EGF-TM7 receptors in rheumatoid arthritis

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Identification of the epidermal growth factor-TM7 receptor EMR2 and its ligand dermatan sulfate in rheumatoid synovial tissue
ABSTRACT

Objective: EMR2 and CD97 are closely related members of the epidermal growth factor (EGF)-TM7 family of adhesion class 7-span transmembrane (TM7) receptors. Chondroitin sulfates (CS) have recently been identified as ligands for EMR2 and CD97. CS have been implicated in the pathogenesis of rheumatoid arthritis (RA). We undertook this study to determine the expression of EMR2 and the distribution of EMR2 and CD97 ligands within RA synovial tissue (ST).

Methods: ST samples were obtained by arthroscopy from 19 patients with RA, 13 patients with inflammatory osteoarthritis (OA), and 13 patients with reactive arthritis (ReA). Immunohistochemistry was performed with a monoclonal antibody against EMR2, and stained STs were analyzed by digital image analysis. Coexpression of EMR2 with cell lineage– and activation-specific markers was determined by double immunofluorescence microscopy. To evaluate the expression of EMR2 and CD97 ligands in RA synovium, binding assays were performed using EMR2- and CD97-specific multivalent fluorescent probes.

Results: EMR2 expression in the synovial sublining was found to be significantly higher in RA patients compared with OA and ReA control patients. Most EMR2+ cells were macrophages and dendritic cells expressing costimulatory molecules and tumor necrosis factor α. Dermatan sulfate was shown to be the ligand of the largest isoforms of EMR2 and CD97 in rheumatoid synovium. In addition, the smaller isoforms of CD97, but not those of EMR2, bound CD55 on fibroblast-like synoviocytes.

Conclusion: The EGF-TM7 receptors EMR2 and CD97 are abundantly expressed on myeloid cells in ST of RA patients where their cognate ligands dermatan sulfate and CD55 are detected. These results suggest that these interactions may facilitate the retention of activated macrophages in the synovium.
Identification of the epidermal growth factor-TM7 receptor EMR2 and its ligand dermatan sulfate in rheumatoid synovial tissue

INTRODUCTION

EMR2 and CD97 belong to the epidermal growth factor (EGF)–TM7 family of adhesion class 7-span transmembrane (TM7) receptors. The EGF-TM7 receptors are predominantly leukocyte-restricted cell surface proteins that possess extended extracellular regions containing variable numbers of N-terminal EGF-like domains. CD97 is found on a broad range of leukocytes, whereas expression of EMR2 is restricted to myeloid cells, including monocytes, macrophages, dendritic cells (DCs), and granulocytes. Interestingly, the EGF domains of EMR2 and CD97 are nearly identical (97% amino acid identity), and due to alternative RNA splicing, isoforms with 2, 3, 4, and 5 EGF domains are expressed.

Increased expression of CD97 at sites of inflammation previously led us to investigate its distribution in rheumatoid arthritis (RA), and a close association was found between CD97+ macrophages and CD55+ fibroblast-like synoviocytes in the intimal lining layer. This observation suggests a possible role of the CD97–CD55 interaction in macrophage retention and activation at this site. Interestingly, aberrant CD97 expression in the synovium is accompanied by detectable levels of soluble CD97 in the synovial fluid. The EGF-TM7 receptors interact via the EGF domains with cellular ligands. Recently, both EMR2 and CD97 have been shown to bind chondroitin sulfate (CS) through EGF domain 4. In addition, EGF domains 1 and 2 of CD97, but not those of EMR2, specifically interact with CD55. Thus, the composition of the EGF domain region defines the ligand specificity of EMR2 and CD97 isoforms. Whereas CS is exclusively bound by the largest isoform of both molecules, the affinity for CD55 varies with the different isoforms of CD97.

