

Expression of Interleukin-1 and Interleukin-1 Receptor Antagonist by Human Rheumatoid Synovial Tissue Macrophages¹

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Interleukin-1 (IL-1) has protean effects in the pathogenesis of rheumatoid arthritis (RA). These effects include production of prostaglandins and collagenase from rheumatoid fibroblasts as well as upregulation of adhesion molecule expression on these cells. IL-1 can activate monocytes and neutrophils, as well as promote the growth of fibroblasts and endothelial cells. Recently, a novel interleukin-1 receptor antagonist protein (IRAP) has been isolated, purified, cloned, and expressed, which may modulate the effects of IL-1. In this study, we present data demonstrating that macrophages isolated from human RA synovial tissues express both IL-1 and IRAP genes. In addition, RA synovial tissue macrophages and lining cells display IL-1 and IRAP antigenic expression by immunohistochemistry. In contrast, osteoarthritis synovial tissues, as compared to RA, have fewer IL-1 and IRAP-positive macrophages. Thus, the production of IL-1 balanced by IRAP may affect the joint destruction found in these diseases. © 1992 Academic Press, Inc.

INTRODUCTION

The rheumatoid (RA) synovial tissue is characterized by infiltrating mononuclear cells, proliferating fibroblasts, and endothelial cells (1). The pathogenesis of synovial inflammation is mediated by both cellular and humoral interactions (2). A number of humoral mediators including cytokines such as interleukin-1 (IL-1) have been implicated in the evolution of the erosive arthritis associated with RA (3).

IL-1 is a 17-kDa cytokine that is produced by immune and nonimmune cells that has pleiotropic effects on a variety of cells (3, 4). Both IL-1- α and IL-1- β bind the same receptor; however, IL-1- β seems to be the

predominant form synthesized and released in the supernatants of stimulated cells (3, 4). IL-1 can mediate a variety of effects that may contribute to the pathogenesis of RA. These effects include stimulation of endothelial cell-derived adherence proteins and production of fibroblast-derived prostaglandin E₂ and collagenase (4–8).

Joint inflammation and subsequent evolution to joint destruction may result from a delicate balance between the production of proinflammatory cytokines and cytokine inhibitors. Recently an IL-1 receptor antagonist protein, termed IRAP, has been isolated, purified, cloned, and expressed (9, 10). Exposure of peripheral blood monocytes to adherent IgG or granulocyte-macrophage colony stimulating factor promote the expression of IRAP from these cells (3, 11–13). This protein functions as a competitive inhibitor of IL-1 at the level of the receptor. Although the production of IL-1 and IRAP by blood monocytes has been assessed, little is known regarding the production of this protein by cells obtained from inflammatory lesions. In this study we report that RA synovial tissue macrophages express mRNA for IL-1 and IRAP. Furthermore, RA synovial tissue macrophages and macrophage-derived synovial lining cells were demonstrated to have IL-1- β and IRAP antigen expression by immunohistochemical localization.

MATERIALS AND METHODS

Patients Studied and Cell Isolation

Synovial tissues were obtained from eight patients with RA at the time of total joint replacement. For comparison with the RA samples, synovial tissues were obtained from six patients with osteoarthritis (OA). All patients met the American College of Rheumatology criteria for RA (14) or OA (15), and all samples were obtained with institutional review board approval. To obtain RA synovial macrophages, the tissues were minced and digested in a solution of

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disperse, DNAase, and collagenase as described previously (16, 17). The resultant single-cell suspensions were fractionated into density-defined subpopulations by isopycnic centrifugation through continuous preformed Percoll gradients (Pharmacia, Piscataway, NJ). Macrophages were enriched by adherence to fibronectin-coated collagen gels and selective trypsinization (incubation with trypsin:EDTA for 5–10 min) (15, 16). Macrophages were harvested from the collagen gels by treatment with clostridial collagenase and found to be $\geq 90\%$ pure, as assessed by F_c receptor-mediated phagocytosis of IgG opsonized sheep red blood cells, esterase staining, and staining with commercial anti-macrophage monoclonal antibodies OKM1 (Ortho Diagnostics, Raritan, NJ) and anti-LeuM3 (Becton-Dickinson, Mountain View, CA). The endotoxin concentration of the tissue culture medium was < 0.05 ng/ml as determined by the Limulus assay (Associates of Cape Code, Woods Hole, MA).

