CD97 and EMR2: receptors on the move
Kwakkenbos, M.J.

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Chapter 6

Identification of the EGF-TM7 receptor EMR2 and its ligand dermatan sulphate in rheumatoid synovial tissue

Submitted for publication

Else N. Kop¹, Mark J. Kwakkenbos², Gwendoline J. D. Teske¹, Maarten C. Kraan¹, Tom J. Smeets¹, Martin Stacey³, Hsi-Hsien Lin³, Paul P. Tak¹, and Jörg Hamann²

¹Division of Clinical Immunology and Rheumatology
²Laboratory for Experimental Immunology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands
³Sir William Dunn School of Pathology, University of Oxford, Oxford, U. K.
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ABSTRACT

Background. EMR2 is a member of the EGF-TM7 family closely related to CD97. Chondroitin sulphates (CS) have recently been identified as ligands for EMR2 and CD97. CS has been implicated in the pathogenesis of rheumatoid arthritis (RA).

Objective. 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 patients with RA (n=19), inflammatory osteoarthritis (OA) (n=13), and reactive arthritis (ReA) (n=13). Immunohistochemistry was performed with an anti-EMR2 monoclonal antibody (mAb) and stained synovial tissues were analyzed by digital image analysis. Co-expression 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 fluorescent beads coated with EMR2-Fc or CD97- Fc fusion proteins.

Results. 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 (DC), expressing co-stimulatory molecules and TNF-α. Dermatan sulphate (DS) 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 fibroblast-like synoviocytes.

Conclusions. The EGF-TM7 receptors EMR2 and CD97 are abundantly expressed on myeloid cells in ST of RA patients where their cognate ligands DS and CD55 are detected. These results suggest that these interactions may facilitate the retention of activated macrophages in the synovium.

INTRODUCTION

EMR2 and CD97 are epidermal growth factor seven-span membrane (EGF-TM7) receptors, which belong to a subgroup of class B GPCR receptors.\(^1\) The EGF-TM7 receptors are predominantly leukocyte restricted cell-surface proteins which possess extended extracellular regions containing variable numbers of N-terminal EGF-like domains.\(^2,3\) CD97 is found on a broad range of leucocytes,\(^4,5\) whereas expression of EMR2 is restricted to myeloid cells, including monocytes, macrophages, DCs and PMNs. Interestingly, the EGF domains of EMR2 and CD97 are nearly identical (97% amino acid identity)\(^1\) and due to alternative RNA splicing, isoforms with two, three, four, and five EGF domains are expressed. Increased expression of CD97 at sites of inflammation\(^3\) previously led us to investigate the distribution in RA\(^8\) 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.\(^3,8\)

The EGF-TM7 receptors interact via the EGF domains with cellular ligands.\(^9\) Recently, both EMR2 and CD97 have been shown to bind chondroitin sulphate (CS) through EGF domain 4.\(^9\) In addition, EGF domains 1 and 2 of CD97 but not EMR2 specifically interact with CD55.\(^1,10,11\) Thus, the composition of the EGF domain region defines the ligand specificity of EMR2 and CD97 isoforms. Whereas CS is exclusively
Identification of EMR2 and dermatan sulphate in rheumatoid synovial tissue 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) abundantly present in both extracellular matrix and in the synovial fluid of RA patients. CS occurs in a number of forms varying in site and degree of sulphation. Three types are recognized: CSA, CSB (dermatan sulphate (DS)), and CSC. DS is an isomer of chondroitin-4-sulphate in which a variable number of glucuronic acid residues are replaced with iduronic acid. Several changes in GAG expression in ST and cartilage of RA and OA patients have been described. In ST of RA patients DS has been shown to be the primary molecular species of CS in inflammatory areas compared to fibrotic areas where CSA/C expression dominates. Basic activity of the disease and proliferation of the synovium correlates with an increased percentage of DS of total GAG content in the synovium. Furthermore, RA chondrocytes are known to synthesize an increased proportion of proteoglycans, enriched in DS. Recently, it was shown that infiltrating cells can bind GAGs in rheumatoid synovial tissue. Of importance, normal or traumatic ST did not exhibit GAG-binding.

