Research article

Differential binding of chemokines to macrophages and neutrophils in the human inflamed synovium

Angela M Patterson, Caroline Schmutz, Scott Davis, Lucy Gardner, Brian A Ashton and Jim Middleton

Leopold Muller Arthritis Research Centre, Centre for Science and Technology in Medicine, Keele University at Robert Jones and Agnes Hunt Orthopaedic Hospital, Oswestry, Shropshire, UK.

Correspondence: Dr Jim Middleton, LMARC, Robert Jones and Agnes Hunt Orthopaedic Hospital, Oswestry, Shropshire SY10 7AG, UK.
Tel: +44 (0)1691 404149; fax: +44 (0)1691 404170; e-mail: Middleton1@hotmail.com

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease of the synovial joints, which can result in articular cartilage and bone destruction, leading to significant disability. Central pathophysiological features of the disease occur in the synovium, with thickening of the lining layer (or intima) and infiltration of the sublining layer, mainly by macrophages and lymphocytes [1]. In addition, the fluid-filled joint cavity contains numerous neutrophils, particularly during acute flares of RA [2].

The normal intima of the synovium is one to two cells thick and consists mainly of macrophage-like and fibroblast-like cells [3]. In inflammation, macrophages make a large contribution to intimal thickening, where these cells increase in number and proportion [3]. Evidence indicates that

Abstract

In chronic inflammatory foci, such as the rheumatoid joint, there is enhanced recruitment of phagocytes from the blood into the tissues. Chemokines are strongly implicated in directing the migration of these cells, although little is known regarding the chemokine receptors that could mediate their chemotaxis into the joint tissue. Therefore the objective of the study was to identify chemokine binding sites on macrophages and neutrophils within the rheumatoid synovium using radiolabeled ligand binding and in situ autoradiography. Specific binding sites for CCL3 (macrophage inflammatory protein-1α), CCL5 (RANTES), CCL2 (monocyte chemoattractant protein-1) and CXCL8 (IL-8) were demonstrated on CD68+ macrophages in the subintimal and intimal layers. The number and percentage of intimal cells that bound chemokines were greater in inflamed regions compared to noninflamed regions. The intensity of intimal binding varied between chemokines with the rank order, CCL3 > CCL5 > CCL2 > CXCL8. Neutrophils throughout the synovium bound CXCL8 but did not show any signal for binding CCL2, CCL3 or CCL5.

Immunohistochemistry showed that both CXCR1 and CXCR2 are expressed by macrophages and neutrophils in the rheumatoid and nonrheumatoid synovia, suggesting that both of these receptors are responsible for the CXCL8 binding. The chemokine binding sites described on phagocytes may be involved in the migration of these cells into the inflamed joint.

Keywords: chemokines, inflamed, receptors, synovium

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intimal macrophages arrive via the circulation as monocytes [4], and it is proposed that increased numbers of intimal macrophages in RA are largely attributed to enhanced monocyte recruitment from blood vessels in the synovium [1,5]. Neutrophils are also recruited in RA and can be found in abundance in synovial fluid, yet they occur in relatively low numbers in the synovium [5]. In synovial fluid their number can reach 10^6 per joint, particularly during an active phase of the disease [6]. Neutrophils occur at the pannus-cartilage junction [7] and it is presumed that these cells reach the joint cavity by migrating from the blood vessels in the synovium, following a chemotactic gradient.

Chemokines are a family of proinflammatory cytokines, which are strongly implicated in inducing migration of leukocytes from the blood into inflammatory lesions. Evidence has accumulated to suggest that chemokines, such as CXCL8 (IL-8), CXCL5 (epithelial cell-derived neutrophil-activating protein), CCL2 (monocyte chemotactant protein-1), CCL3 (macrophage inflammatory protein-1α) and CCL5 (RANTES), direct the migration of leukocytes into the RA synovium and joint fluid [8]. Chemokines occur in significantly elevated levels in human RA synovial fluid, where they are biologically active, and are expressed by cells of the synovium and cartilage. Tumour necrosis factor-α blockade reduces synovial expression of CCL2 and CXCL8 and reduces inflammatory cell migration into RA joints [9]. In addition, these chemokines, produced by RA synovial stromal cells, have been shown to stimulate monocyte migration [10]. The functional importance of chemokines in joint inflammation has also been established in animal models of RA where antibodies to these mediators significantly reduce both the number of leukocytes recruited and the severity of disease. In addition, administration of chemokines to the joints of animal models results in inflammation and leukocyte recruitment.