CS is a class of glycosaminoglycan (GAG) that is abundantly present both in extracellular matrix and in the synovial fluid of RA patients. CS occurs in a number of forms varying in site and degree of sulfation. Three types are recognized: CSA, CSB (dermatan sulfate), and CSC. Dermatan sulfate is an isomer of chondroitin 4-sulfate in which a variable number of glucuronic acid residues are replaced with iduronic acid. Several changes in GAG expression in synovial tissue (ST) and cartilage of RA and osteoarthritis (OA) patients have been described. In ST of RA patients, dermatan sulfate has been shown to be the primary molecular species of CS in inflammatory areas compared with fibrotic areas, where CSA/C expression dominates. Basic activity of the disease and proliferation of the synovium correlate with an increased percentage of dermatan sulfate of the total GAG content in the synovium. Furthermore, RA chondrocytes are
known to synthesize an increased proportion of proteoglycans, enriched in dermatan sulfate. Recently, it was shown that infiltrating cells can bind GAGs in rheumatoid ST. Importantly, ST from healthy individuals or from patients who had joint trauma did not exhibit GAG binding. We hypothesized that the interaction between EMR2 and CS is involved in the retention of inflammatory cells in the inflamed synovium. To test this hypothesis, we investigated the expression of EMR2 as well as that of CD97 and identified their ligands in ST.

**PATIENTS AND METHODS**

**Patient selection**

Nineteen patients with RA and active arthritis of the knee joint underwent synovial biopsy. All patients fulfilled the American College of Rheumatology (formerly, the American Rheumatism Association) 1987 revised criteria for the classification of RA. In addition, synovial biopsy specimens were obtained from 13 patients with inflammatory OA and from 13 patients with reactive arthritis (ReA) of the knee joint. Laboratory assessment included measurement of serum levels of rheumatoid factor and the erythrocyte sedimentation rate.

**Specimen collection**

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

**Immunohistology and double immunofluorescence**

All patients were studied for expression of EMR2 and CD97 and for coexpression of EMR2 with CD68 or tumor necrosis factor α (TNFα). In 7 RA patients, 4 OA patients, and 4 ReA patients with marked expression of EMR2 in their ST, doublelabeling experiments were performed for EMR2 in combination with CD3, CD22, CD38, CD40, CD55, CD80, CD83, and CD86.

Serial sections were stained with monoclonal antibodies (mAb) against EMR2 (2A1; final concentration 1.7 µg/ml) or CD97 (CLB-CD97/3, directed against the stalk region of CD97; final concentration 2.5 µg/ml) or with mAb CLBCD97/1 (directed against the first EGF domain of both EMR2 and CD97; final concentration 5 µg/ml) as previously described. Briefly, following a primary incubation step for 1 hour at room temperature, bound mAb was detected by a 3-step immunoperoxidase method using horseradish peroxidase (HRP)–conjugated goat anti-mouse antibody (Dako, Glostrup, Denmark), HRP-conjugated swine anti-goat antibody (BioSource International, Camarillo, CA), and aminoethylcarbazole (AEC; Vector, Burlingame, CA). In negative control sections, the primary mAb was replaced by an appropriate isotype control mAb.
EMR2

To stain for coexpression of TNFα and EMR2, EMR2 was detected as described above. After development with AEC and preincubation with mouse serum (Central Laboratory of The Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands), an anti-TNFα mAb (clone Mab1; PharMingen, Alphen aan den Rijn, The Netherlands) was added to the slides, followed by HRP-conjugated goat antimouse antibody (Perkin Elmer, Boston, MA), biotinylated tyramide (Dako), and streptavidin–alkaline phosphatase (Dako), and finally developed by the addition of FastBlue (Vector). Using double immunofluorescence techniques, we determined the expression of EMR2 on B cells, T cells, macrophages, DCs, fibroblast-like synoviocytes, and cells that express costimulatory molecules. The staining procedure was modified from a previously described method.22 First, EMR2 mAb (IgG1) was incubated on serial sections, followed by incubation with tetramethylrhodamine isothiocyanate (TRITC)–conjugated goat antimouse IgG1 (BioSource International). Then, after incubation of the slides with mouse serum, fluorescein isothiocyanate (FITC)–conjugated anti-CD3 (clone SK7; Becton Dickinson, Verviers, Belgium), anti-CD22 (clone Rfb-4; BioSource International), anti-CD38 (clone HIT2; PharMingen), anti-CD55 (clone IA10; PharMingen), or anti-CD86 (clone 2331; PharMingen) mAb were applied. For the detection of CD40 (clone 5L3; PharMingen) and CD80 (clone L307.4; PharMingen), the signal was augmented by subsequently adding rabbit anti-FITC antibody (Dako), HRP-conjugated swine anti-rabbit antibody (Dako), biotinylated tyramide, and streptavidin–FITC (Dako). CD83 expression was detected using an IgG2a mAb (HB15A; Immunotech, Montreal, Quebec, Canada), followed by biotin-conjugated goat anti-mouse IgG2a antibody (Nordic, Tilburg, The Netherlands) and streptavidin–FITC.

