Immunoregulatory Role of Interleukin 10 in Rheumatoid Arthritis

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Summary

The presence and the role of interleukin 10 (IL-10), a potent cytokine synthesis inhibitory factor and antiinflammatory cytokine, were investigated in rheumatoid arthritis (RA). The expression of both mRNA and protein for IL-10 could be demonstrated in RA and osteoarthritis (OA) joints. Human IL-10 mRNA could be demonstrated by polymerase chain reaction amplification of cDNA made by reverse transcription of total RNA extracted directly from synovial tissue in five out of five RA and four out of five OA patients. IL-10 protein was demonstrated by specific immunoassay and immunohistology. IL-10 protein was spontaneously produced in all 11 RA and 17 OA synovial membrane cultures investigated, and this production was sustained for up to 5 d in culture in the absence of any extrinsic stimulation. IL-10 protein could also be detected by immunohistology in all five RA and four OA synovial membrane biopsies investigated, but not three normal synovial membranes. Immunohistology revealed that the IL-10 was localized to the synovial membrane lining layer and mononuclear cell aggregates. Immunofluorescence double staining revealed that the sources of IL-10 were monocytes in the lining layer, and T cells in the mononuclear cell aggregates. We found evidence that the IL-10 expression was functionally relevant, as neutralization of endogenously produced IL-10 in the RA synovial membrane cultures resulted in a two- to threefold increase in the protein levels of proinflammatory cytokines tumor necrosis factor α (TNF-α) and IL-1β, although IL-6 and IL-8 levels were not affected. The addition of exogenous recombinant IL-10 to the RA synovial membrane cultures resulted in a two- to threefold decrease in the levels of TNF-α and IL-1β. IL-8 levels were reduced by day 5; however, IL-6 levels were not affected by exogenous IL-10. Neutralization of the endogenous IL-10 in two out of seven RA synovial membrane cultures resulted in the expression of detectable levels of interferon γ (561–1,050 pg/ml). Taken together, the above findings suggest that IL-10 is spontaneously produced in RA and OA and is an important immunoregulatory component in the cytokine network of RA, regulating monocyte and in some cases T cell cytokine production.

R heumatoid arthritis (RA)[1] is an autoimmune disorder that is characterized by a chronic synovitis which often leads to joint destruction (1). It is now established that proinflammatory cytokines such as TNF-α, IL-1, GM-CSF, and IL-6 are all produced by the synovial membrane in RA, and are considered to be important participants in the pathophysiology of the disease (2–9). However, in addition to these proinflammatory cytokines, a compensatory antiinflammatory response is also observed in RA synovial membranes. Thus there is expression of high levels of IL-1 receptor antagonist (IL-1RA), soluble TNF receptors (of both the 55-

and 75-kD receptor) and TGF-β in the RA synovium (8, 10–12, and Brennan, F. M., manuscript in preparation), which suggests that homeostatic mechanisms exist in the rheumatoid joint by which the immune system attempts to contain the inflammation and limit joint destruction.

In contrast to the abundance of monocyte-derived proinflammatory cytokines described above, T cell–derived cytokine proteins have often proven difficult to detect in RA synovium (13, 14). This is rather paradoxical, since the synovial membrane–infiltrating T cells appear phenotypically activated (15, 16). However, mRNA for T cell cytokines such as IFN-γ and IL-2 has been demonstrated (17), and this suggests that there may be T cell cytokine inhibitory factors operative in the rheumatoid synovium and/or cytokine consumption. In addition, T cell proliferative responses to mitogens are impaired in RA (18–20), again suggesting the presence of inhibitors. The report that most T cell clones derived from

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[1] Abbreviations used in this paper: IL-1RA, IL-1 receptor antagonist; OA, osteoarthritis, RA, rheumatoid arthritis.

the RA synovium are of the Th1 cell type (21, and Cohen, S., unpublished observations), which produce predominantly IFN-γ and IL-2 (22), raises the possibility that one of the inhibitory factors present in RA could be IL-10, originally defined as "a cytokine synthesis inhibitory factor," capable of suppressing cytokine synthesis by murine Th1 CD4$^+$ cells (23, 24). However the presence of IL-10 in RA synovium remains to be documented.

IL-10 is a 35-kD homodimeric cytokine, which is produced by human T cells, B cells, and monocytes (23, 25, 26). In humans, IL-10 exhibits a cytokine synthesis inhibitory activity (CSIF), inhibiting IFN-γ, GM-CSF, IL-4, and IL-5 production by T cells and can reduce Ag-specific T cell proliferation (23, 24). The latter chiefly involves inhibiting the capacity of monocytes to present Ag, a phenomenon mediated at least in part through the downregulation of HLA class II on monocytes (27). IL-10 has also been shown to have potent antiinflammatory properties. IL-10 inhibits the synthesis of proinflammatory cytokines such as TNF-α, IL-1, and GM-CSF, as well as HLA class II expression by human monocytes stimulated with LPS (25).

The reported antiinflammatory and CSIF properties of IL-10 led us to investigate whether IL-10 is produced in RA and osteoarthritis (OA), and whether it plays an important immunoregulatory role, regulating monokine and T cell–derived cytokine production. Here we describe the presence of IL-10 mRNA and protein in RA and OA synovial membrane biopsies. We also report spontaneous IL-10 production in RA and OA synovial membrane cultures, and evidence that suggests that it has an important role in the synovium as a regulator of production of the inflammatory cytokines TNF-α and IL-1. IL-10 may thus have a possible role in new therapeutic approaches to inflammatory arthritis.