The biological effects of chemokines are mediated by binding to G-protein-coupled, seven transmembrane domain, chemokine receptors [11]. CC receptors (CCR) bind members of the class of chemokines with two adjacent cysteine (C) residues near the NH2-terminus (CC; e.g. CCL2, CCL3 and CCL5) and CXC receptors (CXCR) bind members of the class of chemokines with C separated by an amino acid defined as X (CXC; e.g. CXCL5 and CXCL8). There have been some reports on the expression of such receptors on T cells in synovial tissue and fluid [12–16]. Little is known about the expression of chemokine receptors on phagocytes in the human RA synovium. One recent study has shown the protein expression of several chemokine receptors on monocytes/macrophages in RA blood, synovium and synovial fluid [17]. The aim of the present study, therefore, was to examine the expression of in situ chemokine binding sites on macrophages and neutrophils that could mediate the migration of these cells into the inflamed synovium.

Materials and methods

Tissue source

Tissue samples were obtained from patients with RA (n = 6) who fulfilled the American Rheumatism Association criteria for RA (Supplementary Table 1). Synovia were taken from these subjects at total knee replacement (patients 1, 3–5) or knee synovectomy operations (patients 2 and 6). Non-RA patients (n = 6) had knee joint symptoms for suspected articular cartilage, meniscal or anterior cruciate ligament damage (patients 7–12). Knee joint synovial biopsies were obtained from these non-RA individuals, with informed consent, at arthroscopy. For RA and non-RA patients, samples were taken from the suprapatellar pouch and medial gutter, which is reported to provide representative sampling of synovial membrane pathology [18].

In situ binding autoradiography

The method of Hub and Rot [19] was followed, with minor modifications (see Supplementary Material).

Identification of macrophages and neutrophils

Representative sections of synovia that had been incubated with 125I-labeled chemokines were immunostained for CD68 as a marker for macrophages. Slides were deparaffinized, rehydrated and treated with 1% pronase for seven minutes at room temperature. Following blocking of endogenous peroxidase, sections were treated with normal horse serum and incubated with 2.5 μg/ml anti-CD68 monoclonal antibody (PG-M1, Dako A/S, Glostrup, Denmark) in PBS/normal horse serum. The bound antibody was detected using biotinylated second antibody, avidin-peroxidase conjugate and diaminobenzidine substrate (Vectastain ABC Elite Kit; Vector Labs, Burlingame, USA). Slides were then coated with emulsion, exposed, developed and stained in haematoxylin, as for in situ binding autoradiography.

Neutrophils were identified by their characteristic multilobed nuclei in haematoxylin and eosin stained sections.

Immunohistochemistry of CXCR1 and CXCR2

Synovial samples that had been examined for chemokine binding were also sectioned and incubated with rabbit polyclonal antibodies to the carboxy termini of CXCR1 and CXCR2 (both from Santa Cruz Biotechnology, Santa Cruz, USA) [20]. Antibody binding was detected using an immunoperoxidase method (Vectastain ABC Elite Kit; Vector Labs, Burlingame, USA). See Supplementary Material for more detailed methods.

Quantitation of synovial sections

See Supplementary Material.
Results

Tissue sampling
In all six RA subjects the histological appearance of synovia showed classic inflammatory pathology with mononuclear cell infiltrates and a thickened intimal layer. In two of these individuals a minority of synovial samples also showed microscopically normal regions with minimal infiltration of inflammatory cells and an intimal layer of normal thickness (one to two cells thick). The synovia of the six non-RA patients showed inflamed and noninflamed regions. These inflamed regions displayed intimal layer thickening and some infiltration of mononuclear cells, although not as severe as in RA samples, and noninflamed sites showed a normal intimal layer and no leukocyte infiltrates.

