TREM-1 expression is increased in the synovium of rheumatoid arthritis patients and induces the expression of pro-inflammatory cytokines

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Objectives. To investigate the expression and function of triggering receptor expressed on myeloid cells-1 (TREM-1) in the synovium of human RA patients as well as the level of soluble TREM-1 in the plasma of RA patients.

Methods. Twenty-four RA synovial samples were analysed by gene expression oligonucleotide microarrays. Expression levels of TREM-1 mRNA in murine CIA paws were determined by quantitative PCR (qPCR). TREM-1 protein expression was detected by immunohistochemistry in five RA synovial samples and two OA synovial samples. TREM-1-positive cells from five RA synovial tissues were analysed by FACS staining to determine the cell type. Activation of TREM-1 was tested in five RA synovial samples. Soluble TREM-1 was measured in serum from 32 RA patients.

Results. The expression of TREM-1 mRNA was found to increase 6.5-fold in RA synovial samples, whereas it was increased 132-fold in CIA paws. Increased numbers of TREM-1-positive cells were seen in RA synovium sections and these cells co-expressed CD14. Using a TREM-1-activating cross-linking antibody in RA synovial cultures, multiple pro-inflammatory cytokines were induced. The average amount of soluble TREM-1 in plasma from RA patients was found to be higher than that in plasma from healthy volunteers.

Conclusions. These findings suggest that the presence of high levels of functionally active TREM-1 in RA synovium may contribute to the development or maintenance of RA, or both. Inhibiting TREM-1 activity may, therefore, have a therapeutic effect on RA. High levels of soluble TREM-1 in the plasma of RA patients compared with healthy volunteers may indicate disease activity.

KEY WORDS: Triggering receptor expressed on myeloid cells-1, DNAX-activation protein 12, Rheumatoid arthritis, RA synovium.
significantly elevated in the disease samples. We have validated these results by demonstrating increased TREM-1 mRNA expression in paws from a mouse model of arthritis, and by immuno-histochemistry (IHC) of RA synovial tissue. Furthermore, we identified the major TREM-1 expressing cell type in dissociated RA SM cultures as CD14+ cells and show that activating TREM-1 in this system induces the production of multiple pro-inflammatory cytokines, including TNF-α, IL-1β, GM-SCF and IL-8. Finally, we have observed that the levels of soluble TREM-1 in plasma samples from RA patients are significantly higher than plasma from healthy volunteers. The elevated expression of both cell surface and soluble TREM-1 in RA samples, together with its functional presence in RA SM cultures, suggests that TREM-1 may contribute to the chronic inflammation associated with RA. TREM-1 blockade may, therefore, prove beneficial in the treatment of RA and other chronic inflammatory disorders.

Materials and methods

Gene expression analysis

Twenty-four synovial tissue samples were obtained from 17 patients with RA, as defined by ACR criteria. All tissue samples used in this study were taken from fully anonymized patients who had given informed consent for its use in medical research. Ethical approval was granted by the Riverside Research Ethics Committee (RREC 1752), Charing Cross Hospital, London, UK. Samples consisted of 10 specimens of joint synovium and 14 of tenosynovium. Among the 14 tenosynovia, 7 were encapsulated tenosynovia from 7 patients and 7 were matched invasive tenosynovia taken from the same patients. The encapsulated tenosynovium and invasive tenosynovium were differentiated at surgery as described in Jain et al. [17, 18]. For comparison, eight additional uninvolved synovial tissues were obtained from three non-RA patients who required amputation due to blunt trauma (non-RA synovia). Synovial samples from RA patients and non-RA patients were harvested and immediately flash frozen in liquid nitrogen and stored at −80°C until processed. Total RNA was isolated and analysed on Affymetrix® (Santa Clara, CA, USA) Hg_U95A & B (human samples) GeneChip™ oligonucleotide microarrays, as described in [19]. Expression measurements from the arrays were generated by the Affymetrix MAS4 algorithm and normalized to estimates of transcripts per million by reference to spiked-in standards [20]. The fold-change of expression was normalized to normal synovium specimens, which were set to a value of 1. Statistical analysis of expression data was executed on log-2 transformed expression measurements. The significance of differential expression between groups was determined by a permutation test [21, 22].