Materials and Methods

Patient Samples and Cell Cultures. 11 patients with RA fulfilling the revised American Rheumatology Association (ARA) criteria (28), and 17 patients with OA, all of whom underwent joint replacement surgery, were included in this study. Synovial membrane cell suspension cultures were prepared by collagenase and DNase digestion of membranes as previously described (12). In brief, synovial membrane tissue was digested in RPMI 1640 containing 5% FCS (Flow, High Wycombe, UK), 1 mg/ml collagenase type A (Boehringer, Mannheim, Germany), and 0.15 mg/ml DNase type 1 (Sigma Chemical Co., Poole, UK) for 2 h at 37°C. After incubation, the tissue was pipetted through a 200-μm^2 nylon mesh into a sterile beaker. Cells were then washed three times with RPMI 1640 plus 5% FCS. Synovial membrane cells were cultured at 10^6/ml/well in RPMI plus 5%/FCS in 24-well plates (Nunc, Uxbridge, UK) at 37° in a 5% CO$_2$ incubator, and supernatants were harvested at 24, 72, and 120 h. Synovial membrane cell cultures were treated with 2 μg/ml of neutralizing rat monoclonal anti-IL-10 antibody 9D7, rat isotype control antibody GL113, or 10 ng/ml rIL-10. Supernatants were frozen at −20°C until assayed. Synovial fluid exudates from nine RA and three OA patients were aspirated during needle biopsy, cells were spun down at 1,000 rpm, and supernatants were assayed at a 1:2 dilution in PBS in the IL-10 and isotype control ELISA. Synovial fluids were also assayed for IL-10 after treatment with hyaluronidase. In brief, synovial fluids were

incubated with 4 U/ml hyaluronidase (Sigma Chemical Co.) for 30 min at 37°C (11), after centrifugation at 2,200 rpm for 12 min. Supernatants were then assayed in the IL-10 and isotype control ELISA. All media and reagents were found to have <0.1 EU/ml endotoxin contamination by the chromogenic Limulus Amoebocyte Lysate assay (BioWhittaker, Walkersville, MD). The only exception was the rat monoclonal anti-IL-10 neutralizing antibody 9D7 which at the concentration of 2 μg/ml used had 0.13 EU/ml endotoxin contamination. This led to the inculsion of 10 μg/ml Polymyxin B (Sigma Chemical Co.) controls in three of the cultures, to exclude the effect of endotoxin. In three other cultures, the endotoxin-free rat monoclonal neutralizing anti-IL-10 antibody 12G8 at 2 μg/ml was also included as another control for the 9D7 antibody.

ELISAs and Antibodies. Recombinant human IL-10, rat monoclonal anti-IL-10 neutralizing IgG1 antibody 9D7, rat monoclonal isotype control IgG1 antibody GL113, rat monoclonal neutralizing IgG2 antibody 12G8, and specific ELISA for human/viral IL-10, were generously provided by Drs. K. Moore and J. Abrams (DNAX, Palo Alto, CA). The sensitivity of the IL-10 ELISA was 100 pg/ml. A control ELISA which differed from the IL-10 ELISA only in that the coating antibody was the nonspecific isotype control mAb GL113 was also used to exclude the effect of RF. A specific TNF-α ELISA was kindly provided by Dr. W. Buurman (University of Limburg, Maastricht, The Netherlands). The IL-1β ELISA was commercially purchased (R & D Systems, Inc., Minneapolis, MN). The IL-6, IL-8, and IFN-γ ELISAs were gifts from Drs. F. di Padova (Sandoz Pharma AG, Basel, Switzerland), W. Lee Wong (Genentech Inc., S. San Francisco, CA), and D. Novick (Weismann Institute, Rehovot, Israel), respectively. Neutralizing anti-TNF-α and anti-IL-1β mouse mAbs A$_2$ and IL-1RA were kind gifts from Dr. P. Daddona (Centocor, Malvern, PA) and Dr. D. Tracey (Upjohn, Kalamazoo, MI). Apart from the IL-10 ELISA, the neutralizing anti-IL-10 antibodies did not interfere in any of the above ELISA assays.

Immunohistology. Synovial membrane biopsies of patients with RA (n = 5) and OA (n = 4) were taken at arthroscopy of knee joints and snapfrozen in *iso*-pentane cooled on liquid nitrogen. Normal synovial membrane specimens (n = 3) were obtained from the knee joint of patients undergoing amputation for osteogenic sarcoma at a site distant for the knee joint. The synovial membranes were sectioned at 6 μm and immunohistochemical staining was performed as described previously (8). The sections were fixed in acetone at −20°C and blocked with 20% normal goat serum, then incubated with 10 μg/ml rat monoclonal IgG1 anti-IL-10 antibody 2A5 (DNAX) overnight at 4°C. Binding was detected by incubating the sections twice with goat anti–rat Ig alkaline phosphatase conjugate (Dako, High Wycombe, UK) for 30 min at room temperature, followed by alkaline phosphatase–antialkaline phosphatase (APAAP) complexes (Dako) for 30 min at room temperature and visualized with Fast red (4-chloro-2-methylbenzenediazonium zinc chloride). Normal rat monoclonal IgG1 (Serotec, Kidlington, UK) at the equivalent protein concentration served as a negative control. The staining of the 2A5 anti-IL-10 antibody was abrogated by preincubating the antibody with 10 μg/ml human rIL-10 for 2 h at 37°C before application to the sections. For double immunofluorescence staining, the anti-IL-10 antibody was detected with a biotinylated donkey anti–rat IgG with minimum cross-reactivity to mouse serum proteins (Jackson Immunoresearch, Luton, UK) and followed by streptavidin-fluorescein (Amersham, Aylesbury, UK). Mouse anti–human CD3, CD20, and CD68 mAbs (Dako) were detected with Texas red–conjugated donkey anti–mouse IgG with minimum cross-reactivity with rat serum proteins (Jackson Immunoresearch).

