\(^2,3\) The age of onset is typically between 30 and 55 years with a female : male ratio of 3:1.

According to criteria co-developed by the American College of Rheumatology and the European League against Rheumatism (EULAR)\(^2\), classification of ‘definite RA’ is based on the confirmed presence of synovitis in at least one joint, absence of an alternative diagnosis better explaining the synovitis, and achievement of a total score of 6 or greater (of a possible 10) from the individual scores in four domains:

- number and site of involved joints (range 0-5),
- serological abnormality (range 0-3),
- elevated acute phase response (range 0-1), and
- symptom duration (two levels; range 0-1).

Although RA involves autoimmune reactions, with presence of autoantibodies like rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA), the precise cause is unknown.\(^3,4\) Multiple different factors such as environmental factors (for instance smoking), as well as hormonal, infectious, and other variables probably interact in genetically susceptible hosts to initiate polyarticular synovitis in a multistage manner (Figure 1).\(^5,6\) In the first stage, a genetic predisposition (shared epitope HLA-DR genes, protein tyrosine phosphatase nonreceptor type 22 (PTPN22), peptidyl arginine deiminase type IV (PADI4), cytotoxic T lymphocyte antigen 4 (CTLA4), Fc receptors for IgG (FcγRs), and various cytokine and cytokine receptor loci) along with environmental factors (like smoking in ACPA positive RA) results in an adaptive immune response with the generation of auto-reactive T cells, B cells, and production of autoantibodies. Presence of RF (antibodies specific for the Fc chain of IgG) and ACPA might precede the clinically detectable onset of RA by years.\(^7\) A non-specific second event (for instance trauma or viral infection leading to synovitis)\(^8\) could function as a secondary event leading to joint inflammation, resulting in increased expression levels of citrullinated proteins in the synovium. In the presence of pre-existing circulating ACPA, this could contribute to epitope spreading and autonomous disease progression. Other mechanisms may be operative in autoantibody negative RA, ultimately leading to activation of common final pathways.
The enhanced immune response can then lead to increased production of cytokines and soluble inflammatory mediators and perpetuation of the synovial inflammation. Finally, this inflammation-driven process can cause joint destruction.

On a cellular level, T cells, B cells, monocytes, macrophages, neutrophils, fibroblasts and their products are thought to be important in the pathogenesis of RA. The first event in the development of RA is probably antigen-dependent T cell activation. Notably, large numbers of CD4-positive T cells accumulate in the joints of patients with RA. However, notwithstanding tremendous efforts, no single initiating antigen has been identified up to now. In addition to antigen recognition through the T cell receptor, activation of CD4+ T cells requires a second, co-stimulatory signal, which is provided by the interaction of B7-1 and B7-2 on antigen-presenting cells (APC) with CD28 on T cells. The T cells found in synovial tissue (ST) display a memory phenotype and produce a number of cytokines like interferon (IFN)-γ, interleukin (IL)-2, and IL-17, although at relatively low levels. These cytokines induce further T cell proliferation and differentiation. Through production of these cytokines and through cell-surface interactions, the activated T cells stimulate monocytes, macrophages, and synovial fibroblasts to produce IL-1, IL-6, and tumor necrosis factor (TNF), and to secrete matrix metalloproteinases (MMP). Furthermore, activated T cells may express the receptor activator for nuclear factor κB ligand (RANKL), stimulating osteoclastogenesis. They can also bind CD40 ligand on B cells, which can result in the production of immunoglobulins. Activated B cells and autoantibody-producing plasma cells are

Figure 1. Multistage pathophysiological model of RA development. See text for details. Figure adapted from Van der Woude et al. 2005.
The locally produced immune complexes may fix complement in the joint, leading to the release of chemotactic factors and the subsequent recruitment of inflammatory cells, for instance neutrophils. Immune complexes can also activate macrophages and monocytes by binding to their Fc receptors. Alternatively, macrophages can be activated by damage-associated molecular patterns (DAMP), binding to pattern recognition receptors such as Toll-like receptors, which are highly expressed in ST of RA patients. Joint destruction is thought to be largely mediated by the action of proinflammatory or regulatory cytokines and growth factors (eg IL-1, IL-6, IL-10, IL-13, IL-15, IL-18, TNF, and granulocyte–macrophage colony-stimulating factor (GM-CSF)), chemokines and chemoattractants (eg IL-8, macrophage inflammatory protein (MIP)-1, and monocyte chemoattractant protein (MCP)-1), matrix metalloproteinases (MMPs), and neopterin generated by activated macrophages. Immune-mediated inflammation results in activation of macrophages and fibroblast-like synoviocytes (FLS), producing degrading enzymes like MMPs, and increased RANKL expression by T cells and synovial fibroblasts. Together, these and other mechanisms lead to joint destruction and ultimately disability.