Mononuclear cells were isolated from normal volunteers by Ficoll-Hypaque density centrifugation as described (18).

Northern Blot Analysis

Total cellular RNA was obtained from 2.5×10^6 RA macrophages using a modification of Chirgwin and associates and Jonas and associates (19–21). Briefly, cells were placed in a solution containing 25 mM Tris, pH 8.0, 4.2 M guanidine isothiocyanate, 0.5% Sarkosyl, and 0.1 M 2-mercaptoethanol. After homogenization, the suspension was added to a solution containing an equal volume of 100 mM Tris, pH 8.0, 10 mM EDTA, and 1% SDS. The mixture was then extracted with chloroform-phenol and chloroform-isoamyl alcohol. The RNA was alcohol precipitated and the pellet dissolved in diethylpyrocarbonate-treated H_2O . Total RNA was separated by Northern analysis using formaldehyde, 1% agarose gels, transblotted onto nitrocellulose, baked, prehybridized, and hybridized with a ^{32}P -5' end-labeled oligonucleotide probe. A 30-mer oligonucleotide probe was synthesized using the published cDNA sequence for human-derived IRAP (10). The probe was complementary to nucleotides 438–468 and had the sequence 5'-TGT-GCA-GAG-GAA-CCA-ACC-GGG-GCA-GGC-GGC-3'. A 30-mer oligonucleotide probe was synthesized using the published cDNA sequence for IL-1- β (22). The probe was complementary to nucleotides 166 through 195 and had the sequence 5'-CGC-GGC-CTG-CCT-GAA-GCC-CTT-GCT-GTA-GTG-3'. Equivalent amounts of total RNA/gel were assessed by monitoring 28S and 18S rRNA.

Immunohistochemistry of Synovial Tissues

Antibodies used. Murine monoclonal anti-IL-1- β and anti-IRAP (monoclonal antibody I4) were obtained from the Upjohn Co. (Kalamazoo, MI). Irrelevant

mouse monoclonal antibodies were used as negative controls.

Immunoalkaline Phosphatase Staining

Immunoalkaline phosphatase staining was performed on formalin-fixed, paraffin-embedded RA synovial tissues (23). Tissue sections (5–7 μ m) mounted on poly-L-lysine-coated glass slides were deparaffinized and rehydrated in a graded series of xylene and ethanol solutions. The slides were treated with avidin for 15 min at room temperature followed by biotin (1:50 in goat blocking serum, room temperature) to block binding sites for these molecules (Vector Laboratories, Mountain View, CA). The slides were exposed to optimal dilutions of specific antibody or control antibody. After incubating for 20 min at 37°C, the slides were

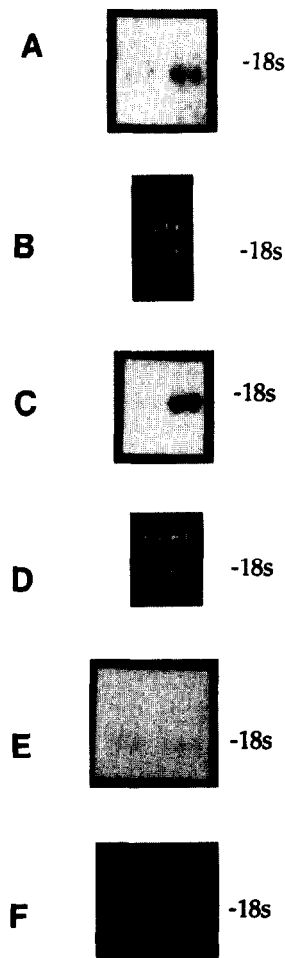


FIG. 1. (A) A representative experiment showing IRAP gene expression by isolated RA synovial tissue macrophages from two patients. One of these samples was obtained from RA patient number 4 (Table 1). (B) 18S and 28S rRNA corresponding to (A). (C) A representative experiment showing IL-1- β gene expression by isolated macrophages from the same two patients. (D) 18S and 28S rRNA corresponding to (C). (E) A representative experiment showing lack of IRAP gene expression in normal peripheral blood mononuclear cells. (F) 18S and 28S rRNA corresponding to (E).