PCR Amplification and Northern Blot. RNA was extracted directly from synovial membrane tissues by the guanidinium isothiocyanate method (29). To synthesize cDNA, 1 μg of total RNA was incubated with 2 μg oligo (dt)$_{(12-18)}$ primers (Pharmacia, Milton Keynes, UK), 5 ng BSA, 0.5 mM dNTPs, 10 mM DTT, 10 μl x10 RT buffer (Y00146; GIBCO BRL, Uxbridge, UK), and 20 U murine Moloney leukemia virus reverse transcriptase (GIBCO BRL) (30). PCR reactions were performed using 5 μl of this cDNA in the presence of 50 mM KCl, 10 mM Tris, pH 8.3, 1.5 mM MgCl$_2$, gelatin (0.001%), 0.2 mM dNTPs, 1 μM of each primer, and 1.25 U Taq Polymerase (Perkin Elmer Cetus, Beaconsfield, UK) in a total of 50 μl. PCR analysis was performed using two human IL-10 27-mer primers, spanning nucleotides 323–349 (sense strand) and 648–674 (antisense strand) (23). These primers do not amplify the viral homologue of IL-10, BCRF1. Samples were overlaid with 50 μl of light mineral oil (Sigma Chemical Co.) and were incubated at 94°C for 1 min, 55°C for 1 min, and 72°C for 2.5 min. This cycle was repeated 35 times using a DNA thermal cycler (Perkin Elmer Cetus) (31). 10 μl PCR products were electrophoresed through a 6% polyacrylamide gel and visualized by ethidium bromide staining. 10 μl amplified DNA was digested with 1 U HaeIII restriction enzyme (New England Biolabs, Bishop's Stortford, UK) for 1 h at 37°C, and yielded the expected 123- and 229-bp size products.

For Northern blot analysis, total RNA was extracted as above from single cell suspension cultures of two RA synovial membranes which were treated for 24 h with either nothing, 2 μg/ml anti-IL-10 neutralizing rat mAb 9D7, 2 μg/ml isotype control rat mAb GL113, or 10 ng/ml rIL-10. Total RNA was size fractionated by electrophoresis after denaturation with glyoxal and DMSO and then transferred to a Hybond-N nylon membrane (Amersham). After transfer, the membrane was baked for 2 h at 80°C. Before hybridization, glyoxal was removed from RNA by washing the filter with 20 mM Tris-HCl, pH 8.0, at 65°C. The membrane was prehybridized in prehybridization buffer: 6× SSC (1× SSC = 150 mM NaCl, 15 mM Na Citrate, pH 7.0), 5× Denhardt's reagent (1× Denhardt's = 0.05% Ficoll 400, 0.05% polyvinylpyrrolidone, 0.05% BSA), 0.5% SDS, 100 μg/ml salmon sperm DNA (Sigma Chemical Co.), and 50% formamide (BDH, Poole, UK) for 2 h at 42°C. Membranes were hybridized overnight at 42°C with a cDNA probe to TNF-α (kind gift H. M. Shepard, Genentech) labeled with α-[^{32}P] dCTP (Amersham). Membranes were washed with 2× SSC and 0.5% SDS at room temperature, 2× SSC and 0.1% SDS at room temperature, 0.1× SSC, and 0.5% SDS at 37°C, and 0.1× SSC and 0.5% SDS at 65°C. Membranes were then exposed to Hyperfilm-MP (Amersham) at −70°C. The TNF-α probe was stripped from membranes by immersing membranes in 1 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, and 0.1× Denhardt's reagent for 2 h at 75°C. Membranes were prehybridized and then hybridized with a probe to the 7B6 housekeeping gene (32). Autoradiographs were scanned on a dual wavelength TLC scanner CS-930 (Shimadzu, Kyoto, Japan).

Statistics. Results were analyzed with Wilcoxon's signed rank test.

Results

IL-10 Is Produced by RA and OA Synovial Membranes. IL-10 was produced by all 11 RA and 17 OA synovial membrane cultures tested. The IL-10 levels were measured by specific ELISA, and at 24 h ranged from 358 to 5,501 pg/ml for RA
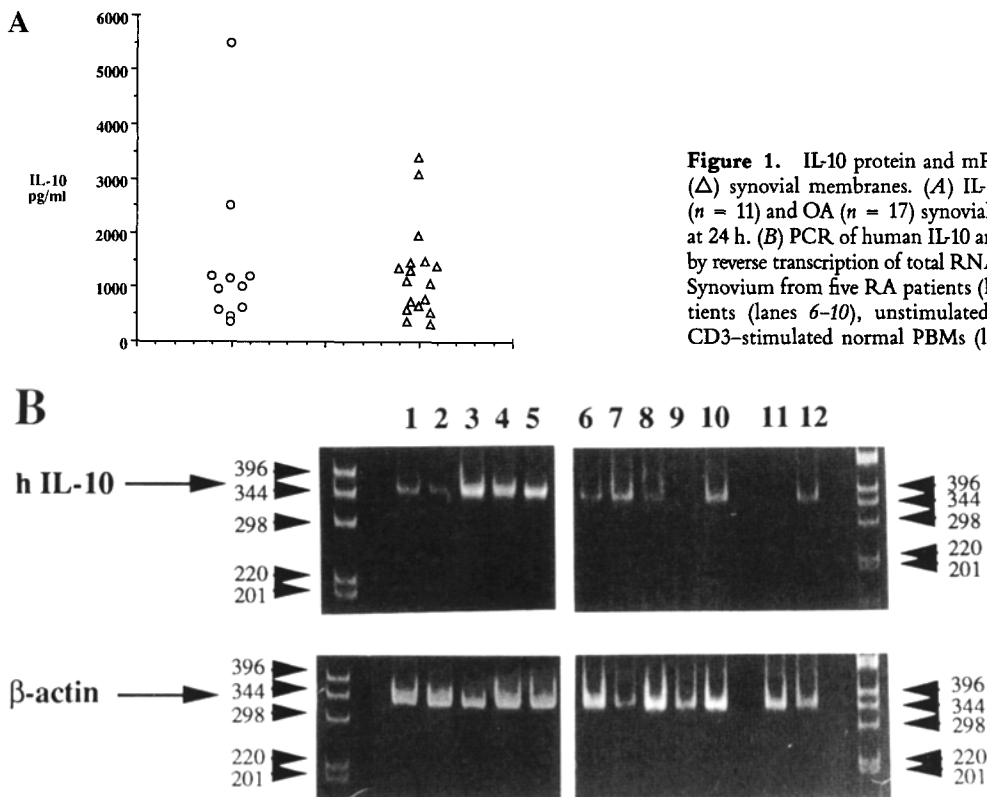

