EGF-TM7 receptors in rheumatoid arthritis

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EMR3, a member of the EGF-TM7 receptor family: a possible role in regulating synovial neutrophils and dendritic cells of patients with rheumatoid arthritis, psoriatic arthritis, and inflammatory osteoarthritis

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Submitted for publication.
ABSTRACT

Objectives: Previous work has suggested a role for the EGF-TM7 receptors CD97 and CD312 (EMR2) in the pathogenesis of arthritis. There are as yet no data on their family member EMR3. We undertook this study to determine the expression of EMR3 in peripheral blood (PB), synovial fluid (SF), and synovial tissue (ST), and to assess the effect of SF and various cytokines on EMR3 expression in vitro.

Methods: ST samples were obtained from patients with rheumatoid arthritis (RA), psoriatic arthritis (PsA), and inflammatory osteoarthritis (OA). EMR3 was detected by immunohistochemistry, and stained sections were evaluated by digital image analysis. Co-expression of EMR3 with cell-lineage markers was determined by double immunofluorescence microscopy. Expression of EMR3 in paired PB and SF leukocyte samples of arthritis patients was analyzed by flow cytometry. Neutrophils incubated with EMR3 mAb were analyzed for adhesion, rolling and clustering on TNFα-activated endothelial cells in a flow chamber model.

Results: EMR3 was expressed by granulocytes and dendritic cells (DCs) in inflamed ST. SF granulocytes expressed significantly more EMR3 than PB granulocytes. EMR3 expression on blood cells increased after addition of RA SF. Stimulation of granulocytes with TNFα, CXCL8 (IL-8), or GM-CSF also resulted in an increase in EMR3 expression. Ligation of EMR3 does not affect neutrophil mediated rolling.

Conclusions: EMR3 is the first EGF-TM7 receptor that is mainly expressed on neutrophils. 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.
EMR3, a member of the EGF-TM7 receptor family: a possible role in regulating synovial neutrophils and dendritic cells of patients with rheumatoid arthritis, psoriatic arthritis, and inflammatory osteoarthritis

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease that mainly targets the synovial membrane, cartilage, and bone. Joints affected by RA are characterized by an influx of inflammatory cells in synovial tissue (ST) and dramatically elevated numbers of leukocytes (predominantly neutrophils) in the synovial fluid (SF). Neutrophils have great capacity to inflict tissue damage by producing degradative proteinases, cytokines (amongst others TNFα, IL-1, IL-15, IL-16, and IL-18), and reactive oxygen species, contributing to joint destruction and signs and symptoms associated with inflammation. Commonly used disease-modifying antirheumatic drugs (DMARDs) like methotrexate and anti-TNF affect neutrophil function, implying a functional role of neutrophils in perpetuating the chronic inflammation in ST of RA patients. However, little is known about the molecular mechanisms involved in the neutrophils’ contribution to the inflammatory process in the synovial compartment.

EMR3 is a molecule with an as yet unknown function that is expressed predominantly by mature granulocytes and, to a lower extent, by CD16-positive monocytes and myeloid dendritic cells (DCs). Its tissue distribution has not been determined yet. A ligand for EMR3 was described to be located at the surface of monocyte-derived macrophages and activated granulocytes. EMR3 is a member of the EGF-TM7 family of Adhesion-type G-protein-coupled receptors (GPCRs), consisting of CD97, EMR1, EMR2, EMR3, and EMR4. Epidermal growth factor (EGF)-like domains at the N-terminus of CD97 and EMR2 have been demonstrated to bind cellular ligands. Previous research has shown CD97 and EMR2 and their ligands CD55 and chondroitin sulfate to be upregulated in ST of RA patients. Furthermore, CD97 antibody treatment in a mouse model for RA, collagen-induced arthritis (CIA), proved beneficial. Both CD97- and CD55-negative mice have increased resistance to actively and passively induced arthritis (CIA and K/BxN serum transfer model). Functional studies suggested that CD97 may be involved in granulocyte migration. 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.