Although the immune response in RA is systemic, its main target lies within the ST. The synovium is normally a relatively acellular membrane that attaches to skeletal tissues at the bone-cartilage interface. In RA, the ST shows a marked intimal lining hyperplasia due to an increase in FLS and macrophages that during disease progression is associated with accumulation of macrophages, dendritic cells, T cells, B cells, plasma cells, natural killer cells, mast cells, and neutrophils in the synovial sublining (Figure 2). Recruitment of inflammatory cells, local retention, cell proliferation, and impaired apoptosis may all contribute to the increased cellularity of the ST in RA.

The thickened intimal lining layer in RA consists of intimal macrophages and FLS. Both cell types play a key role as effector cells in RA by producing a variety of cytokines, MMP, and other soluble mediators that promote joint inflammation and destruction. At least two-thirds of the synoviocytes are macrophages, 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. Their relative importance in sustaining inflammation in RA is supported by the observation that they disappear once the disease ameliorates. Furthermore, macrophage numbers in ST of patients with progressive erosive disease correlate with radiographic outcome in RA, underlining their involvement in inducing erosions and bone damage.

Due to the availability of new effective drugs and the insight that aggressive treatment ameliorates the outcome of disease, RA treatment has changed dramatically over the past 20 years. The EULAR recommends to treat RA in a multi step manner. The treatment target is clinical remission or, if remission is unlikely to be achievable, at
least low disease activity. In summary: initially (phase I), methotrexate plus or minus glucocorticoids are started. If contraindications for methotrexate exist, leflunomide, intramuscular gold or sulfasalazine are started. If this regimen is not successful after three months (phase II) and prognostically unfavorable factors are present, a biological drug (especially a TNF-inhibitor) is added. In less severe disease, a second synthetic disease-modifying anti-rheumatic drug (DMARD) is started. If this regimen also fails (phase III), the biological treatment is changed; one either switches to a second TNF-blocking drug (+ DMARD) or replaces a TNF-blocking drug by abatacept (T cell costimulation blocker) (+ DMARD), rituximab (anti-CD20 antibody) (+ DMARD), or tocilizumab (IL-6 receptor blockade) (+ DMARD).

Progressive understanding of the critical effector pathways operating in RA has led to these new treatments, but although successful, treatment-free remission for patients with RA remains an indefinable goal. Elucidating the pathogenesis of RA can present novel opportunities for controlling the disease.

**CD97-CD55 interaction - a new target for the treatment of RA?**

A potential novel target for the treatment of RA is the interaction between CD97 and CD55. Our research group previously described a close association between CD55+ FLS and CD97+ intimal macrophages in RA synovium\(^{29}\), and we hypothesized that the interaction between CD97 and CD55 might be involved in the retention of immune cells in the inflamed synovium, which is known to sustain the chronic inflammation in RA. FLS are mesenchymal cells, which under physiological conditions produce extracellular matrix. In RA, FLS display pathological characteristics as they accumulate to form pannus tissue that can display local tumor-like destructive and invasive features.\(^{30-32}\) FLS express decay-accelerating factor (DAF, CD55) at high levels.\(^{29,33-35}\) CD55 is a glycosol phosphatidylinositol-anchored cell surface protein that protects autologous cells against complement attack.\(^{36}\) The name ‘DAF’ refers to the role of CD55 in promoting the dissociation of C3 and C5, two convertases that are crucially involved in activation of the complement cascade. FLS express much more CD55 than leukocytes, endothelial cells, and epithelia, making it a suitable histological marker for this cell type within the intimal lining layer.