We hypothesize that the interaction between EMR2 and CS is involved in the retention of inflammatory cells in the inflamed synovium. To find support for this hypothesis, we investigated the expression of EMR2 as well as CD97 and identified their ligands in ST.

PATIENTS AND METHODS

Patients
19 patients with RA and active arthritis of the knee joint underwent synovial biopsy. All patients fulfilled the American College of Rheumatology criteria for RA. In addition, synovial biopsies were obtained from 13 patients with inflammatory osteoarthritis (OA) and 13 patients with reactive arthritis (ReA) of the knee joint. Laboratory assessment included serum levels of rheumatoid factor (RF) and the erythrocyte sedimentation rate (ESR).

Specimen collection
Biopsy specimens were taken from the knee joint with a Parker-Pearson needle, as previously described. The different tissue samples (at least 6 per patient) were snap-frozen together in TissueTek OCT (Miles, Elkhart, IN) by immersion in liquid nitrogen and stored until sectioned for staining. 5-μm sections were cut in a cryostat and mounted on glass slides (Star Frost adhesive slides, Knittelgläser, Braunschweig, Germany). Slides were stored at -70°C until immunohistochemical analysis was performed.

Immunohistology and double immunofluorescence
All patients were studied for expression of EMR2, CD97, and double-labelling for EMR2/CD68 and EMR2/TNF-α. In 7 RA, 4 OA, and 4 ReA patients with marked expression of EMR2 in their ST double-labelling 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), CD97 (CLB-CD97/3) (directed against the stalk region of CD97), or CLB-CD97/
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(directed against the first EGF domain of both EMR2 and CD97) as previously described. In brief, following a primary incubation step, bound mAb was detected by a 3-step immunoperoxidase method using horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (Dako, Glostrup, Danemark), HRP-conjugated swine anti-goat antibody (Biosource, Camarillo, CA), and amino ethylcarbazole (AEC) (Vector, Burlingame, CA). In negative control sections, the primary mAb was replaced by an appropriate isotype control Ab.

To stain for co-expression of TNF-α and EMR2, EMR2 was detected as described above. After developing with AEC and pre-incubation with mouse serum (CLB, Amsterdam, the Netherlands), an anti-TNF-α mAb (clone Mab1, PharMingen, Alphen aan den Rijn, The Netherlands) was added to the slides, followed by HRP-conjugated goat anti-mouse antibody (Perkin Elmer, Boston, MA), biotinylated tyramide (Dako), streptavidin-AF (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, dendritic cells (DC), fibroblast-like synoviocytes, and cells that express co-stimulatory molecules. The staining procedure was modified from a previously described method. First, EMR2 mAb (IgG1) was incubated on serial sections, followed by incubation with tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgG1(Biosource). Then, after incubation of the slides with mouse serum, fluoresceine isothiocyanate (FITC)-conjugated CD3 (clone SK7, BD), CD22 (clone Rfb-4, Biosource, Camarillo, CA), CD38 (clone HIT2, PharMingen), CD55 (clone IA10, PharMingen), or CD86 (clone 2331, PharMingen) mAb was applied. For the detection of CD40 (clone 5L3, PharMingen) and CD80 (clone L307.4, PharMingen), the signal was augmented by adding subsequently 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, Canada), followed by biotin-conjugated goat-anti mouse IgG2A antibody (Santa Cruz, Nordic, Tilburg, The Netherlands) and streptavidin-FITC (Dako).

EMR2 staining in combination with CD68 was performed by incubating sections with 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). The sections were examined under a fluorescence photomicroscope (Leica, Germany).

**Microscopic and digital image analysis**

To evaluate staining for EMR2 and CD97, digital image analysis was used, as previously described. All sections were coded and analyzed in a random order by an independent observer (ENK) who was blinded for the clinical diagnoses. Slides were analyzed in 2 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 be only related to higher cell numbers. Therefore, we also calculated the integrated optical density (IOD) per cell (expressed as (IOD)/nucleus/mm²).