Figure 1. IL-10 protein and mRNA is expressed in RA (O) and OA (Δ) synovial membranes. (*A*) IL-10 is produced spontaneously by RA (*n* = 11) and OA (*n* = 17) synovial membrane cultures, assayed by ELISA at 24 h. (*B*) PCR of human IL-10 and β-actin amplified from cDNA made by reverse transcription of total RNA extracted directly from synovial tissue. Synovium from five RA patients (lanes *1–5*), synovium from five OA patients (lanes *6–10*), unstimulated normal PBMs (lane *11*), and anti-CD3–stimulated normal PBMs (lane *12*).
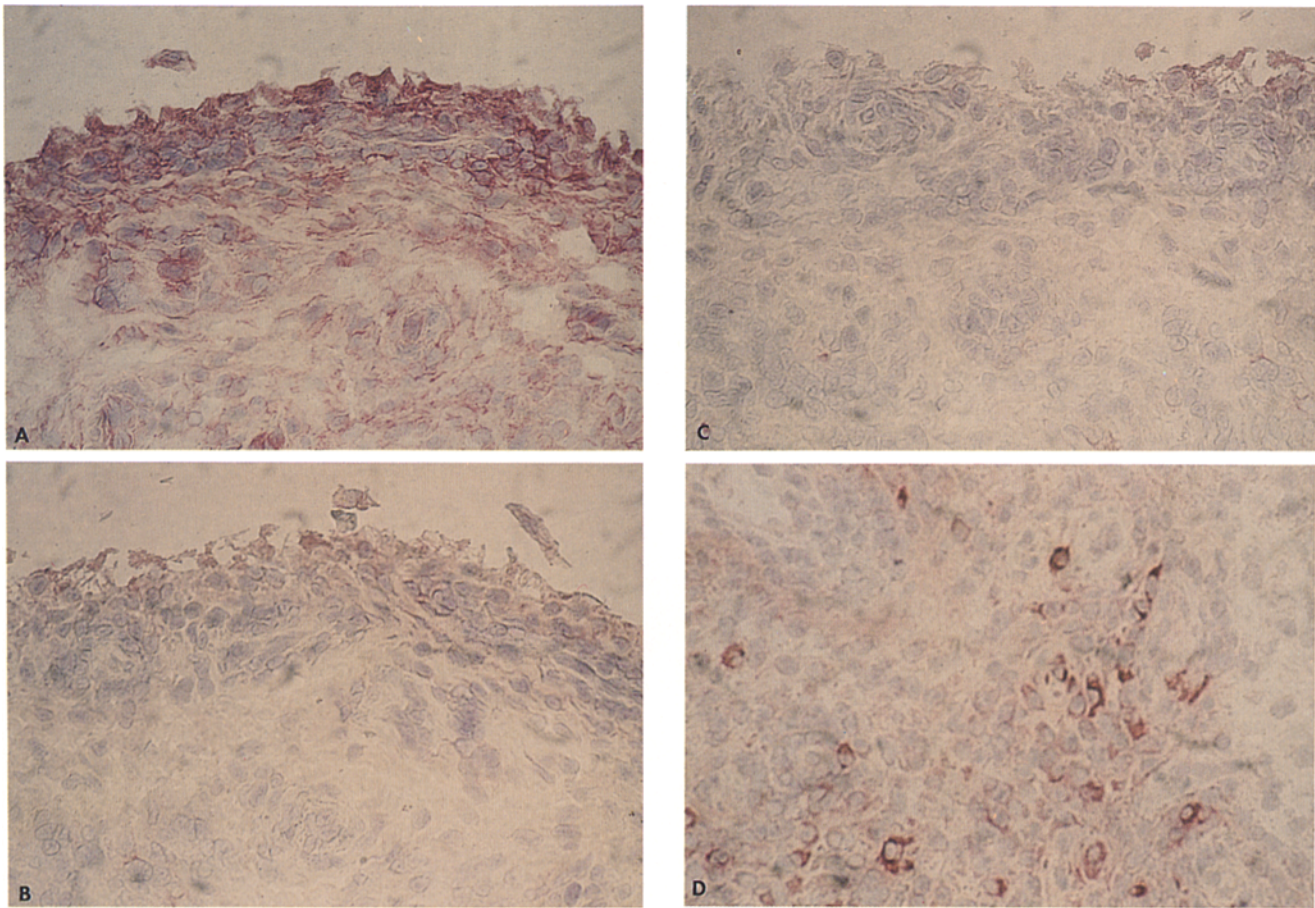
Figure 2. Immunohistological staining for IL-10 in RA synovial membranes. (*A*) IL-10 staining in the synovial membrane lining layer. (*B*) Preincubation of anti-IL-10 antibody with rIL-10 results in abrogation of staining. (*C*) Lack of staining with isotype control rat mAb. (*D*) IL-10 staining in the RA synovial membrane mononuclear aggregates. (*E*) No IL-10 staining detected in normal synovial tissue. (*F* and *G*) IL-10 and monocyte immunofluorescence double staining in RA synovial membrane lining layer. (*F*) IL-10 staining (9D7 anti-IL-10 rat mAb, followed by biotinylated donkey anti-rat IgG and streptavidin-fluorescein). (*G*) Monocyte/macrophage staining (anti-human CD68 mouse mAb, followed by Texas red-conjugated donkey anti-mouse IgG antibody). (*H* and *I*) IL-10 and T cell immunofluorescence double staining in RA synovial membrane mononuclear aggregate. (*H*) IL-10 staining (9D7 anti-IL-10 rat mAb, followed by biotinylated donkey anti-rat IgG and streptavidin-fluorescein). (*I*) T cell staining (anti-human CD3 mouse mAb, followed by Texas red-conjugated donkey anti-mouse IgG antibody). (*Arrows*) Individual cells that double stain for IL-10 and CD3.