Quantitative real-time PCR

CIA was induced in male DBA/1 mice (Jackson Laboratories, Bar Harbor, ME, USA) using bovine collagen type II (Chondrex, Redmond, WA, USA) as described in [23]. Mice were monitored for disease progression at least three times a week. Individual limbs were assigned a clinical score based on the following index: 0, normal; 1, visible erythema accompanied by one to two swollen digits; 2, pronounced erythema characterized by paw swelling and/or multi-digit swelling; 3, massive swelling extending into ankle or wrist joint; and 4, difficulty in use of limb or joint rigidity. The sum of all limb scores for any given mouse yielded a potential maximum total body score of 16. Animals were euthanized, and paws were harvested at various disease stages. RNA from diseased animals was prepared from three score 3 paws and one score 4 paw, and RNA from normal animals was prepared from four naïve paws. Primers used for mTREM-1 were CAGATGTGTTCACTCCTGTCATCA (forward; 413–436), CTGGGGTACATTTTGTGTTAATA- AGG (reverse; 494–468) and CCTATTACAGGCTCA- GAGCGTCCCA (probe; 439–466). Primers used for mDAP12 were CCTGGTCTCCCGAGGTCTCA (forward; 255–273), GGCAGACTCTGCTCAGAATG (reverse; 323–302) and TTGTTTCCGGTCCCTCCGCT (probe; 300–279). RNA levels to calculate fold-changes were normalized to glyceralde-hyde-3-phosphate dehydrogenase (GAPDH) mRNA. Primers used for mGAPDH were CGTGTTCCTACCCCAATGTT (forward; 752–771), GTACATCATTGGCAAGTCTCCT (reverse; 823–800) and CGTGGATCTGACGTGCCCGC (probe; 779–798). Real-time PCR (RT-PCR) reactions and analyses were performed according to manufacture’s recommendation (Applied Biosystems, Foster City, CA, USA).

Cytokine analysis in dissociated RA SM cultures

The synovium culture assay was performed as described by Brennan et al. [24]. Synovial tissues were obtained from joint replacement surgery of RA patients (Charing Cross Hospital, London, UK), and samples were placed in Roswell Park Memorial Institute (RPMI) 1640 with 5% fetal calf serum (FCS) for transport. To disrupt tissue and release cells, tissue samples were dissected into small pieces using sterile scissors and forceps in a minimum volume of medium. Tissue pieces were separated from dissection media using a cell strainer (BD Falcon, Sparks, MD, USA), and then transferred to 20 ml of RPMI 1640 with 10% FCS containing 5 mg/ml collagenase IV (Invitrogen, Carlsbad, CA, USA) and 0.15 mg/ml DNase I (Sigma, St Louis, MO, USA). Tissue samples were incubated at 37°C for 90 min, with frequent mixing, and residual tissue debris was removed by passing through a 100-μm nylon mesh. Cells were washed and resuspended in RPMI 1640 with 5% FCS and counted for plating.

For antibody activation of TREM-1, 96-well tissue culture-treated plates were coated with 100 μl per well mouse anti-hTREM-1 antibody (clone mAb5, Wyeth, Cambridge, MA, USA) or an isotype-matched control antibody (ε-E. tenella, Wyeth). Antibody dilutions were performed in endotoxin-free PBS at the indicated concentrations and plated in triplicate. Coated plates were incubated for 3 h at 37°C and washed with 250 μl PBS just prior to the addition of cells. Cells/well (1 x 105) were plated in a total volume of 200 μl RPMI 1640 supplemented with 5% FCS. Cell-free supernatants were harvested after 18 h and stored frozen at −80°C before being assayed for the indicated cytokines using multiplexed ELISA plates (Meso Scale Discovery, Gaithersburg, MD, USA). Cell viability was assessed using the methylthiazolyldiphenyl-tetrazolium bromide assay (Sigma) (data not shown), and none of the antibodies displayed toxicity as determined by this endpoint.