Co-expression of EMR2 with CD3, CD22, CD38, CD40, CD55, CD68, CD80, CD83, CD86, and TNF-α was quantified by counting at least 50 and, if possible, up to
Identification of EMR2 and dermatan sulphate in rheumatoid synovial tissue

200 EMR2+ cells by two independent observers (GJDT and ENK). The percentage of double-staining cells was noted. The percentages were stratified in 5 groups; 0-5%, 6-25%, 26-50%, 51-75%, 76-100%. Conversely, we also counted the cells positive for CD3, CD22, CD38, CD40, CD55, CD68, CD80, CD83, CD86, and TNF-α co-expressing EMR2+. If there were less than 15 cells positive for any of these markers per section, the results were discarded to prevent disproportionately high percentages. TNF-α 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. In short, 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. Then, HEK293 cells were transfected with 40 μg DNA per 175-cm² flask and cultured for 5 days in conditioned Opti-MEM 1 medium (Life Technologies Ltd, Paisly, Scotland). Secreted soluble recombinant protein was purified using a protein A (Sigma) column 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 avidin-coated fluorescent beads (Spherotech Inc., Libertyville, IL) were washed with PBS/0.5% BSA and incubated with saturating amounts (>1 μg) of biotinylated recombinant protein. After 1 h, non-binding 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 human pooled serum to prevent non-specific binding. The different bead-protein complexes (10 μl complex plus 40 μl PBS) were added to the ST sections. After incubation for 1 h 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 U/ml chondroitinase AC or B (Sigma), 50 μl CD55 mAb (10 μg/ml, CLB-CD97/L111) or 50 μl 5 mM EGTA, for 30 min before addition of the beads. Furthermore, beads were preincubated with 50 μl chondroitin A, B or C (10 μg/ml, Sigma) before addition to the slides. Slides were coverslipped after addition of 100 μl of Vectashield (Vector) or Immosl-Mount (Klinipath, Duiven, the Netherlands). 2μl 4',6-diamidino-2-phenylindole (DAPI) (5 mg/ml Sigma) was added per slide for nuclear staining.

Statistical analysis
The means and standard deviations 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.
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RESULTS

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

Table 1. Clinical features of rheumatoid arthritis, osteoarthritis, and reactive arthritis patients included in the study.

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<th>OA patients (n = 13)</th>
<th>ReA patients (n = 13)</th>
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* RF = rheumatoid factor; ESR = erythrocyte sedimentation rate.

EMR2 expression is increased in rheumatoid synovial tissue
Representative images of the distribution of EMR2 and CD97 in the ST of patients with RA, OA, and ReA are depicted in Figure 1 (See appendix, page 133) and 2. EMR2 was expressed in the intimal lining layer and synovial sublining of all patients with RA, 12 out of 13 patients with OA, and 11 out of 13 patients with ReA. EMR2 expression was significantly higher in the synovial sublining of RA patients compared with disease controls, 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 to 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 study, where 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 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.

EMR2 is expressed by activated macrophages and dendritic cells in synovial tissue
Whereas CD97 is found on most hematopoietic cells, expression of EMR2 has been shown to be restricted to cells of the myeloid lineage. 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 co-stimulatory molecules (CD40, CD80, CD86) or the inflammatory cytokine TNF-α (Table 2). These experiments enabled us to determine whether EMR2
Identification of EMR2 and dermatan sulphate in rheumatoid synovial tissue

Figure 2. Expression of EMR2 and CD97 in the intimal lining layer and synovial sublining of patients with rheumatoid arthritis (n = 19), osteoarthritis (n = 13), and reactive arthritis (n = 13) shown as IOD per cell per mm². CLB-CD97/1 recognizes both EMR2 and CD97, while 2A1 and CLB-CD97/3 are monospecific mAbs. Bars signify the median values. * indicates statistical significance P<0.05.

has a similar expression pattern in various arthritides. First, we studied EMR2+ cells and counted the percentage of double staining with phenotypic markers (Table 2). Second, cells defined by the expression of phenotypic markers were studied to determine co-expression with 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 expressed EMR2 in all groups (Table 3).

To study the activation state of the cells expressing EMR2, double staining for co-stimulatory molecules and TNF-α was performed. EMR2+ cells in RA ST co-expressed CD40 in 34 ± 8%, CD80 in 23 ± 8%, and CD86 in 7 ± 0.1%. Furthermore, 50 ± 6% of the EMR2+ cells in RA ST expressed TNF-α, irrespective of the localization in the intimal lining layer or synovial sublining. In conclusion, EMR2+ cells in the synovium are either activated macrophages or mature DC.