EMR2 staining in combination with CD68 was performed by incubating sections with anti-CD68 mAb (clone PG-M1, IgG3; Dako) and EMR2, followed by incubation with FITC-conjugated goat anti-mouse IgG3 antibody (Nordic) and TRITC-conjugated goat anti-mouse IgG1 antibody (Nordic).23,24 The sections were examined under a fluorescence photomicroscope (Leitz, Wetzlar, Germany).

Microscopic and digital image analysis

To evaluate staining for EMR2 and CD97, digital image analysis was used as previously described.25 All sections were coded and analyzed in a random order by an independent observer (ENK) who was blinded to the clinical diagnoses. Slides were analyzed in two ways. First, the number of EMR2+ or CD97+ cells per mm² was counted. Since ST of RA patients is characterized by an increase in cell numbers, higher expression of EMR2 and CD97 could theoretically only be related to higher cell numbers. Therefore, we also calculated the integrated optical density (IOD) per cell (expressed as IOD/nucleus/mm²). Coexpression of EMR2 with CD3, CD22, CD38, CD40, CD55, CD68, CD80, CD83, CD86, and TNFα was quantified by having 2 independent observers (GJDT and ENK) count at least 50 and, if possible, up to 200 EMR2+ cells. The percentage of double-staining cells was noted. The percentages were stratified into 5 groups: 0–5%, 6–25%, 26–50%,

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51–75%, and 76–100%. Conversely, we also counted the cells that were positive for CD3, CD22, CD38, CD40, CD55, CD68, CD80, CD83, CD86, and TNFα and that coexpressed EMR2. If there were <15 cells positive for any of these markers per section, the results were discarded to prevent disproportionately high percentages. TNFα expression was measured separately for the intimal lining layer and the synovial sublining.

**Generation of multivalent fluorescent probes**

Generation of EMR2- and CD97-specific multivalent fluorescent probes was performed as described previously. Briefly, sequences encoding the EGF domain regions of EMR2 and CD97 isoforms were cloned upstream of the coding sequence for truncated mouse IgG2b and the peptide recognition sequence for the *Escherichia coli* biotin holoenzyme synthetase BirA. HEK 293 cells were then transfected with 40 µg DNA per 175-cm² flask and cultured for 5 days in conditioned Opti-MEM 1 medium (Life Technologies, Paisley, UK). Secreted soluble recombinant protein was purified using a protein A column (Sigma-Aldrich, St. Louis, MO) and biotinylated using the BirA enzyme (Avidity, Denver, CO) according to the manufacturer’s protocol. Biotinylated proteins were aliquoted and stored at 80°C after quantification by the Bradford assay.

To generate multivalent probes, 10 µl of avidin-coated fluorescent beads (Spherotech, Libertyville, IL) was washed with phosphate buffered saline (PBS)/0.5% bovine serum albumin and incubated with saturating amounts (>1 µg) of biotinylated recombinant protein. After 1 hour, nonbinding protein was removed by washing with PBS. The bead–protein complexes were sonicated immediately before use.