FACS analysis of TREM-1-positive cells

Cells were isolated from RA SMs as described above. For FACS staining, cells were pre-blocked in 1 ml of FACS buffer.
(PBS containing 5% normal human serum, 0.05% sodium azide and 2 mM ethylenediaminetetra-acetic acid) and then incubated with the relevant antibodies for 30 min at 4°C in the dark. The surface expression of TREM-1 was measured on freshly isolated synovial cells by direct staining with phycoerythrin (PE)-conjugated anti-TREM-1 mAb and non-specific binding was corrected using an isotype-matched control antibody (R&D Systems). These mixed populations were co-incubated with anti-CD45 (CD45-RPE-Cy5, Dako, Denmark or CD45-FITC, BD Biosciences Pharmingen, San Diego, CA, USA) together with directly conjugated antibodies recognizing either CD3, CD14 (APC-labelled), CD19, CD56-(FITC-labelled) (BD Biosciences Pharmingen). Dendritic cells were distinguished as negative for the Lin 1 antibody cocktail (anti-CD3, -CD14, -CD16, -CD19, -CD20 and -CD56 mix) and positive for HLA-DR using HLA-DR-Pacific Blue (BioLegend, San Diego, CA, USA) and FITC-labelled Lin-1 (BD Biosciences Pharmingen). Cells were washed in FACS buffer and fixed in BD Cytofix/Cytoperm (BD Biosciences Pharmingen) prior to analysis. Flow cytometry was performed using a BD FACSCanto II and the data were analysed using the FlowJo software (Tree Star, Ashland, OR, USA).

Determination of soluble TREM-1 in human plasma by ELISA assay

An ELISA was developed for the detection of soluble TREM-1 from human clinic plasma samples from the DuoSet ELISA development system (R&D systems). Briefly, ELISA was performed using a sandwich format with 4.0 μg/ml of capture antibody and 200 ng/ml of detection antibody. The plasma samples were diluted in the ratio 1:2 in Calibrator Diluent GF1 buffer from Meso Scale Discovery (MSD) (Gaithersburg, MD, USA). The standard was diluted in a 1:2 dilution of neat plasma in GF1 buffer. All samples were subjected to a false-positive test using an irrelevant secondary antibody to eliminate interference from serum components like RF. The limit of the detection was 1.37 pg/ml using a four-parameter curve fit (XL-Fit IDBS, Burlington, MA, USA) with R² of 0.999 in the range of 1.37–1000 pg/ml.

Plasma from RA patients was collected from a phase II, double-blinded, placebo-controlled, parallel, randomized, multicentre, outpatient, comparative study in subjects with active RA and an inadequate response to stable dosages of MTX (7.5–20 mg/week). Subjects were enrolled at 81 sites worldwide. At selected sites, 32 blood samples were obtained from subjects who agreed to participate in voluntary sample collection for exploratory biomarker studies. We report data from plasma samples taken on Day 1 (pre-dose). The control group plasma was collected from 25 subjects enrolled in a healthy volunteer multi-centre, prospective, non-interventional observational study. Each clinic site’s institutional review board or ethics committee approved these studies, and prior informed consent was obtained from all participants. Twenty serum samples from patients with bacteria infections were purchased from SeraCare Life Sciences (Milford, MA, USA).