and 310 to 3,390 pg/ml for OA cultures (Fig. 1 *A*). IL-10 levels remained detectable up to 5 d in culture. No difference in IL-10 was observed between synovial cultures of RA and OA patients. IL-10 was not detected (<100 pg/ml) in either PBS-diluted or hyaluronidase-treated synovial fluids of nine RA and three OA patients (data not shown). All samples were tested in the control ELISA (isotype-matched, antibody-coated plate) to exclude RFs and showed no binding. IL-10 mRNA was demonstrated by PCR amplification of reverse transcribed cDNA in five out of five RA patients, and four out of five OA patients (Fig. 1 *B*). IL-10 was also detected by immunohistological staining of synovial membrane tissue in all five RA and four OA membranes examined. Cell staining with IL-10 was found in the synovial membrane limiting layer and the mononuclear aggregates. (Fig. 2). This staining was shown to be specific for IL-10 since it could be abrogated by preincubation of 2A5 staining antibody with recombinant human IL-10, and the isotype control mAb yielded no staining.

(Fig. 2). There was no IL-10 staining in the three normal synovial membranes examined (Fig. 2). By immunofluorescence double staining, IL-10 was shown to be produced by monocytes in the lining layer and T cells in the lymphocytic aggregates (Fig. 2).

Regulatory Role of IL-10 in Proinflammatory Cytokine Production in Rheumatoid Synovial Membranes. Blocking IL-10 with the neutralizing rat monoclonal anti-IL-10 antibody 9D7 resulted in an increase in the proinflammatory cytokines produced by the rheumatoid synovial membrane cultures. After 24 h in culture, the TNF-α and IL-1β levels measured by specific ELISAs increased to 208 ± 16% and 198 ± 25% (x ± SEM) of the control levels respectively (p <0.01 and p <0.01). The effect of anti-IL-10 was sustained up to day 5, when TNF-α was increased to 322 ± 91% and IL-1β to 235 ± 65% of control levels. In contrast, blocking IL-10 had no significant effect on IL-6 and IL-8 production (Table 1, Figs. 3 and 4). Northern blot analysis was performed on