**Binding assays with multivalent fluorescent probes**

Slides were thawed, fixed in acetone, washed in ice-cold PBS, and preincubated with pooled human serum to prevent nonspecific binding. The different bead–protein complexes (10 µl complex plus 40 µl PBS) were added to the ST sections. After incubation for 1 hour at 4 °C, unbound protein–bead complexes were removed by washing with PBS. To determine the specificity of the binding, slides were pretreated with 50 µl 0.8 units/ml chondroitinase AC or B (Sigma-Aldrich), 50 µl anti-CD55 mAb (CLB-CD97/L1 [9]; 10 µg/ml), or 50 µl 5 mM EGTA for 30 minutes before addition of the beads. Furthermore, beads were preincubated with 50 µl chondroitin sulfate A, B, or C (10 µg/ml; Sigma-Aldrich) before addition to the slides. Slides were coverslipped after addition of 100 µl of Vectashield (Vector) or Imsol-Mount (Klinipath, Duiven, The Netherlands). Two microliters of 4’,6-diamidino-2-phenylindole (5 mg/ml; Sigma-Aldrich) was added per slide for nuclear staining.

**Statistical analysis**

Means and SDs were calculated, and the Kruskal-Wallis test was used to compare measures between all diagnostic groups (RA, OA, and ReA). The Mann-Whitney U test was used to compare differences between 2 groups.
Table 1. Clinical features of rheumatoid arthritis, osteoarthritis, and reactive arthritis patients included in the study.

<table>
<thead>
<tr>
<th></th>
<th>RA patients (n = 19)</th>
<th>OA patients (n = 13)</th>
<th>ReA patients (n = 13)</th>
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</thead>
<tbody>
<tr>
<td>Sex (no. males/females)</td>
<td>6/13</td>
<td>3/10</td>
<td>8/5</td>
</tr>
<tr>
<td>Age (years, mean ± SD)</td>
<td>58 ± 12</td>
<td>74 ± 9</td>
<td>45 ± 15</td>
</tr>
<tr>
<td>RF (no. pos./neg.)*</td>
<td>13/6</td>
<td>1/12</td>
<td>1/12</td>
</tr>
<tr>
<td>ESR (mm/h, mean ± SD)*</td>
<td>30 ± 15</td>
<td>25 ± 18</td>
<td>18 ± 13</td>
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</table>

* RF = rheumatoid factor; ESR = erythrocyte sedimentation rate.

RESULTS

Patients
Clinical data on the patients are presented in Table 1. The mean duration of disease was 58 months (range 1–336 months) in RA patients, 57 months (range 2–240 months) in OA patients, and 11 months (range 1–42 months) in ReA patients.

Increased EMR2 expression in rheumatoid ST
Figure 1 depicts representative images of the distribution of EMR2 and CD97 in the ST of patients with RA and OA. Figure 2 shows the distribution of EMR2 and CD97, expressed as IOD/nucleus/mm², in the ST of patients with RA, OA, and ReA. EMR2 was expressed in the intimal lining layer and synovial sublining of all patients with RA, 12 of 13 patients with OA, and 11 of 13 patients with ReA. EMR2 expression was significantly higher in the synovial sublining of RA patients compared with OA and ReA control patients, even after correction for cell numbers (P <0.002). A similar trend was noted in the intimal lining layer, although the difference did not reach statistical significance. EMR2 expression did not correlate with measures of disease activity (data not shown).

Expression of EMR2 was generally more restricted compared with that of CD97. Detection of CD97 with the monospecific mAb CLB-CD97/3 showed expression in all compartments of the synovium, but particularly in the lymphocyte aggregates and in the intimal lining layer. This expression pattern was similar to the CD97 staining pattern we observed in a previous study⁷, in which we used CLB-CD97/1 (directed to the first EGF domain), which is cross-reactive with EMR2.

Considering the great similarity between the staining patterns of CLB-CD97/1 and CLB-CD97/3, and in light of the fact that CD97 expression is much more abundant than EMR2 expression, we might conclude that this cross-reactivity did not significantly affect the staining pattern.

Expression of EMR2 by activated macrophages and DCs in ST
While CD97 is found on most hematopoietic cells, expression of EMR2 has been shown
to be restricted to cells of the myeloid lineage (2,5,6,20). To gain more insight into the distribution of EMR2 in inflamed synovium, we determined which cells express EMR2 by performing double staining with markers for macrophages (CD68), DCs (CD83), T cells (CD3), B cells (CD22), plasma B cells (CD38), fibroblast-like synoviocytes (CD55), and cells expressing costimulatory molecules (CD40, CD80, CD86) or the inflammatory cytokine TNF (Table 2). These experiments enabled us to determine whether EMR2 has a similar expression pattern in various arthritides. First, we studied EMR2 cells and counted the percentage of cells that also showed staining for phenotypic markers (Table 2). Second, cells defined by the expression of phenotypic markers were studied to determine coexpression of EMR2 (Table 3).