Chemokine binding to macrophages
To examine in situ binding of chemokines, synovia were treated with 125I-CXCL8, 125I-CCL2, 125I-CCL3 and 125I-CCL5 followed by autoradiography. For direct comparison, each chemokine was added at the same specific radioactivity and concentration, and autoradiography was performed for an identical exposure time. All four chemokines bound to synovial tissue cells in the subintima and the intima in every RA and non-RA individual (Supplementary Fig. 1a for CCL5). Radiolabelled chemokine binding was absent in synovia treated with 1000-fold excess homologous nonradiolabelled chemokine, indicating that the binding sites were specific (Supplementary Fig. 1b). Histological examination indicated that macrophages accounted for a high proportion of the chemokine binding, so to confirm this observation, representative sections were immunostained with CD68 as a marker of monocytes/macrophages, then processed for autoradiography. All four chemokines bound to CD68+ cells in the subintima in every RA and non-RA synovial sample. No major differences in chemokine binding were apparent between the six RA patients, despite variation in disease duration and drug treatment (Supplementary Table 1). The positive cells were located close to blood vessels in leukocyte infiltrates and non infiltrated sites, and in more remote sites within the subintimal stroma (Fig. 1). In addition CD68+ macrophages bound all four chemokines in the intimal layer of inflamed (Fig. 1) and noninflamed samples. In control experiments there was no immunostaining when isotype-matched control mouse IgG was used instead of the anti-CD68 antibody.

Quantitation of intimal binding
The number of intimal cells that bound chemokines were counted and the results for RA and non-RA patients were expressed as means (Table 1). There was a significant increase of chemokine-positive cells in inflamed intima compared to noninflamed intima, for both RA and non-RA synovia. The number of positive cells varied between chemokines and in RA tissue the rank order was, CCL3 > CCL5 > CCL2 > CXCL8. In non-RA tissue the order was, CCL3 > CCL5 > CXCL8 > CCL2. The number of CCL3 and CCL5 positive intimal cells did not differ between RA and non-RA tissue. However, the number of intimal cells binding CCL2 in RA was greater than non-RA synovium. In addition, there were more cells binding CXCL8 in inflamed, non-RA intima compared to inflamed RA tissue.

Data were also expressed as the percentage of intimal cells binding chemokines (Supplementary Table 2). Similar results to data expressed as the number of cells binding chemokines were obtained (Table 1 and Supplementary Table 2). In both RA and non-RA synovia there was a significantly higher percentage of positive cells in inflamed compared to noninflamed intima. The rank order of
Chemokine binding was almost identical whether the data were expressed as percentage or number of cells binding chemokines. In the cases of CCL2, CCL3 and CCL5 there were no differences in the percentages of positive cells between RA and non-RA tissue. A higher percentage of cells, however, bound CXCL8 in non-RA inflamed intima compared to RA inflamed intima.

Chemokine binding to neutrophils
Neutrophils were present in the synovia of four out of the six RA patients, but they were less abundant than mononuclear cells. Neutrophils were absent in non-rheumatoid synovia. Neutrophils were positive for 125I-CXCL8 binding (Fig. 2) which was displaced in the presence of 1000-fold excess nonradiolabelled chemokine. These cells bound IL-8 in all locations within the synovium: within blood vessels; close to blood vessels in the extravascular tissue; and close to (or at) the intimal surface. Neutrophils did not show any signal for binding CCL2, CCL3 and CCL5 (Fig. 2). No major differences in neutrophil chemokine binding were apparent between the four patients who had these cells in their synovia.

Immunolocalisation of CXCR1 and CXCR2
To define the molecular identity of the 125I-CXCL8 binding sites on synovial macrophages and neutrophils, immunohistochemistry was performed to examine expression of CXCR1 and CXCR2 by these cells. Sections from the same blocks of synovia used for radiolabelled chemokine binding were treated with antibodies to CXCR1, CXCR2 and CD68. Samples from every RA and non-RA patient showed expression of both CXCR1 and CXCR2 by macrophages in the subintima and intima (Supplementary Fig. 2 and Fig. 3). Similarly, in RA samples that possessed neutrophils, immunoreactive CXCR1 and CXCR2 was displayed by these cells throughout the subintimal and intimal layers; the staining intensity for CXCR2 was more strong than for CXCR1 (Fig. 3c). Sections treated with rabbit IgG instead of CXCR1 or CXCR2 were negative for macrophage and neutrophil staining (Fig. 3d).