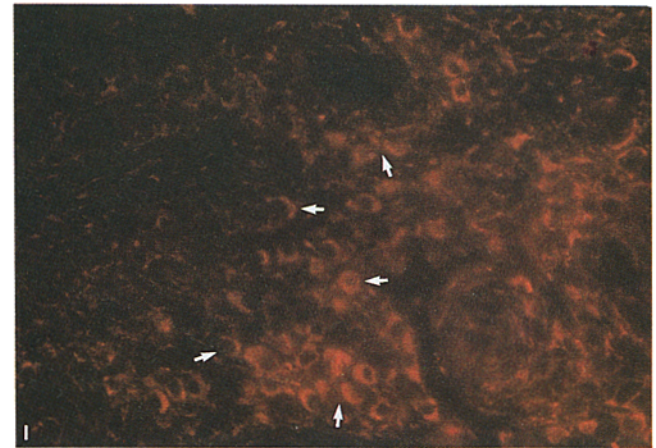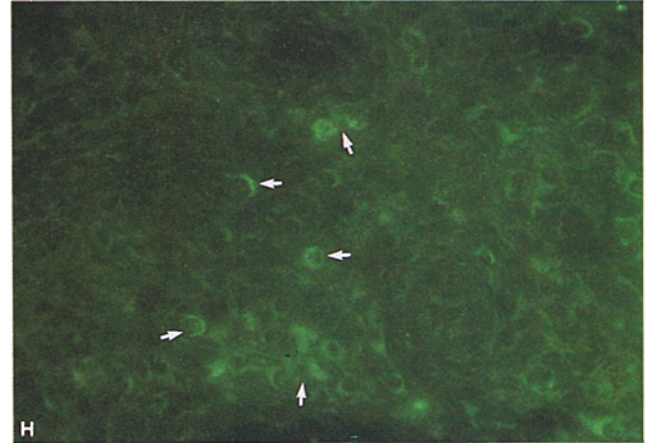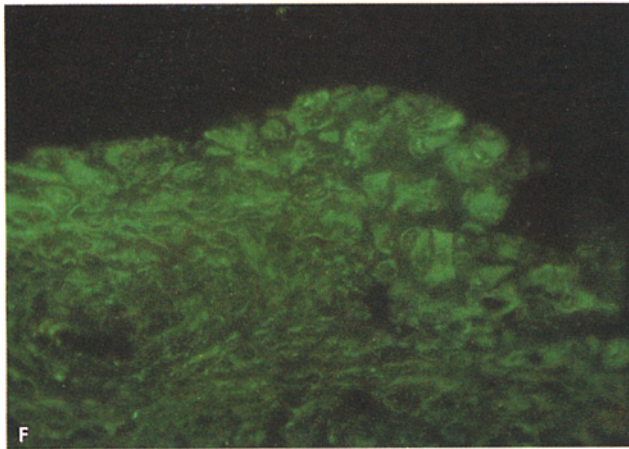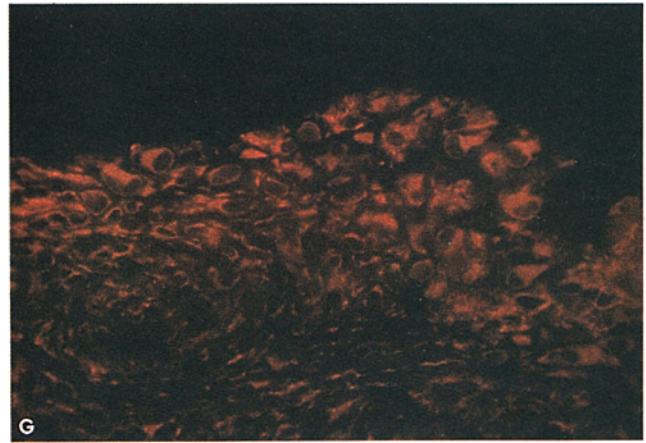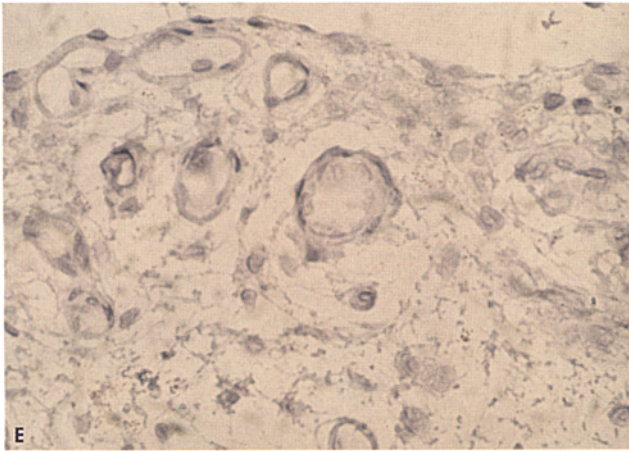
two 24-h RA synovial membrane cultures. Treatment of these two cultures with anti-IL-10 resulted in a twofold increase in TNF-α protein produced, however the TNF-α mRNA levels did not change (data not shown). Addition of 10 μg/ml polymyxin B to three RA synovial membrane cultures treated with the 9D7 antibody, and treatment of three RA membranes with the endotoxin-free neutralizing anti-IL-10 antibody 12G8 gave identical results to the 9D7 treatment (data not shown). The rat isotype control mAb GL113 had no effect on TNF-α and IL-1β produced by the synovial membranes (Fig. 3). It was of interest, in view of the original function of IL-10 described (cytokine synthesis inhibition) that anti-IL-10 treatment resulted in detectable levels of IFN-γ in two RA membranes (561 and 1,050 pg/ml, respectively) out of seven 24-h cultures that were assayed. In contrast, in the control cultures, or isotype control antibody–treated cultures, IFN-γ levels were undetectable (<100 pg/ml).

Exogenous Addition of IL-10 to Synovial Membrane Cultures Inhibits Proinflammatory Cytokine Production. The levels of IL-10 produced by the synovium membrane cultures were all, with the exception of one (5 ng/ml), <2 ng/ml. We therefore investigated whether the IL-10/IL-10 receptor system in these cultures was saturated, and whether addition of exogenous IL-10 would have any effect on monokine produc-

tion. Addition of 10 ng/ml of IL-10 resulted in marked decrease in cytokine production. At 24 h, TNF-α was reduced to 49 $\pm$ 6% and IL-1β to 64 $\pm$ 11% of control (p <0.01 and p = 0.02, respectively), and this reduction was even more marked by day 5 (TNF-α to 42 $\pm$ 9% and IL-1β to 51 $\pm$ 9% of control) (Table 2, Figs. 3 and 4). Exogenous IL-10 did not reduce TNF-α mRNA levels at 24 h in two RA cultures in which the IL-10 protein levels were reduced by twofold (data not shown). IL-6 levels were not affected by

Table 1. *Effect of Anti-IL-10 Treatment on Cytokine Production by Synovial Membrane Cultures*