rinsed with phosphate-buffered saline ($\times 3$), overlaid with biotinylated secondary antibodies (Biogenex, San Ramon, CA), and incubated another 20 min at 37°C, followed by a phosphate-buffered saline (PBS) wash. Sections were treated with alkaline phosphatase-labeled streptavidin (Biogenex) for 20 min at 37°C, rinsed in PBS, and overlaid with Vector red substrate chromogen containing 150 mM NaCl and 10 mM levamisole (Vector) for 20 min at room temperature. Mayer's hematoxylin was used as a counterstain.

Microscopic Analysis of Immunohistochemistry

Each tissue was assigned an inflammatory score from 1 to 4, with 4 representing the greatest amount of mononuclear cell infiltrate. In addition, a macrophage score was determined based on cell morphology to indicate the relative amount of macrophages present in a tissue. Macrophage scores ranged from 1 to 4, with 4 representing the greatest number of macrophages as previously described (24). Each tissue was reviewed by two pathologists and scored in a blinded fashion to determine the approximate percentage of macrophages, synovial lining cells, and endothelial cells reactive with the monoclonal antibodies.

Statistical Analysis

Correlation coefficients were determined by regression analysis. Differences between the mean values of groups was determined using a paired Student *t* test. *P* values < 0.05 were considered significant.

RESULTS

Gene Expression of IL-1- β and IRAP by RA Synovial Tissue Macrophages

Northern Blot analysis of mRNA isolated from purified macrophages from patients with RA is shown in Fig. 1. Figure 1A shows steady state IRAP mRNA from two patients with RA. Figure 1B shows 18S and 28S rRNA corresponding to (A). Figure 1C shows steady state IL-1- β mRNA from the same patients along with 18S and 28S rRNA (D). Normal peripheral blood monocytes do not express IRAP (E and F) and require exogenous stimulation prior to expression of IRAP mRNA. In contrast, RA macrophages express both IRAP and IL-1 mRNA without exogenous stimuli.

Antigenic Expression of IL-1- β and IRAP by RA Synovial Tissue Macrophages

Since RA synovial macrophages were found to express both IL-1- β and IRAP mRNA, we determined whether this mRNA was translated to protein. Formalin-fixed, paraffin-embedded sections of synovial tissues were obtained from seven patients and six OA patients. Sections were immunostained with anti-IRAP (Table 1) and in selected cases with anti-IL-1- β . In general, the RA tissues had higher inflammatory scores than the OA tissues (2.3 ± 0.3 versus 1.2 ± 0.2) (means \pm SE). In addition, the numbers of macrophages present within the RA synovial tissues were