The binding partner of CD55, CD97, is a founding member of the EGF-TM7 family of Adhesion-GPCRs.\(^{37-39}\) These predominantly leukocyte-restricted cell-surface proteins possess large extracellular regions containing multiple N-terminal epidermal growth factor (EGF)-like domains.\(^{37}\) CD97 is expressed by a wide range of leukocytes\(^{40,41}\), including activated lymphocytes, granulocytes, monocytes, macrophages, and dendritic cells. Due to alternative RNA splicing, CD97 is expressed as three isoforms containing three, four, or five EGF-like domains (Figure 3).\(^{39}\) All isoforms, albeit with different affinity, bind CD55 via the first two EGF domains.\(^{42}\)
In addition to CD55, CD97 interacts with two other cellular ligands: the glycosaminoglycan chondroitin sulfate B (CSB, dermatan sulfate) and the integrin α5β1 (VLA-5). Integrin α5β1 and possibly αvβ3 bind the RGD motif in the stalk of human CD97. Integrin α5β1 is one of the predominant β1 integrins expressed by rheumatoid synovial pannus and is expressed by cells in the intimal lining layer and endothelial cells, especially in venules and capillaries associated with lymphocyte aggregates. The interaction with
Figure 3. (A) Cartoon representation of human CD97 interacting with its cellular ligands. At the cell surface, CD97 is expressed as a non-covalently associated dimer consisting of an extracellular α and a membrane-spanning β chain. The two chains result from autocatalytic processing of a CD97 propeptide. Alternative splicing generates isoforms with three, four or five EGF domains. Shown here are the smallest and the largest isoform. While EGF domain 1 and 2 interact with CD55, EGF domain 4, which only is present in the largest isoform, binds chondroitin sulfate B. Integrins bind a RGD motif in the stalk region of human CD97. (B) Mouse CD97 has a structure similar to human CD97 albeit that the maximal number of EGF domains is four. Shown here is the middle isoform. In the largest isoform, the EGF domains 2 and 3 are separated by 45 amino acids. (C) Characteristics of human and mouse CD97 isoforms. Depicted is the composition of the EGF domain region, the relative amount of transcripts present in leukocytes, and the ligand specificity. In humans, affinity for CD55 correlates inversely with the number of EGF domains. An interaction of EGF domain 3 of mouse CD97 (the homolog of EGF domain 4 in humans) with chondroitin sulfate B still needs to be proven. The binding site of mAbs recognizing specific EGF domains in mouse CD97 is indicated. Reproduced with permission from Hamann et al. 2010.
CSB involves the fourth EGF domain of CD97 and is restricted to the largest isoform of CD97. In rheumatoid synovial tissue, CSB has been shown to be the primary molecular species of chondroitin sulfates in inflammatory areas. Taken together, both CD97 and its ligands are abundantly present on cells that are implicated in sustaining the chronic inflammation in RA.

CD97 is, as mentioned above, a member of the EGF-TM7 family. Other members are EMR2, EMR3, EMR1, and EMR4. Expression of the different EMR molecules is restricted to myeloid cells. EMR2 (CD312) is expressed by monocytes, macrophages, dendritic cells, and granulocytes. Expression is upregulated during differentiation and maturation of macrophages and is downregulated during dendritic cell maturation. EMR2, like CD97, binds CSB through EGF domain 4. Recently, a role of EMR2 in regulating human neutrophil function has been described. EMR3 is a molecule with an as yet unknown function that is expressed predominantly by granulocytes, mature monocytes and myeloid dendritic cells. A ligand for EMR3 was described to be located at the surface of monocyte-derived macrophages and activated granulocytes. EMR1 is exclusively expressed in eosinophils. Finally, EMR4 is not expressed in humans.

At the starting point of this project, the identification of EGF-TM7 receptors and their ligands was the focus of intensive research. This research led to the characterization of EMR1 to 4 and to the discovery of CSB and integrins as ligands of CD97 and EMR2. Moreover, antibodies and knockout mice were developed that allowed functional studies on CD97 in vivo. Studies with blocking mAbs against CD97 in mice suggested that CD97 was involved in leucocyte trafficking.
Aim and outline of the thesis

The aim of this thesis was to expand our knowledge on the expression of EGF-TM7 family members CD97, EMR2, and EMR3 in RA ST. Furthermore, we aspired to gain insight into the functional consequences of CD97-ligand interaction in RA. The following research questions were asked:

1. Are EGF-TM7 family members EMR2 and EMR3 expressed in ST of RA patients?
2. What are the functional consequences of CD97 ligation in \textit{in vitro} models?
3. What are the functional consequences of CD97 ligation in \textit{in vivo} models of RA?

Answers to these questions are provided in the three parts of this thesis. The preceding Chapter 2 provides a review of the molecular structure, chromosomal localization, gene organization, evolution, expression, ligand binding, and function of all members of the EGF-TM7 family.

Part 1 \textit{In situ} studies
To evaluate the potential role of other EGF-TM7 receptor family members in the pathogenesis of RA, we investigated the expression of EMR2 (Chapter 3) and EMR3 (Chapter 4) in RA ST. Moreover, we visualized the distribution of CD97 and EMR2 ligands.

Part 2 \textit{In vitro} studies
To analyze the role of CD97-ligand interactions \textit{in vitro}, several models were used. In Chapter 5, the effect of CD97-directed mAbs, interfering with different ligand binding sites, on T cell proliferation, cytokine production and integrin expression was studied using mixed lymphocyte cultures. The effect of the same mAbs on neutrophil migration was investigated using a flow chamber model and is described in Chapter 6.

Part 3 \textit{In vivo} studies
To study the role of the CD55-CD97 interaction in the pathogenesis of RA \textit{in vivo}, we used two approaches. Firstly, we applied EGF domain-specific mAbs to block the interaction between CD97 and CD55 in the collagen-induced model of RA (Chapter 7). Collagen induced arthritis (CIA) shares many clinical, histologic, and immunologic features with human RA, like symmetric joint involvement, synovitis, and cartilage and bone erosions. Secondly, we studied CIA and the passive K/BxN serum-transfer model in knockout mice lacking CD97 or CD55 (Chapter 8).
References