Since CD97 and EMR2 and their ligands are both abundantly expressed in RA, we studied the expression of EMR3 in the synovial compartment of RA patients and other arthritides in situ as well as the effect of SF and pro-inflammatory cytokines on
its expression *in vitro*. Furthermore, given the potential role of EMR2 and CD97 in leukocyte trafficking, we investigated the effects of EMR3 antibody binding in an *in vitro* flow model of cell migration.

### PATIENTS AND METHODS

#### Patients
Ten patients with RA, 10 patients with psoriatic arthritis (PsA), and 10 patients with inflammatory osteoarthritis (OA) underwent synovial biopsy; all patients had active arthritis. In addition, paired blood samples and SF samples were collected from 9 RA patients and 16 non-RA patients with active arthritis. The study was approved by the local medical ethics committee, and written informed consent was obtained from all patients.

#### Specimen collection
Biopsy specimens were taken from the knee joint with a Parker-Pearson needle. The different tissue samples (at least 6 per patient) were processed as described previously in detail.

#### Antibodies
The following monoclonal antibodies (mAbs) directed against EGF-TM7 receptors were used: 3D7 (anti-EMR3), 2A1 (anti-EMR2), and 1B5 (anti-CD97) were generated previously in our laboratory; MEM180 (anti-CD97) was a kind gift of Prof. Vaclav Horejsi (Prague, Czech Republic).

#### Immunohistology and double immunofluorescence
ST of 10 RA, 10 PsA, and 10 inflammatory OA patients was studied for the expression of EMR3. Serial frozen tissue sections were stained with mAb 3D7 against EMR3 (biotin-labeled F(ab’)2 fragment, dilution 1:20). In brief, following a primary incubation step for 1h at room temperature, bound mAb was detected by a 4-step immunoperoxidase method using streptavidin-horse radish peroxidase (HRP) (Dako, Glostrup, Denmark), biotinylated tyramide (Dako), streptavidin-HRP, and amino ethylcarbazole (AEC) (Vector, Burlingame, CA). In negative control sections, biotin-labeled 3D7 F(ab’)2 fragment was replaced by non-labeled 3D7 F(ab’)2 fragment.

Using double immunofluorescence techniques, we determined the expression of EMR3 on T cells, macrophages, myeloid DCs, granulocytes, and fibroblast-like synoviocytes in ST of 8 RA patients. The staining procedure was modified from a previously described method. First, 3D7 (biotin-labeled F(ab’)2 fragment) was incubated on serial tissue sections, followed by incubation with streptavidin-HRP, biotinylated tyramide (Perkin Elmer Life Sciences, Boston, MA) and streptavidin-tetramethylrhodamine
isothiocyanate (TRITC) (Zymed laboratories, San Francisco, CA). Then, fluoresceine isothiocyanate (FITC)-conjugated mAb to CD3 (clone SK7; Becton Dickinson, Franklin Lakes, NJ), CD55 (clone M2192; CLB, Amsterdam, The Netherlands), or BCDA1 (CD1c) (clone AD5-8E7; Miltenyi Biotec, Bergisch Gladbach, Germany) was applied. CD15 (clone C3D-1; Dako) and CD68 (clone EBM11; Dako) expression was detected using an ALEXA 488-conjugated goat anti-mouse mAb (clone A11001; Invitrogen, Breda, The Netherlands) as secondary reagent. Stained sections were examined under a fluorescence photomicroscope (Leica, Wetzlar, Germany).

**Microscopic and digital image analysis**

To evaluate staining for EMR3, digital image analysis was used, as previously described. Co-expression of EMR3 with cellular markers was quantified by counting up to 100 EMR3+ cells by two independent observers. The mean percentage of double-staining cells was noted. The percentages were stratified in 5 groups: 0%, 1-25%, 26-50%, 51-75%, and 75-100%. Conversely, we also counted the cells positive for CD1c, CD3, CD15, CD55, and CD68 co-expressing EMR3.