Results

TREM-1 mRNA expression in RA synovium

We analysed the gene expression profiles of 24 RA (10 joints and 14 tendons) and eight normal synovial tissues by microarray analysis (see ‘Materials and methods’ for details). The complete data for this profiling analysis will be published elsewhere (Feldman et al., submitted). TREM-1 mRNA was increased in RA samples (n=24) compared with uninvolved samples (n=8) with an average fold-change of 6.5 (P-value < 0.001) (Fig. 1A). The relative expression levels of TREM-1 in each individual samples were shown in Fig. 1B. The average expression of TREM-1 in RA samples is 42.7 ± 5.3 (n=24) transcripts per million, with a range of 6–90 transcripts per million, whereas the average expression of uninvolved samples is 7.2 ± 2 (n=8) transcripts per million, with a range of 2–16 transcripts per million. Expression levels of TREM-1 in 20 RA samples were above the range of the uninvolved samples. In addition, the mRNA of DAP12, an adaptor of TREM-1 signalling, was also increased with an average fold-change of 2 (P-value of 7.83 x 10⁻⁷). TREM-1 and DAP12 mRNAs were more highly expressed in invasive tenosynovium (n=7) when compared with encapsulated tenosynovium (n=7) with fold-changes of 2.64 and 1.4, respectively (P-values 1.36 x 10⁻⁴ and 1.67 x 10⁻², respectively). These results show that TREM-1 and its signalling-associated adaptor protein DAP12 are detectably increased in RA synovium particularly in the invasive synovium.

The inflammation sites of RA normally contain a large number of infiltrated inflammatory cells such as neutrophils, monocytes and macrophages. TREM-1 is normally expressed on these cells, but its expression can also be induced by TLR ligands such as LPS [25]. Therefore, the increased expression of TREM-1 in RA synovium could be contributed by either the increased number of TREM-1 expressing cells or the induction of the TREM-1 expression. To test if the increased expression of TREM-1 in RA synovium could be explained solely by infiltrating neutrophils, monocytes or macrophages, we analysed the expression levels of marker transcripts that are selectively expressed in neutrophils, monocytes and macrophages (Table 1). As expected, these marker transcripts were more highly expressed in RA synovium, consistent with an increase of the number of neutrophils, monocytes and macrophages in the RA samples. However, the observed increase of TREM-1 expression (6.5-fold) was found to be greater than the increase in any of the marker gene sets (mean fold-change 1.22–1.47). Therefore, in addition to the contribution of the increased number of TREM-1-positive cells in the RA synovium,
TREM-1 expression is increased in RA synovium

To establish the identity of the cell type expressing TREM-1 in RA, we did the FACS analysis on dissociated RA synovia from five additional donors. CD45 staining was used to gate the haematopoietic and non-haematopoietic populations and the level of TREM-1 expression was determined by measuring the difference in mean fluorescence intensity (ΔMFI) between PE-labelled anti-TREM-1 antibody and an isotype-matched control. On this basis, no TREM-1 expression was detected in the non-haematopoietic compartment of these tissues, but in 3 of the 5 patients examined, TREM-1 expression was detected in the CD45+ population. Further analysis revealed that the majority of the CD45+/TREM-1+ cells were also positive for CD14. Figure 3B shows a representative staining pattern of surface TREM-1 expression in one RA donor, and the levels of TREM-1 on CD14+ cells from individual samples are shown in Table 3. Consistent with our IHC data, a similar spread in human acute monocytic leukemia cell line (TREM-1) expression levels can also be observed in FACS analysis with a barely detectable surface expression of TREM-1 in two of the five donors tested. No TREM-1 expression was measured on CD3+ and CD19+ populations in any donor tested (data not shown). We also stained these samples for CD56+ (NK cells) and HLA-DR-positive/Lin-1-negative (dendritic) cells, but the cell numbers isolated from RA SMs were too low to perform meaningful analyses (data not shown).

TREM-1 in RA synovial cultures

Activation of TREM-1 with a cross-linking antibody has been shown to trigger the production of pro-inflammatory cytokines in both human monocytes and neutrophils [26]. Therefore, we tested whether TREM-1 activation had a similar pro-inflammatory effect in RA synovial cultures. We generated a mouse anti-human TREM-1 monoclonal antibody (mAb5) using a recombinant protein containing the extracellular domain of TREM-1. This antibody recognizes cell surface TREM-1 in human acute monocytic leukemia cell line (THP-1) cells. Cell cultures were prepared from knee joint ablative arthroscopies of RA patients. These cultures spontaneously produce TNF-α, IL-8, -1β and GM-CSF (Fig. 4). In addition, TREM-1 activation using mAb5 induced further significant increase of these cytokines and chemokines as compared with the IgG1 isotype control. The increases were dose dependent on the amount of mAb5. We therefore conclude that TREM-1 is functionally present in primary RA synovial cultures.