In all patient groups, expression of EMR2 was mainly restricted to macrophages and DCs (Table 2). Little if any expression was found on fibroblast-like synoviocytes or lymphocytes in any form of arthritis. A significant proportion of macrophages in all groups expressed EMR2 (Table 3). To study the activation state of the cells expressing EMR2, double staining for costimulatory molecules and TNF \( \alpha \) was performed. Of the EMR2+ cells in RA ST, a mean ± SD of 34 ± 8% coexpressed CD40, 23 ± 8% coexpressed CD80, and 7 ± 0.1% coexpressed CD86. Furthermore, 50 ± 6% of the EMR2+ cells in RA ST expressed

**Figure 1.** Expression of EMR2 and CD97 in synovial tissue from patients with rheumatoid arthritis (A and B) and osteoarthritis (C and D). Sections were stained with monoclonal antibody (mAb) 2A1 for EMR2 (A and C), with mAb CLB-CD97/3 for CD97 (B and D), and with control Ig (E). Monostaining peroxidase technique was used, followed by counterstaining with Mayer’s hemalum. (Original magnification x200)
EMR2

Figure 2. Expression of EMR2 and CD97 in the intimal lining layer and synovial sublining of 19 patients with rheumatoid arthritis (RA), 13 patients with osteoarthritis (OA), and 13 patients with reactive arthritis (ReA), shown as integrated optical density per nucleus per mm² (IODp/nucleus/mm²). Monoclonal antibody (mAb) CLB-CD97/1 recognizes both EMR2 and CD97, while mAb 2A1 and CLB-CD97/3 are monospecific. Horizontal bars represent median values. * = P<0.002.

TNFα, irrespective of the localization in the intimal lining layer of the synovial sublining. The above results indicate that EMR2+ cells in the synovium are either activated macrophages or mature DC’s.

**Dermatan sulfate in ST is a ligand of the largest isoforms of EMR2 and CD97**

Having demonstrated the expression of EMR2 and CD97 in rheumatoid ST, we aimed to detect the ligands of these members of the EGF-TM7 family in situ in the synovium. Therefore, we generated multivalent probes. Recombinant soluble protein of the extracellular part of EMR2 and CD97 was biotinylated *in vitro* and coupled to avidin-oated fluorescent beads. Isoform-specific beads enabled us to study the ligand distribution of all isoforms of EMR2 and CD97 in RA, OA, and ReA synovial tissue. The largest isoforms, EMR2(EGF1,2,3,4,5) and CD97(EGF1,2,3,4,5), broadly bound throughout the entire synovial sublining in a largely similar manner (Figure 3). However, the staining obtained using the other isoforms clearly differed between EMR2 and CD97. Whereas no ligands for EMR2(EGF1,2), EMR2(EGF1,2,5), and EMR2(EGF1,2,3,5) were detected (results not shown), CD97(EGF1,2,5) and, to a lesser extent, CD97(EGF1,2,3,5) specifically attached to the intimal lining layer. No apparent difference in the ligand distribution was observed between RA, OA, and ReA (results not shown).
To confirm that EMR2 and CD97 isoform beads bind to their specific ligands, control experiments were performed (Figures 3 and 4). The addition of EGTA prevented binding of all EMR2 and CD97 isoforms, emphasizing that ligand interactions were mediated through the EGF domains. It has been previously documented that the smaller isoforms of CD97 bind CD55, which is a defining marker of synovial fibroblast-like synoviocytes in the intimal lining layer. Incubating the synovium with an anti-CD55 mAb prior to the addition of beads completely prevented binding (Figures 3B and D). In addition, we found that CD97(EGF1,2,5) beads, but not control beads, bound to in vitro–cultured fibroblast-like synoviocytes (results not shown). The largest isoforms of CD97 and EMR2 have been recently shown to bind dermatan sulfate. The specificity of this interaction was confirmed by a clear decrease in bead binding after addition of chondroitinase B or after pretreating the beads with dermatan sulfate (Figure 4). While preincubating the slides with chondroitin sulfate A had no effect, pretreatment with chondroitinase AC or chondroitin sulfate C resulted in a decrease in bead binding.