Discussion
The results of the present study show the presence of chemokine binding sites on macrophages and neutrophils

Table 1

| Chemokine | Rheumatoid (n = 6) | Nonrheumatoid (n = 6) |
|-----------|-------------------|----------------------|
|           | Inflamed | Noninflamed | Inflamed | Noninflamed |
| CXCL8     | 13 ± 1*  | 3 ± 1      | 22 ± 2*  | 5 ± 1       |
| CCL2      | 23 ± 3*  | 10 ± 1†    | 15 ± 3*  | 5 ± 1       |
| CCL5      | 29 ± 2*  | 14 ± 3     | 25 ± 3*  | 10 ± 2      |
| CCL3      | 33 ± 2   | –          | 36 ± 3*  | 13 ± 2      |

The number of positive cells per standard length of intimal surface was counted (3360 µm total length per patient); data are expressed separately for inflamed (>2 cells thick) and noninflamed tissue (1–2 cells thick). Data are expressed as mean ± SE. n, number of patients; *significant difference between inflamed and noninflamed; †significant difference between rheumatoid and nonrheumatoid.

Figure 2
Chemokine binding to neutrophils in rheumatoid synovia. (a) CXCL8 binds to marginated neutrophils within a blood vessel. (b) CXCL8 binds to neutrophils in the subintimal tissue. There was a lack of binding of CCL5 (c) and CCL3 (d) to neutrophils in the subintima. Magnification ×470 (a)–(d).
in the RA synovium. These sites were evident around blood vessels of the subintima, throughout the subintimal stroma and in the intima. In RA there is increased recruitment of monocytes, which migrate into the synovial subintima and to the intima, presumably following a chemotactic gradient [3]. The increase in synovial macrophage numbers is mainly due to enhanced recruitment since there appears to be little contribution from in situ proliferation of these cells [21], although deficient apoptosis may have an effect on increasing cell number [1]. There is also increased recruitment of neutrophils in RA, migrating from subintimal venules, across the synovium and into the synovial fluid where they can occur in large numbers. The binding of CCL2, CCL3, CCL5 and CXCL8 to macrophages and CXCL8 binding to neutrophils suggests that these receptors may be involved in signaling the migration of the phagocytes through the RA synovium into tissue microcompartments and fluid.

The finding that the proportion and percentage of chemokine binding sites on macrophages increases in the inflamed intima of RA and non-RA synovia suggests that these receptors may function in chronic severe synovitis in RA and more mild synovitis associated with joint damage/degeneration in non-RA pathology. In the cases of CCL2, CCL3 and CCL5 there were no differences in the percentage of chemokine-positive cells in inflamed regions of the RA and non-RA intima. However, there were fewer CXCL8 positive cells in the inflamed RA intima than in the inflamed non-RA intima. The RA synovia were obtained from patients with long-standing disease whereas the non-RA samples were essentially from patients with joint symptoms of shorter duration (Supplementary Table 1). One explanation is that there may be temporal changes in chemokine receptor expression, which could explain this difference between RA and non-RA CXCL8 binding. In this respect, temporal changes in chemokine expression have been reported in human RA and an animal model of RA [22,23]. Alternatively the difference in CXCL8 binding could relate to variation in inflammatory mechanisms or a response to drug treatment in the RA group.

The present experimental approach was taken with the aim of finding out if receptors on phagocytes bind chemokines in situ, and showed that the receptors are available and functional in terms of binding chemokines. Our results agree with in situ studies in human skin, where macrophages also express binding sites for radiolabelled CCL3, CCL5 and CXCL8 [24,25]. The disadvantage with the radioligand technique is the lack of definition regarding the molecular nature of the receptors. In contrast, immunohistochemistry or flow cytometry have the advantage of molecular identification, but can not show definitively if the receptors bind ligands. Immunohistochemistry showed that both CCR1 and CCR2 were expressed by synovial macrophages and neutrophils, suggesting that the CXCL8 binding sites on these cells were CCR1 and CCR2.

A recent immunohistochemical study has shown the presence of CCR1, CCR2 and CCR5 on CD68+ macrophages in the synovium of RA patients.
macrophages in the RA synovium, but not CCR4 [17]. Since CCL2 is a ligand for CCR2, and both CCL3 and CCL5 are ligands for both CCR1 and CCR5 [11], these receptors are probably the CC chemokine binding sites on the macrophages in the present study. The lack of CCL2, CCL3 and CCL5 binding to synovial neutrophils suggests little or no expression of the corresponding CC chemokine receptors on these cells. This is in agreement with other studies showing lack of CCL2 binding to blood neutrophils and general lack of chemotactic response of these cells to CC chemokines [26], although recent reports indicate that neutrophils, in response to certain cytokines, are capable of expressing CC chemokine receptors [27,28].