		TNF-α	IL-1β	IL-6	IL-8
		(pg/ml)	*(pg/ml)*	*(ng/ml)*	*(ng/ml)*
	Control	1,161 ± 370 (n = 10)*	1,549 ± 659 (n = 9)	151 ± 61 (n = 7)	242 ± 75 (n = 7)
Day 1	Anti-IL-10	2,236 ± 649	2,899 ± 991	160 ± 61	271 ± 76
	Isotype control	1,168 ± 380	1,542 ± 660	142 ± 56	258 ± 79
	Control	301 ± 133 (n = 8)	1,630 ± 1,159 (n = 6)	264 ± 83 (n = 7)	473 ± 148 (n = 7)
Day 3	Anti-IL-10	1,025 ± 505	2,513 ± 1,665	280 ± 80	514 ± 142
	Isotype control	295 ± 134	1,761 ± 1,284	276 ± 83	510 ± 161
	Control	185 ± 100 (n = 6)	472 ± 182 (n = 5)	269 ± 66 (n = 6)	438 ± 100 (n = 6)
Day 5	Anti-IL-10	1,024 ± 781	1,170 ± 516	272 ± 59	535 ± 121
	Isotype control	173 ± 92	381 ± 210	282 ± 73	419 ± 95

* Mean ± SE, n = number of cultures.

exogenous IL-10 addition to the RA cultures. IL-8 production was only affected by day 5, reduced to 68 ± 7% of control (p = 0.05). (Table 2, Figs. 3 and 4).

Blocking of TNF-α and IL-1 Downregulates IL-10 Production by the Synovial Membrane Cultures. IL-1RA (10 μg/ml) consistently inhibited IL-10 production, IL-10 was reduced at 24 h to 68 ± 8%, at day 3 to 57 ± 7%, and at day 5 to 50 ± 13% of control. (data not shown). Anti-TNF-α neu-

tralizing mAb antibody A2 (5 μg/ml) also resulted in a decrease, albeit lesser, in IL-10 levels produced by the synovial membrane cultures at 24 h to 80 ± 8% (p <0.05), at day 3 to 76 ± 8%, and at day 5 to 75 ± 11% of control (data not shown). However, anti-TNF-α and IL-1RA, when used in combination, showed no additive or synergistic effect (data not shown), suggesting that TNF-α and IL-1 regulate IL-10 levels by a common pathway.
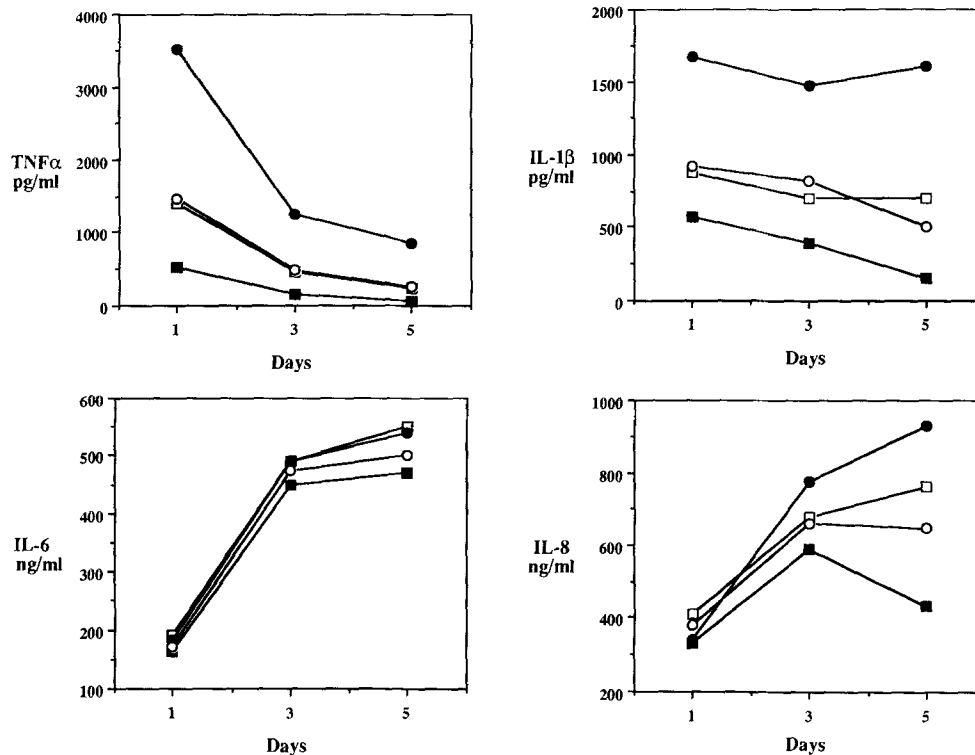

Figure 3. RA synovial membrane cultures: effect of neutralizing endogenously produced IL-10 and of the addition of exogenous IL-10. Representative experiment showing TNF-α, IL-1β, IL-6, and IL-8 levels produced by RA synovial membrane culture SM1376. Synovial membrane culture was treated with either neutralizing rat monoclonal anti-IL-10 antibody 9D7 (2 μg/ml), isotype-matched control rat monoclonal GL113, or 10 ng/ml of rIL-10 for 24, 72, and 120 h. (Similar studies have been performed on 5–10 membranes; see Table 1). (—□—) Control; (—●—) anti-IL-10; (—O—) isotype control; (—■—) IL-10.
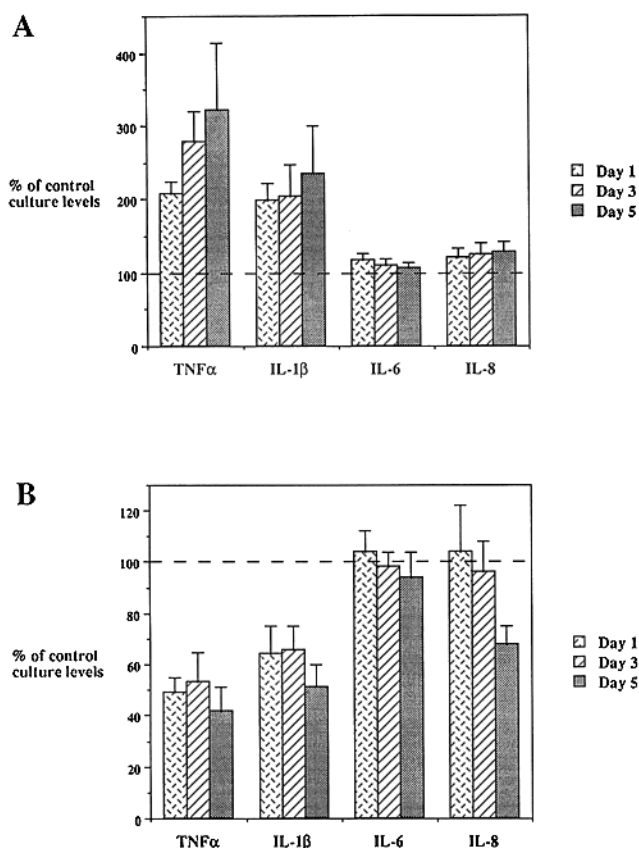
A