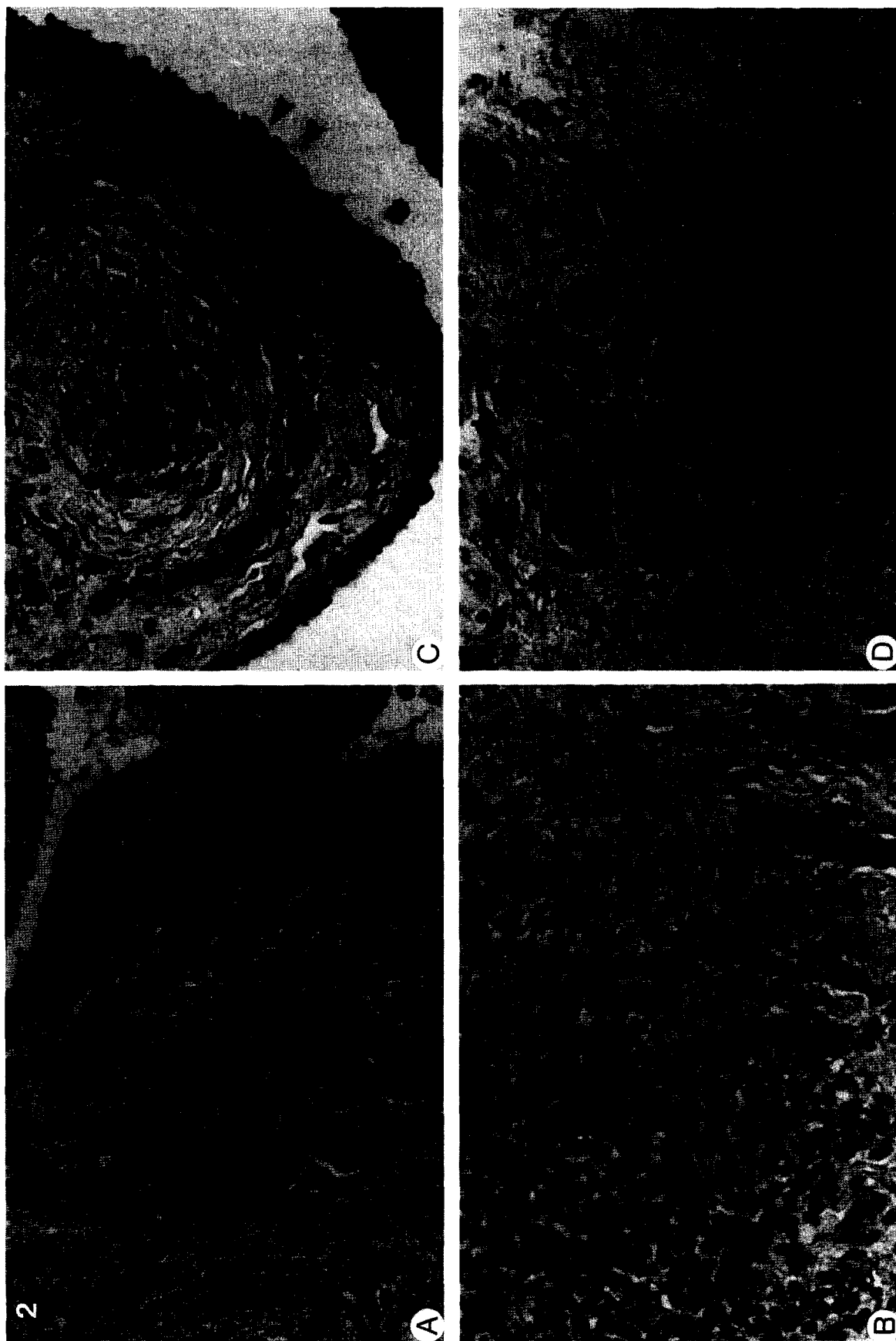
TABLE 1
IRAP and IL-1- β Expression on Formalin-Fixed Human Synovial Tissues

Patient	Age/sex	Inflammatory score ^a (1-4)	M ϕ score ⁺ (1-4)	IRAP		IL-1- β	
				% of m ϕ s +	% of lining cells +	% of m ϕ s +	% of lining cells + ^b
Rheumatoid arthritis							
1	32/M	2	3	95	95	55	60
2	65/F	4	4	80	100	85	85
3	33/F	1	2	0	1	ND	ND
4	73/F	2	4	20	10	90	95
5	40/F	2	2	40	15	5	30
6	67/F	2	3	0	0	ND	ND
7	-/F	3	4	60	90	ND	ND
Mean \pm SE	52	2.3 ± 0.3	3.1 ± 0.3	42 ± 13	44 ± 16	58 ± 17	68 ± 13
Osteoarthritis							
1	60/F	1	1	7	50	30	90
2	65/F	1	1	8	5	25	35
3	73/F	1	1	30	25	ND	ND
4	70/F	1	1	2	60	10	*
5	89/F	1	1	0	70	60	70
6	88/F	2	1	10	95	5	30
Mean \pm SE	67	1.2 ± 0.2	1.0	9.6 ± 4	50.8 ± 11	26 ± 8	56 ± 12

Note. F, female; M, male; ND, not done. M ϕ = macrophage.