### Table 2. Co-expression of phenotypic markers by EMR2+ cells in synovial tissue from patients with rheumatoid arthritis, osteoarthritis, and reactive arthritis.

<table>
<thead>
<tr>
<th>Marker</th>
<th>% of EMR2+ cells coexpressing (n)</th>
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<tbody>
<tr>
<td></td>
<td>RA patients</td>
</tr>
<tr>
<td>CD68</td>
<td>51-75 (16)</td>
</tr>
<tr>
<td>CD83</td>
<td>6-25 (3)</td>
</tr>
<tr>
<td>CD3</td>
<td>0-5 (7)</td>
</tr>
<tr>
<td>CD22</td>
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<tr>
<td>CD40</td>
<td>26-50 (6)</td>
</tr>
<tr>
<td>CD80</td>
<td>26-50 (3)</td>
</tr>
<tr>
<td>CD86</td>
<td>6-25 (3)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>26-50 (8)</td>
</tr>
</tbody>
</table>

### Table 3. Expression of EMR2 by various cell types in synovial tissue from patients with rheumatoid arthritis, osteoarthritis, and reactive arthritis.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>% of EMR2+ cells coexpressing (n)</th>
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<tr>
<td></td>
<td>RA patients</td>
</tr>
<tr>
<td>CD68+</td>
<td>26-50 (15)</td>
</tr>
<tr>
<td>CD83+</td>
<td>6-25 (3)</td>
</tr>
<tr>
<td>CD3+</td>
<td>0-5 (5)</td>
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<tr>
<td>CD22+</td>
<td>0-5 (5)</td>
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<tr>
<td>CD38+</td>
<td>0-5 (7)</td>
</tr>
<tr>
<td>CD55+</td>
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<tr>
<td>TNF-α</td>
<td>6-25 (12)</td>
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These observations are consistent with previously reported data. In conclusion, we can state that the largest isoforms of EMR2 and CD97 bind specifically to dermatan sulfate, while the smallest and intermediate isoforms of CD97 bind CD55 in RA ST.

**DISCUSSION**

Rheumatoid synovium is characterized by intimal lining layer hyperplasia and marked infiltration of the synovial sublining by inflammatory cells. The intimal lining layer is formed by two types of cells: intimal macrophages expressing the EGF-TM7 receptor CD97 and fibroblast-like synoviocytes expressing its ligand CD55. The cells mainly found in the synovial sublining are macrophages, T cells, and plasma cells, in addition to lower numbers of B cells, mast cells, natural killer cells, DCs, and neutrophils. The importance of macrophages is supported by the clinical observation that macrophage numbers in the synovium are associated with clinical signs of disease activity, as well as by the success of therapies targeting macrophagederived cytokines.

In RA, two-thirds of the intimal lining layer is formed by macrophages, which are thought to be recruited from bone marrow–derived monocytes from the bloodstream and which subsequently enter the synovial sublining through the vascular endothelium. These cells might be trapped by fibroblast-like synoviocytes as well as by extracellular matrix components. In the present study, we confirmed previous observations suggesting that the CD97/CD55 pair might be involved in the interaction between intimal macrophages and fibroblast-like synoviocytes, thereby supporting the specific architecture of the intimal lining layer.

The current investigation focused on the distribution of the related EGF-TM7 receptor EMR2. EMR2 was detected on intimal macrophages as well as on macrophages and DCs in the synovial sublining. Interestingly, expression in RA was higher than in OA and ReA. These observations are consistent with earlier findings demonstrating that EMR2 expression is restricted to the myeloid lineage, with the highest levels on more mature cells. A substantial proportion of EMR2 macrophages in RA were found to be activated, as shown by coexpression of costimulatory molecules such as CD40 and CD80 and the inflammatory mediator TNF. Whether macrophages are activated as a consequence of EMR2 expression remains to be shown.