**Flow cytometry**

To study whether EMR3 and its family members are expressed on SF cells, fresh SF and corresponding blood samples of 9 RA and 16 non-RA patients were analyzed immediately after they were obtained in a 2-step staining procedure as described previously in detail. In summary cells were incubated with appropriate concentrations of mAbs directed against CD97 isoforms (biotin-labeled 1B5, non-labeled MEM180), EMR2 (biotin-labeled 2A1), and EMR3 (biotin-labeled 3D7 F(ab’)2 fragment). Mouse IgG1 (unlabeled), mouse IgG2a (biotin-labeled), and hamster Ig (biotin-labeled) were used as controls (all Pharmingen, San Diego, CA). Secondly cells were incubated with allophycocyanin (APC)-conjugated streptavidin, or phycoerythrin (PE)-labeled goat anti-mouse. All samples were treated with Lysis buffer (BectonDickinson, San Diego, CA) according to the manufacturer’s instructions. Cells were analyzed on a dual laser flow cytometer (FACSCalibur, BectonDickinson). Data analysis was performed using Cellquest software (BectonDickinson). Geometric mean fluorescence intensity (MFI) was measured.

**In vitro stimulation of whole blood cells by SF and chemokines**

To determine whether factors in SF regulate EMR3 expression, blood samples of healthy donors were incubated with frozen pooled SF (n=3, pools with 5 patients per pool) or fresh SF (n=1) for 30 or 60 min, and analyzed for EMR3 expression. Furthermore, we stimulated blood cells of healthy donors with 0.01 μg/ml CXCL8 (n=9), TNFα (n=7), GM-CSF (n=9), SDF (n=2), IL-1β (n=3), or IL-6 (n=3) to determine whether EMR3 upregulation would occur. If upregulation occurred stimulation with
different concentrations was performed. (0.001, 0.01, 0.1, and 0.5 μg/ml) for 30 or 60 min (n=4-6). Values are expressed as fold increase, in which the medium control is defined as 1. All stimulation experiments were done in triplo. Cells were analyzed by flow cytometry.

**Isolation of neutrophils**
Neutrophils were isolated by the isotonic Ficoll gradient method (Pharmacia, Uppsala, Sweden). All preparations contained > 95% neutrophils.

**Endothelial cells**
Human umbilical vein endothelium cells (HUVECs) were isolated from human umbilical cord veins according to Jaffe et al. with some minor modifications. Cell monolayers were grown to confluence in 5-7 days. Endothelial cells of the second or third passage were used in perfusion assays. HUVECs was activated by 100 U/ml TNFα (5-7 h, 37°C) before the perfusion experiments.

**Perfusion chamber**
Perfusions under steady flow were performed in a modified form of transparent parallel plate perfusion chamber. This microchamber has a slit height of 0.2 mm and width of 2 mm. The chamber contains a circular plug on which a coverslip with confluent HUVECs was mounted.

**Neutrophil perfusion and evaluation**
Neutrophils were incubated with mAbs (10 μg/ml) against EMR3, L-selectin (DREG 56; positive control), or HLA-A, -B, -C (W6/32; negative control) at 37°C during 20 min. The individual runs occurred all in a 37°C temperature box. Perfusion experiments were recorded on video tape. The neutrophil suspension was perfused during 5 min at a shear stress of 3 dyn/cm². To automatically determine the percentage of rolling cells, custom-made software was developed in Optimas 6.1. Furthermore, video images were evaluated for the number of adhered cells with a Quantimet 570C image-analysis system (Leica Cambridge LTD).