Soluble TREM-1 levels in the circulating plasma of RA patients

A splice variant or shed version of TREM-1 is detectable in soluble form in plasma during acute inflammation [27]. We developed a highly sensitive ELISA method to measure soluble TREM-1 levels in human plasma with sensitivity of 1.37 pg/ml. All samples were subjected to and passed a false positive test using an irrelevant secondary antibody to eliminate the interference from serum components such as RF. Using this method, we determined the amount of soluble TREM-1 in plasma from 32 RA patients and 25 healthy volunteers. As seen in Fig. 5, the average amount of soluble TREM-1 in plasma from RA patients was 10.0±1.6 pg/ml, whereas the average amount of soluble TREM-1 in healthy volunteers was 2.5±0.6 pg/ml. The level of soluble TREM-1 in RA plasma is therefore found to be ~4-fold higher than in plasma from healthy volunteers, with a P-value of <0.0001 (unpaired t-test). As a control, we also measured the soluble TREM-1 in the sera of 20 patients suffering from bacterial infections. Consistent with published reports, we detected increased levels of soluble TREM-1 in the sera of these patients with the mean value of 34.1±4.5 pg/ml (P-value <0.0001).

Identification of TREM-1 expressing cells in RA synovium

Table 1. Expression levels of neutrophil, monocyte and macrophage marker genes in the RA synovium

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Number of marker genes</th>
<th>Mean fold-change</th>
<th>Fold-change range, mean ± s.d.</th>
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<tbody>
<tr>
<td>Neutrophil</td>
<td>328</td>
<td>1.22</td>
<td>0.80–1.87</td>
</tr>
<tr>
<td>Monocyte</td>
<td>182</td>
<td>1.36</td>
<td>0.90–2.05</td>
</tr>
<tr>
<td>Macrophage</td>
<td>34</td>
<td>1.47</td>
<td>0.90–2.41</td>
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The mean fold-change column indicates the average fold-change in RA synovium vs uninvolved synovium for each marker gene set.
Discussion

In this study, we have found the increased expression of functionally active TREM-1 in human RA synovium. Elevated levels of mRNA and TREM-1 protein were detected in most of the RA patients. Importantly, a further increase in TREM-1 expression is associated with the invasive synovium as compared with the encapsulated synovium. Encapsulating tenosynovium is the
lubricating sheath of synovial tissue that surrounds tendons. In about one-half of the RA patients, tenosynovium proliferates and becomes invasive, and can cause tendon adhesions, impaired tendon function or tendon rupture [17, 18]. Invasive tenosynovium cells in vitro have been shown to express higher levels of MMPs than encapsulating tenosynovium [18]. However, the details of the molecular differences between invasive and encapsulating tenosynovium remain to be elucidated. The increase of TREM-1 expression in invasive synovium suggests that TREM-1 expressing cells may participate in the invasive phenotype of invasive synovium. Activation of TREM-1 in the RA synovium is able to produce multiple pro-inflammatory cytokines and chemokines, and may thus contribute to the amplification of inflammatory responses at the disease sites. In addition, we have also observed the elevation of soluble TREM-1 in the plasma of RA patients, which could serve as a biomarker of the disease. Furthermore, increased expression of TREM-1 was observed in the CIA model of RA. Taken together, our data strongly suggest that TREM-1 plays a role in the pathogenesis of RA.