To localize ligands of EMR2 and CD97 in RA ST, we used multivalent probes generated by coupling biotinylated recombinant soluble protein (derived from the extracellular part of the receptors) to avidin-coated fluorescent beads. This approach, originally developed by Brown et al., has been very helpful in the analysis of cell–cell interactions within the immune system. We applied this technique here for the first time in an investigation of pathologic tissue. Specificity was convincingly demonstrated by binding of CD97(EGF1,2,5) and, to a lesser extent, CD97(EGF1,2,3,5) beads to CD55 on fibroblast-like synoviocytes. The intensity of staining was in accordance with known affinities between CD55 and different CD97 isoforms.
Beads loaded with the largest isoform of EMR2 or CD97 bound extracellular matrix in the synovial sublining. Dermatan sulfate is abundantly expressed in the extracellular matrix of inflamed ST. The observed binding pattern of EMR2(EGF1,2,3,4,5) and CD97(EGF1,2,3,4,5) fits this extracellular matrix distribution pattern. The varied

Figure 3. Localization of EMR2 and CD97 ligands in rheumatoid arthritis synovial tissue. Shown is the binding of green immunofluorescent beads coated with recombinant soluble protein of the extracellular part of A, the largest isoform of EMR2, B, the intermediate isoform of CD97, C, the largest isoform of CD97, and D, the smallest isoform of CD97 (see Results). Small panels show control binding in the presence of anti-CD55 monoclonal antibody and EGTA. Cell nuclei were stained with 4,6-diamidino-2-phenylindole. (Original magnification 400x)

Figure 4. Evidence that dermatan sulfate is the ligand of the largest isoform of EMR2 in synovial tissue. Shown is the binding of green immunofluorescent beads loaded with recombinant soluble protein of the extracellular part of the largest isoform of EMR2 after pretreatment of the slides with A, medium, B, chondroitinase B, C, chondroitinase AC, D, chondroitin sulfate A, E, dermatan sulfate, and F, chondroitin sulfate C (see Results). Cell nuclei were stained with 4,6-diamidino-2-phenylindole. The largest isoform of EMR2 bound specifically to dermatan sulfate as illustrated by ablation of bead binding after pretreatment with dermatan sulfate or chondroitinase B. (Original magnification 400x)
molecular structure of dermatan sulfate is determined by a number of factors, including polysaccharide chain length, iduronic acid placement, and sulfation. Variability is tightly regulated in a tissue- and cell type–specific manner, generating complex subregional heterogeneity. For example, it has been suggested that in OA cartilage, the sulfation of the terminal residues of dermatan sulfate is altered. Conceivably, such changes in sulfation might alter the capacity of synovial dermatan sulfate to bind to receptors such as EMR2 and CD97.

Using specific mAb, Worrall and colleagues previously showed that dermatan sulfate in normal synovium is homogeneously distributed throughout the interstitium. In ST of RA patients, however, dermatan sulfate is especially found in the deeper layers underlying the intimal lining layer. Previous research has shown that the expression of dermatan sulfate is positively correlated with basic activity of RA and proliferation of the synovium. The results presented here support the notion that increased or altered expression of dermatan sulfate might be involved in the retention of activated EMR2+ macrophages and DCs in the synovium, promoting synovial inflammation. Consistent with this view, involvement of CD97 in leukocyte infiltration has recently been demonstrated in an animal model of colitis as well as in a model of streptococcal infection. We are currently investigating the molecular mechanism by which CD97 and EMR2 affect leukocyte migration.

 Taken together, these results indicate that upregulation of dermatan sulfate might facilitate the recruitment and/or retention of leukocytes in the inflamed synovium. In this process, CD97, which is present on all leukocytes (especially on activated lymphocytes and monomyeloid cells), can function as a primary dermatan sulfate receptor. EMR2, expressed by activated macrophages and DCs, may serve as a second dermatan sulfate receptor contributing to the massive increase in the numbers of both types of cells in rheumatoid ST.

Acknowledgement
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References

17. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American