**Statistics**
EMR3 expression on ST was compared using the Kruskal-Wallis test for non-parametric measures. Difference in expression of CD97, EMR2, and EMR3 on SF cells versus blood cells was analyzed by the Wilcoxon matched pair test, since the Kolmogorov-Smirnov test showed non-Gaussian distribution. Expression of EMR3 on blood cells after cytokine stimulation was expressed as fold increase of medium control and analyzed by one way ANOVA for repeated measures test, and if this was significant, followed by a paired t-test.
RESULTS

Patients
Clinical data on the patients are presented in Table 1.

EMR3 is expressed in ST of patients with inflammatory arthritis by granulocytes and myeloid dendritic cells
Figure 1A,B depicts a representative example of the distribution of EMR3 in ST of a patient with inflammatory arthritis. EMR3 staining was present in all ST compartments, albeit scarcely. EMR3 was expressed in ST of patients with RA, PsA, and inflammatory OA. No significant difference in mean counts per mm² was noted (mean ± SEM: RA 122.6 ± 36.2, PsA 136.6 ± 49.0, and OA 59.5 ± 18.7; P = 0.3) (Figure 1C).
To determine which cell types express EMR3, RA ST was double-stained with markers for T cells (CD3), neutrophils (CD15), macrophages (CD68), DCs (CD1c), and fibroblast-like synoviocytes (CD55). EMR3-positive cells were either neutrophils or myeloid DCs (Table 2). Of the neutrophils 51-75% was EMR3-positive, while 26-50% of the DCs expressed EMR3 (Table 2). T cells, macrophages, and fibroblast-like synoviocytes did not express EMR3.

Table 1. Clinical features of rheumatoid arthritis (RA), osteoarthritis (OA), and psoriatic arthritis (PsA) patients included in the histological study and of RA and a heterogenous group of non-RA patients analyzed for expression on paired blood versus synovial fluid cells.

<table>
<thead>
<tr>
<th></th>
<th>RA patients (n = 18)</th>
<th>OA patients (n = 10)</th>
<th>PsA patients (n = 10)</th>
<th>Non-RA patients (n = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (no. males/females)</td>
<td>6/12</td>
<td>2/8</td>
<td>7/3</td>
<td>6/10</td>
</tr>
<tr>
<td>Age (years, mean ± SD)</td>
<td>55 ± 13</td>
<td>70 ± 10</td>
<td>49 ± 14</td>
<td>43 ± 18</td>
</tr>
<tr>
<td>RF (no. pos./neg.)*</td>
<td>16/2</td>
<td>2/8</td>
<td>0/10</td>
<td>1/8, 7 ND*</td>
</tr>
<tr>
<td>CRP (mg/l, mean ± SD)*</td>
<td>52 ± 41</td>
<td>24 ± 35</td>
<td>14 ± 13</td>
<td>58 ± 69</td>
</tr>
</tbody>
</table>

* RF = rheumatoid factor; CRP = C-reactive protein; ND = not done

Table 2. Expression of EMR3 by various cell types in synovial tissue.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Marker</th>
<th>Percentage of cells positive for EMR3</th>
<th>Percentage of EMR3-positive cells coexpressing the cell type marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cells</td>
<td>CD3</td>
<td>0% (n=4)</td>
<td>0% (n=4)</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>CD15</td>
<td>51-75% (n=6)</td>
<td>26-50% (n=6)</td>
</tr>
<tr>
<td>Macrophages</td>
<td>CD68</td>
<td>0% (n=4)</td>
<td>0% (n=4)</td>
</tr>
<tr>
<td>Myeloid dendritic cells</td>
<td>CD1c</td>
<td>26-50% (n=4)</td>
<td>1-25% (n=4)</td>
</tr>
<tr>
<td>Fibroblast-like synoviocytes</td>
<td>CD55</td>
<td>0% (n=3)</td>
<td>0% (n=3)</td>
</tr>
</tbody>
</table>
All EGF-TM7 receptor family members are upregulated on SF cells compared to blood cells

To study whether EGF-TM7 receptor family members are upregulated on SF immune cells, we compared expression of EMR3, CD97, and EMR2 on blood cells with fresh SF cells in patients with active arthritis by FACS analysis (Figure 2A-C). On SF-derived immune cells, expression of the EGF-TM7 receptors was increased. In general, no different expression patterns were observed between RA compared to non-RA patients.