Conclusion
This study demonstrates the expression of available chemokine binding sites for CCL2, CCL3, CCL5 and CXCL8 on CD68+ macrophages in the subintimal and intimal layers of rheumatoid and nonrheumatoid synovia. Chemokine binding sites for CXCL8, but not for CCL2, CCL3 or CCL5, were found on neutrophils in the rheumatoid synovium. These receptors may mediate the recruitment of phagocytes to the subintima, intima or synovial fluid and could provide potential therapeutic targets.

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Supplementary material

Supplementary Materials and methods

_in situ_ binding autoradiography

Synovia were cut into approximately 2 mm³ pieces. Four samples were placed in tubes containing 0.1 to 4.0 ng of recombinant human ¹²⁵I-labeled (rh ¹²⁵I)-CXCL8 (Mₑ = 8400), rh ¹²⁵I-CCL5 (Mₑ = 8000), rh ¹²⁵I-CCL2 (Mₑ = 8800) or rh ¹²⁵I-CCL3 (Mₑ = 8000) (262 µCi/µg; NEN, Boston, USA) in 400 µl Hank’s balanced salt solution containing 0.1% bovine serum albumin and 20 mM HEPES. The samples were then incubated for one hour at room temperature on a platform shaker [19]. In these experiments, synovial samples from each patient were treated with each of the four chemokines. Initial experiments showed that binding sites were saturable over this concentration-RAnge and the optimum binding/background ratio was obtained using 0.8 ng of chemokine; this amount was used in all the subsequent experiments. Control tubes contained 1000-fold excess of the corresponding unla- beled recombinant human chemokine (molecular mass as for radiolabelled chemokines) (PeproTech EC, London, UK; CXCL8 was kindly donated from Novartis Research Institute, Vienna, Austria). The tissue was washed four times for 10 min in 2 ml Hank’s balanced salt solution on a platform shaker, then fixed overnight in 4% paraformaldehyde. It was then dehydrated in alcohol, embedded in fibrowax and sectioned at 5 µm thickness. Sections were deparafinized, rehydrated, air-dried and coated with Amer- sham LM1 emulsion (Amersham Pharmacia Biotech, Little Chalfont, UK). The slides were exposed at 4°C for two weeks, developed and stained in haematoxylin and eosin.

Immunohistochemistry for CXCR1 and CXCR2

Sections were deparafinized, rehydrated and in the case of CXCR1 pre-treated with 1% pronase for 10 min to unmask epitopes. Following blocking of endogenous peroxidase with 0.3% hydrogen peroxide in methanol for 30 min, slides were rinsed in PBS and treated with a 1/60 dilution of normal goat serum for 30 min. Sections were rinsed in PBS, incubated with CXCR1 or CXCR2 antibodies (at 1/50 and 1/500 dilution respectively) or rabbit IgG (at the same concentration as primary antibodies) for 30 min. After washing with PBS, the antibodies were detected using reagents in the Vectastain ABC Elite Kit (Vector Labs, Burlingame, USA); the sections were treated with biotinylated goat anti-rabbit IgG (at 1/200 dilution) for 30 min, rinsed with PBS, incubated with streptavidin-conjugated peroxidase for 30 min, washed with PBS, then treated with the chromogen 3,3′-diaminobenzidine for 5 min. Sections were washed twice in PBS then water, dried and counter- stained in Mayer’s haematoxylin. All steps in the method were carried out at room temperature.