Our observation of elevated soluble TREM-1 in sera from RA patients suggests that this protein could be used as a biomarker for this disease. However, it should be noted that we saw variation in TREM-1 expression and activation among different synovial samples and individual plasma samples. This is not surprising, as RA is known to be a heterogeneous disease, with disease type, site, stage, gender, age and medical history contributing to this heterogeneity. Five of the 24 synovial samples used in the initial study had TREM-1 mRNA levels in the range of that seen in the uninvolved samples (Fig. 1B). The protein levels of TREM-1 in two out of the five samples used in IHC were undetectable (Table 2), and the same proportion of dissociated RA samples had low to barely detectable TREM-1 expression by FACS (Table 3). We see a similar variation of the level of the soluble TREM-1 in the RA plasma (Fig. 5). Of the clinical markers examined, the strongest association with elevated soluble TREM-1 was with high RF levels, with Spearman’s correlation coefficient of 0.383, P-value of 0.03. By analysing the opposite way, we observed that the correlation between patients being RF positive and having detectable TREM-1 in plasma had a P-value of 0.015. Correlation of other clinical characteristics with TREM-1 levels did not reach the statistical significance. TREM-1 expression is increased in most of the patients and provides another biomarker of RA; however, the variability of TREM-1 expression suggests that it could be used in conjunction with other clinical data. We found that most TREM-1-positive cells isolated from the RA synovium were also CD14+ cells, indicative of infiltrating monocytes and/or macrophages. Taken together, the detection of surface TREM-1 expression on dissociated RA synovial cells and their activation in response to the cross-linking TREM-1 mAb shows that this receptor is indeed functionally present in RA tissue.

Schenck et al. [28] recently reported that TREM-1 expression is increased in the intestinal macrophages of chronic IBD. This study showed that TREM-1 expression correlates with disease activity in human IBD as well as two experimental mouse colitis models. Moreover, macrophages isolated from IBD tissue and exposed to TREM-1 agonizing antibody in vitro mediated enhanced pro-inflammatory cytokine and chemokine production, and TREM-1 blockade using an antagonistic peptide attenuated the clinical manifestation and disease progression when administered before or after disease onset [28]. Taken together with our finding here, these data strongly suggest that TREM-1 not only amplifies acute, but also chronic inflammation. TREM-1 may
contribute to the pathogenesis of RA by the induction of inflammatory cytokines, chemokines and other inflammatory mediators.

Additionally, TREM-1 activation synergizes with LPS and other pattern-recognition receptors [TLR and nucleotide-binding domain, leucine-rich repeats containing protein (NLR)] ligands in the production of pro-inflammatory cytokines. TREM-1 activation in human monocytes can up-regulate the expression of co-stimulatory molecules such as CD40, CD86 and MHC Class II molecules leading to the differentiation of monocytes into immature dendritic cells, which have improved ability to elicit T-cell responses. Thus, TREM-1 potentially participates in the adaptive immune response contributing to the pathogenesis of RA disease [7, 26].

The TREM-1 ligand is not yet known. Interestingly, neutrophils in septic patients have been reported to bind to TREM-1, suggesting that the yet unidentified TREM-1 ligand(s) may also be induced under disease conditions [29]. Similarly, endogenous TREM-1 ligands may also be induced and present in the RA synovium contributing to the expression of inflammatory mediators. A similar IgV domain receptor that has increased expression under disease conditions including RA is receptor for advanced glycosylation end products (RAGEs). RAGE is a multi-ligand receptor, and both RAGE and RAGE ligands are increased in RA [30]. Soluble RAGE can block the progression of mouse CIA, indicating that pro-inflammatory receptors such as TREM-1 and RAGE may play critical roles in maintaining RA disease [31].

**Conclusion**

TREM-1 mRNA and protein levels are increased in synovia of RA patients, and plasma levels of soluble TREM-1 are a potential biomarker for this disease. TREM-1 expression and activation may contribute to the inflammatory cycle that leads to or maintains RA, indicating that TREM-1 blockade is of potential therapeutic value.

<table>
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<tr>
<th>Rheumatology key messages</th>
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<tr>
<td>TREM-1 expression is increased in the synovium of RA patients.</td>
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<tr>
<td>Activation of TREM-1 induces multiple pro-inflammatory cytokines that may contribute to the disease.</td>
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<tr>
<td>Plasma levels of soluble TREM-1 is a potential biomarker of RA.</td>
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