EMR3 was predominantly expressed by granulocytes and to a lesser extent by monocytes. This is in concordance with previously published results.11 Granulocytes on SF cells expressed significantly more EMR3 than their blood cell counterparts (Figure 2A).

To analyze expression of CD97, two mAbs were used: one recognizing the stalk region of CD97 and thus all isoforms of CD97 (MEM180), the other binding the fourth EGF domain present only in the largest isoform of CD97 (1B5). Both antibodies detected CD97 on lymphocytes, monocytes, and granulocytes. In SF, all measured cell types...
expressed significantly more CD97 than the cells derived from blood in both RA and non-RA groups (Figure 2B and data not shown). EMR2 was predominantly expressed on monocytes, consistent with previous observations. A significant increase in the expression on SF-derived immune cells as compared to blood cells was observed for both monocytes and granulocytes (Figure 2C).
EMR3 expression on blood-derived granulocytes increases after stimulation with SF of RA patients likely due to the presence of pro-inflammatory cytokines

To determine whether EMR3 upregulation of SF-derived granulocytes might be the consequence of environmental factors (i.e. factors in SF), blood cells of healthy donors were stimulated with fresh RA SF (n=1) or with pools of previously frozen RA SF (n=3, 5 patients per pool). Upregulation of EMR was found after 30 and 60 min. The mean fold increase ± SEM was 1.3 ± 0.1 (P<0.05) (data not shown).

We next tested whether pro-inflammatory cytokines that are abundantly present in SF of RA patients increase the expression of EMR3 in vitro. Upon stimulation with the cytokines TNFα, GM-CSF, and CXCL8, all potent primers and activators of human neutrophils, EMR3 expression on granulocytes augmented in a dose-dependent manner after stimulation for 30 or 60 min (Figure 3). Stimulation with SDF, IL-1β, and IL-6 had no effect on EMR3 expression (data not shown).

Figure 3. Inflammatory mediators increase the expression of EMR3 on granulocytes. Whole blood granulocytes were stimulated for 30 min (open bars) or 60 min (striped bars) with (A) TNFα (n=5), (B) GM-CSF (n=6), and (C) CXCL8 (n=4) and subsequently analyzed by flow cytometry. EMR3 expression is depicted as fold increase related to non-stimulated cells. * indicates statistical significance P < 0.05.

EMR3 does not affect rolling (A), adhesion (B), or clustering (C) of neutrophils on TNFα-stimulated human umbilical vein endothelium cells (HUVEC). Neutrophils were perfused over TNFα-activated HUVEC in an in vitro flow chamber model, and adhesion, clustering, and rolling were measured. Each experiment (n = 3) was performed in duplo or triplo. Shown are the mean values. Data of the same experiment are connected by lines.

Figure 4. EMR3 does not affect rolling (A), adhesion (B), or clustering (C) of neutrophils on TNFα-stimulated human umbilical vein endothelium cells (HUVEC). Neutrophils were perfused over TNFα-activated HUVEC in an in vitro flow chamber model, and adhesion, clustering, and rolling were measured. Each experiment (n = 3) was performed in duplo or triplo. Shown are the mean values. Data of the same experiment are connected by lines.
The EMR3 mAb 3D7 does not affect rolling, adhesion, or clustering of granulocytes on endothelial cells under flow conditions

Having shown that EMR3 expression on SF-derived neutrophils is augmented, we decided to investigate whether the EMR3 mAb used in this study affects the ability of neutrophils to roll or adhere on endothelium, i.e. affects the initial phases of cell migration into the tissue. No differences between the negative control antibody and 3D7 were observed in rolling, adhesion or clustering (Figure 4A-C).