Quantitation of synovial sections

Sections were used from _in situ_ binding autoradiography experiments and were quantitated blind by one observer. The sample length was 280 µm of synovial intima, the width of a field of view using a ×63 objective lens on a Nikon diaplan light microscope. Intimal cells were scored as being positive for chemokine binding when the autoradiographic signal was greater than that of the adjacent background. The number of positive and negative cells were counted visually in three sample lengths per section (i.e. 840 µm). The number of sections per patient was

Supplementary Table 1

| Details of RA and non-RA patients | | | |
|---|---|---|---|
| Gender | Age | Diagnosis/pathology | Disease duration (years) | Medication |
| 1. Female | 71 | RA | 7 | An |
| 2. Male | 61 | RA | 20 | St |
| 3. Female | 67 | RA | 7 | St, NSAID |
| 4. Male | 73 | RA | 15 | Pen, NSAID, An |
| 5. Female | 52 | RA | 20 | Hyd, St, MTX, Gold, NSAID |
| 6. Female | 56 | RA | 25 | – |
| 7. Female | 47 | articular cartilage damage | 5 | – |
| 8. Male | 43 | acl damage | <1 | – |
| 9. Male | 46 | meniscal tear, articular cartilage damage | 7 | – |
| 10. Female | 46 | meniscal tear, articular cartilage degeneration | <1 | NSAID |
| 11. Female | 63 | meniscal tear, articular cartilage degeneration | <1 | – |
| 12. Male | 43 | acl and articular cartilage damage | >5 | – |

acl, anterior cruciate ligament; An, analgesic; Hyd, Hydroxychloroquine; MTX, methotrexate; NSAID, non-steroidal anti-inflammatory drug; Pen, penicillamine; RA, rheumatoid arthritis; St, steroid.
four, giving a total of 3360 µm of intima measured per patient. The intima was noted as being normal where it was one to two cells thick, and inflamed where greater than two cells thick. All cells were counted except neutrophils, which only comprised <1% of all cells sampled.

From these data, the number and percentage of positive cells in the intimal samples were obtained and expressed per 500 µm of intima. Statistical analyses were by Student’s t-test, The result was significant when \( P < 0.05 \).

Supplementary Discussion

The functional role of chemokine receptors in inflammatory cell recruitment is suggested from animal models of RA. Using the CC chemokine receptor antagonist MCP-1(9-76), a truncated analogue peptide corresponding to MCP, markedly reduces joint histopathology and symptoms in the MRL-lpr mouse model [S1]. The CC receptor antagonist MetRANTES decreased the incidence and severity of arthritis in mouse collagen-induced arthritis [S2]. With both antagonists, mononuclear infiltration is reduced.

CCL2, CCL3, CCL5 and CXCL8 occur in human RA synovial fluid and tissue [8]. Therefore the ligands for the binding sites described here occur endogenously within the RA joint, adding further evidence towards a role for these receptors in vivo. In the RA joint the synovial macrophage is among the principle cell types that produce CCL2, CCL3 and CXCL8 [8,17]. The finding of chemokine receptors on these cells suggests that macrophages may amplify inflammation by attracting cells of the same type, and other cell types, into the joint.

CXCR1 immunohistochemistry of the synovium using immunoperoxidase and diaminobenzidine (brown) staining. (a) Section of a macrophage-rich infiltrate in a rheumatoid synovium treated with a CXCR1 antibody. (b) Adjacent section to (a) treated with an anti-CD68 antibody as a macrophage marker. Note the presence of CXCR1 on macrophages. Magnification ×380 (a) and (b).
thereby setting up a positive feedback loop. Similar mechanisms may also be occurring with neutrophils, since in the RA joint these cells produce CXCL8 [S3] and express CXCL8 binding sites.

### Supplementary Table 2

| Cytokine | Rheumatoid (n = 6) | Nonrheumatoid (n = 6) |
|----------|-------------------|-----------------------|
|          | Inflamed          | Noninflamed           | Inflamed | Noninflamed |
| CXCL8    | 42 ± 5 * †         | 13 ± 4                | 58 ± 5   | 17 ± 2      |
| CCL2     | 56 ± 5 *           | 27 ± 3                | 48 ± 7   | 19 ± 3      |
| CCL5     | 79 ± 3 *           | 49 ± 11               | 66 ± 7   | 33 ± 7      |
| CCL3     | 88 ± 2             | –                     | 87 ± 5   | 44 ± 6      |

The number of positive cells per standard length of intimal surface was counted (3360 μm total length per patient); data are expressed separately for inflamed (>2 cells thick) and noninflamed tissue (1–2 cells thick). Data are expressed as mean ± SE. n, number of patients. *Significant difference between inflamed and noninflamed; †significant difference between rheumatoid and nonrheumatoid.

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