Dermatan sulphate in synovial tissue is a ligand of the largest isoform of EMR2 and CD97

Having shown 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-coated fluorescent beads. Isoform-specific beads enabled us to study the ligand distribution of all isoforms of EMR2 and CD97 in RA, OA, and ReA ST.
Table 2. Co-expression of phenotypic markers by EMR2\textsuperscript{+} cells in synovial tissue from patients with rheumatoid arthritis (RA), osteoarthritis (OA), and reactive arthritis (ReA).

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<th>Diagnosis</th>
<th>CD68</th>
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ND = not done.

Table 3. Expression of EMR2 by various cell types in synovial tissue from patients with rheumatoid arthritis (RA), osteoarthritis (OA), and reactive arthritis (ReA).

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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. See appendix, page 136). 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 (data not shown),
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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 (data not shown).

To confirm EMR2 and CD97 isoform beads to bind to their specific ligands, control experiments were performed (Figures 3 and 4, See appendix, page 136). It has been previously documented that the smaller isoforms of CD97 bind CD55,11 which is a defining marker of synovial fibroblast-like synoviocytes in the intimal lining layer.28 Incubating the synovium with an anti-CD55 antibody prior to the addition of beads completely prevented binding (Figure 3C and D, See appendix, page 136). The addition of EGTA also prevented binding of all EMR2 and CD97 isoforms, emphasizing the crucial role of Ca\textsuperscript{2+} in maintaining the structural integrity of EGF-like domains. The largest isoforms of CD97/EMR2 have been previously shown to bind DS.9 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 DS (Figure 4, See appendix, page 136). While preincubating the slides with chondroitin sulphate A had no effect, pretreatment with chondroitinase AC or chondroitin sulphate C resulted in a decrease in bead binding. These observations are in agreement with previous published data.

In conclusion we can state that the largest isoforms of EMR2 and CD97 bind specifically to DS, while the smallest and medium isoform 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.29 The intimal lining layer is formed by two cell types: intimal macrophages expressing the EGF-TM7 receptor CD97 and fibroblast-like synoviocytes expressing its ligand CD55.8 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, dendritic cells, and neutrophils.29 The importance of macrophages is supported by the clinical observation that macrophage numbers in the synovium are associated with clinical signs of disease activity30 and the success of therapies targeting macrophage-derived cytokines.

In RA two-thirds of the intimal lining layer is formed by macrophages, which is thought to be the result of recruitment from bone-marrow derived monocytes from the bloodstream, entering 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 confirm 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.8

The current investigation focused on the distribution of the related EGF-TM7 receptor EMR2.1 EMR2 was detected on intimal macrophages as well as macrophages and DC in the synovial sublining. Of interest, expression in RA was higher compared to OA and ReA. These observations are in agreement with earlier findings demonstrating that EMR2 expression is restricted to the myeloid lineage with the highest levels on more mature cells.20 A substantial proportion of EMR2+ macrophages in RA were found
to be activated as shown by co-expression of co-stimulatory molecules like CD40 and CD80 and the inflammatory mediator TNF-α. 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 and Barclay, 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 pathological 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. DS 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 molecular structure of DS is determined by a number of factors including, polysaccharide chain length, iduronic acid placement, and sulphation. Variability is tightly regulated in a tissue and cell type-specific fashion generating complex subregional heterogeneity. For example, it has been suggested that in OA cartilage the sulphation of the terminal residues of DS is altered. Conceivably, such changes in sulphation might alter the binding capacity of synovial DS to receptors like EMR2 and CD97.

Using specific mAbs, Edwards and colleagues previously showed that DS in normal synovium is homogenously distributed throughout the interstitium. In ST of RA patients, however, DS is especially found in the deeper layers underlying the intimal lining layer. Previous work has shown that the expression of DS is positively correlated to basic activity of RA and proliferation of the synovium. The results presented here support the notion that increased or altered expression of DS might be involved in the retention of activated EMR2 positive macrophages and DC 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 a model of streptococcal infection.

Taken together, upregulation of DS 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 DS receptor. EMR2, expressed by activated macrophages and DCs, may serve as a second DS receptor contributing to the massive increase in the number of both cell types in rheumatoid ST.

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REFERENCE LIST

Chapter 6