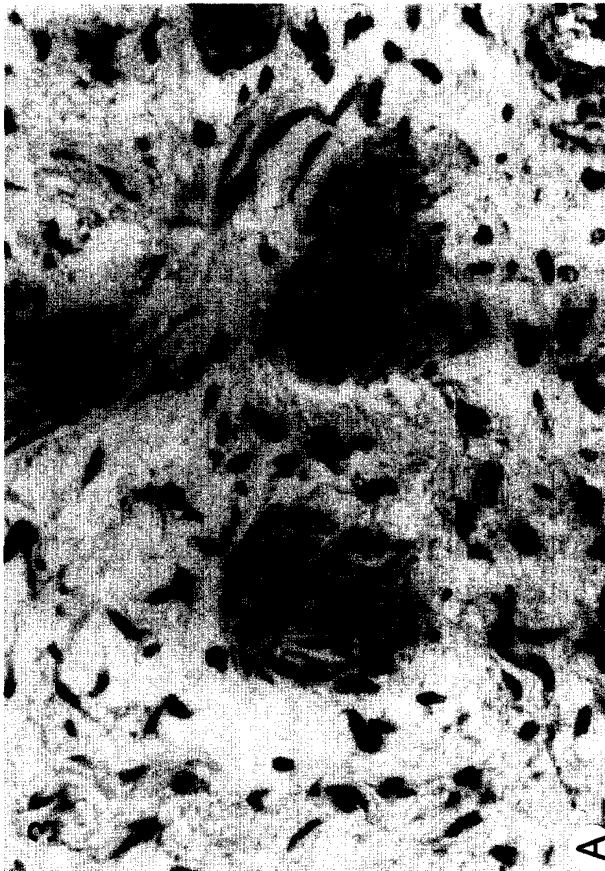
^a Tissues were assigned an inflammatory score with 1 being the least amount of inflammation and 4 being the greatest amount of inflammation.

^b Tissues were assigned a macrophage score with 1 being the least amount of macrophages and 4 being the greatest.





C



A



B

FIG. 2. Immunohistochemical phosphatase staining of a representative formalin-fixed, paraffin-embedded RA synovial tissue from RA patient 1 (Table 1). (A) Irrelevant antibody control (300 \times). Macrophage staining with anti-IL-1- β (150 \times) (arrow). (C) Synovial lining layer (arrow heads) and subsynovial macrophage staining with anti-IRAP (300 \times) (arrow). (D) Subsnoyial macrophages intensely stained with anti-IRAP (300 \times) (arrow).

FIG. 3. Immunohistochemical phosphatase staining of a representative formalin fixed, paraffin-embedded OA synovial tissue from OA patient 6 (Table 1) (250 \times). (A) Irrelevant antibody control. (B) Macrophage staining with anti IL-1- β (arrow). (C) Staining of synovial lining (arrow heads) and the minority of subsynovial macrophages with anti-IRAP (arrow).

greater than found within the OA tissues (3.1 ± 0.3 versus 1.0).

In RA, IRAP expression was noted in the synovial lining layer with $44 \pm 16\%$ of cells reacting with anti-IRAP (Table 1, Fig. 2). OA tissues also exhibited synovial lining layer IRAP reactivity (Table 1, Fig. 3).