DISCUSSION

This is the first study on the expression, distribution, and functional role of EMR3 in human arthritis. We found that EMR3 is expressed in ST of all patients with arthritis, irrespective of the disease etiology. While the pathogenesis of RA, OA, and PsA is different, EMR3 expression patterns in ST from patients suffering from these diseases was comparable. Therefore, EMR3 may exert an effect in a common final effector pathway in joint inflammation.

The cell types expressing EMR3 in inflamed joints were mainly neutrophilic granulocytes and, to a lesser extent, myeloid DCs and monocytes. This is in accordance with a previous study demonstrating that EMR3 expression in peripheral blood is limited to granulocytes and few other cells derived from myeloid precursors, mainly CD16 (FcγRIII)-positive peripheral blood monocytes. Importantly, EMR3 could not be found on RA synovial macrophages. It seems possible that in inflamed joints EMR3 expression is lost during maturation of monocyte into macrophage. Alternatively, EMR3-expressing monocytes may be more likely to differentiate into myeloid DCs than into macrophages.

In ST of patients with arthritis, relatively few neutrophils are present, and in general, these neutrophils are located in the inflamed ST at the interface of cartilage with pannus. In contrast, accumulation of polymorphonuclear leukocytes in SF is a hallmark of arthritis. In RA, neutrophils can constitute more than 90% of the cellular exudate in SF. Yet their role in pathogenesis is incompletely understood. They may simply be responding to chemoattractants generated by other cells or alternatively may have a more primary role in promoting inflammation within the joint.

It has previously been reported that in RA SF-derived neutrophils have a more active phenotype compared to blood neutrophils. By secretion of myeloperoxidase, elastase, lysozyme, collagenase, acid hydrolases, matrix metalloproteinases, IL-1β, and prostaglandins, neutrophils potentiate inflammation in the adjacent synovium and promote joint destruction. Furthermore, the secretion of the chemoattractants
platelet-activating factor (PAF) and leukotriene B4 (LTB₄) facilitates recruitment of more neutrophils to the SF compartment.⁷,⁴⁰ Together, these effects add to elevated levels of proinflammatory cytokines in the SF.⁵⁵ We found that EMR3 is significantly upregulated on SF-derived granulocytes and propose that this upregulation is due to the inflammatory micro-environment in the SF. This proposition is substantiated by the fact that 1) SF derived from RA patients increases EMR3 expression levels on human blood-derived neutrophilic granulocytes and 2) TNFα, GM-CSF, and CXCL8 all exert a similar EMR3-increasing effect on these cells.

TNFα, GM-CSF, and CXCL8 are all potent activators of human neutrophils. Thus, expression of EMR3 is upregulated by cytokines that are involved in neutrophil activation and influx in inflamed joints. In conjunction with the fact that two other EGF-TM7 molecules, CD97 and EMR2, are thought to be involved in leukocyte trafficking¹⁸,²², it seems conceivable that this increased EMR3 expression is instrumental in neutrophil migration in arthritis. The EMR3 mAb used in this study did not affect the initial steps of neutrophil migration in vitro.

In this study we show that the related EGF-TM7 receptors CD97 and EMR2 are also expressed on immune cells in SF. Previously, we hypothesized that macrophages in RA might get trapped in the ST by interaction of CD97 and EMR2 with their ligands CD55 and dermatan sulfate.¹⁶,¹⁷ The observation that neutrophils in SF express vast quantities of EMR2 and CD97, in combination with the sparse presence of granulocytes in ST, indicates that interaction of EMR2 and CD97 and their ligands alone is not sufficient for neutrophils to accumulate in ST.

In summary, the EGF-TM7 receptor EMR3 is the first EGF-TM7 family member that is mainly expressed on neutrophils, and, except for low level expression on myeloid DCs, is virtually absent from other cells in inflamed joints. 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.

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References