In RA synovial tissues, macrophages located in the subsynovial areas expressed IRAP, with $42 \pm 13\%$ of macrophages staining positively as compared to only $9.6 \pm 4\%$ of the macrophages obtained from OA immunostaining positive for IRAP. Although RA macrophages that were immunopositive for IRAP were principally distributed in the subsynovial region, perivascular macrophages were also immunopositive for IRAP. There was a positive relationship between the percentage of macrophages staining with anti-IRAP in RA and the macrophage score ($r = 0.38$) or the inflammatory score ($r = 0.63$), although neither correlation reached statistical significance. The minority of RA tissues displayed IRAP positive vascular endothelial cells. In addition, fibroblasts and lymphocytes located in the synovial tissues of RA patients did not express IRAP.

Representative RA synovial tissues were stained with anti-IL-1- β (Table 1, Fig. 2). IL-1- β reactivity was present on the majority of macrophages ($58 \pm 17\%$) and lining cells ($68 \pm 13\%$). In contrast, a smaller percentage of macrophages in OA (26 ± 8) were IL-1- β positive (Table 1, Fig. 3).

DISCUSSION

IL-1 has been implicated as an important factor mediating inflammation and joint destruction in RA (3). Attempts to measure bioactivity in human synovial fluids have often resulted in conflicting results, perhaps due to the presence of IL-1 inhibitors in these fluids (3, 25, 26). More recently, IL-1 measured by ELISA demonstrated significantly increased levels of IL-1 in RA synovial fluids (3, 25, 27). Synovial fluid cells *in vitro* do not appear to produce increased levels of IL-1 (3); however, rheumatoid synovial tissue *in vitro* synthesizes both IL-1- α and IL-1- β (28). Firestein and associates using *in situ* hybridization techniques have shown that approximately 10% of the cells dispersed from RA synovial tissues expressed IL-1- β mRNA. Furthermore, cells that were expressing constitutive IL-1- β were the macrophage-enriched OKM1-positive cell population (29). Interestingly, these studies failed to demonstrate IL-1- β mRNA expression from synovial fluid monocytes, suggesting potential disparate IL-1- β production between monocytes from synovial fluid and synovial tissue macrophages.

IRAP, a naturally occurring competitive inhibitor of IL-1 at the level of the IL-1 receptor, may serve to modulate IL-1 effects. The relative amounts of IL-1- β or IRAP synthesized by blood monocytes in culture depend upon the immunologic stimulus, and it appears

that the same population of monocytes secrete both proteins (30). IRAP production is stimulated by adherence of monocytes to IgG, but this production can be counteracted by lipopolysaccharide stimulation of the cells (30). When peripheral blood monocytes are differentiated in culture to monocyte-derived macrophages, their production of IRAP increases, particularly in the presence of granulocyte-macrophage colony stimulating factor. In contrast, IL-1- β production is low in these cells (3, 11, 12, 13). Malyak *et al.* have recently analyzed synovial fluids for the presence of IRAP by ELISA and found that IRAP levels are increased in RA as well as other arthritic disorders such as gouty arthritis (31). The origin of the cells liberating IRAP into synovial fluids was not examined.

In this study we have confirmed the production of IL-1- β by purified RA macrophages freshly isolated from the inflamed synovial tissue. In addition, we have shown that this same population of cells constitutively produce IRAP, unlike normal blood monocytes which exhibit little, if any, transcription of either IL-1- β or IRAP mRNA without *in vitro* stimulation. Moreover, immunohistochemical localization of both IL-1 and IRAP antigens are found on RA synovial tissue macrophages as well as synovial lining cells. These findings indicate that tissues containing significant numbers of inflammatory cells, presumably secrete high levels of cytokines and contain an increased percentage of IRAP-positive macrophages. Furthermore, inflammatory cells within OA tissues such as macrophages are likely to secrete lower levels of some cytokines including IRAP. Thus, a greater percentage of RA, as compared to OA, subsynovial macrophages express IRAP. Furthermore, these data indicate that in the context of RA, the same population of synovial tissue macrophages can both mediate erosive joint destruction, via the production of IL-1, and also modulate this destruction by the production of IRAP in an autocrine and paracrine fashion. The net biological effect of IL-1 may be substantially regulated by the production of IRAP by synovial macrophages within the RA joint.

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