B

Figure 4. Neutralization of spontaneously produced IL-10 and addition of exogenous IL-10 in RA synovial membrane cultures. Pooled data from 5–10 RA synovial membrane cultures. (*A*) Neutralization of endogenous IL-10 with the 9D7 neutralizing rat monoclonal anti-IL-10 antibody (2 μg/ml) results in increased levels of TNF-α and IL-1β, but not IL-6 and IL-8. (*B*) Exogenous rIL-10 (10 ng/ml) decreases TNF-α and IL-1β levels in RA cultures. (*Dashed lines*) 100% of control untreated cultures.

Discussion

As judged by quantity, the major participants in the cytokine network of rheumatoid synovitis are the monocyte and fibroblast-derived inflammatory cytokines, including TNF-α, IL-1β, IL-6, GM-CSF, and IL-8. These are produced spon-

taneously in the diseased synovial membrane, and are considered to play an important role in the pathophysiology of the chronic synovitis (33–35). Recently, TGF-β and cytokine inhibitors such as IL-1RA and soluble TNF receptors (both p55 and p75 sTNFR) have also been found in synovial fluids and shown to be produced by synovial membrane cultures from RA patients (8, 10–12, and Brennan, F. M., manuscript in preparation), and presumably represent a homeostatic attempt to contain the inflammation. The recent description of the potent antiinflammatory properties of IL-10 (23, 25) prompted us to investigate whether IL-10 was produced by the synovial membrane in RA, and if it played a regulatory role in the cytokine network in synovitis.

In this study, IL-10 was detected at both the mRNA and protein level in RA synovial membranes. PCR of reverse transcribed total RNA extracted directly from synovial membrane tissue showed that human IL-10 mRNA is present in these membranes. Biologically significant quantities of IL-10 were spontaneously produced in cell suspension cultures of RA synovial membranes, and persisted for the 5-d duration of the synovial cultures. IL-10 protein was also demonstrated in RA and OA synovial tissues by immunohistology, but not in the normal synovium. IL-10 immunostaining showed that monocytes in the synovial membrane lining layer, and T cells in the mononuclear aggregates stained for IL-10. IL-10 however could not be detected in RA and OA synovial fluids by ELISA.

The IL-10 produced spontaneously by the RA cultures was shown by neutralization experiments to play an important regulatory role in RA in the synovial membrane cultures. Blocking IL-10 in these cultures with a neutralizing mAb resulted in a two- to threefold increase in the production of TNF-α and IL-1β. This result probably has pathogenetic consequences, as TNF-α and IL-1β are implicated in the pathology of RA, since they can both induce bone resorption and cartilage destruction, and can stimulate PGE2 release and collagenase production (33), and blocking TNF-α in RA patients with a high affinity neutralizing chimeric anti-TNF-α antibody has been shown to result in clinical benefit (36). The suppression of these cytokines by IL-10 thus appears to

Table 2. *Effect of IL-10 Treatment on Cytokine Production by Synovial Membrane Cultures*

		TNF-α	IL-1β	IL-6	IL-8
		(pg/ml)	(pg/ml)	(ng/ml)	(ng/ml)
Day 1	Control	1,145 ± 418 (n = 9)*	1,708 ± 850 (n = 7)	135 ± 63 (n = 7)	227 ± 77 (n = 7)
	IL-10	563 ± 210	1,098 ± 725	125 ± 56	200 ± 69
Day 3	Control	301 ± 133 (n = 8)	1,563 ± 1,168 (n = 6)	248 ± 86 (n = 7)	479 ± 147 (n = 7)
	IL-10	106 ± 36	1,317 ± 1,041	242 ± 83	449 ± 135
Day 5	Control	185 ± 100 (n = 6)	407 ± 147 (n = 5)	241 ± 65 (n = 6)	438 ± 100 (n = 6)
	IL-10	55 ± 24	204 ± 89	231 ± 60	308 ± 76

* Mean ± SE, n = number of cultures.

be of potential importance in diminishing the synovial inflammation. The observation that IL-10 upregulates the production of IL-1RA and soluble TNF receptors by human monocytes (Joyce, D., D. Gibbons, P. Green, M. Feldmann, and F. M. Brennan, manuscript submitted for publication) also supports this concept. There is thus increasing evidence that in RA, IL-10, IL-1RA, and soluble TNF receptors are constituents of an important antiinflammatory response. It is noteworthy that blocking IL-10 in the RA cultures had no effect on the IL-6 and IL-8 levels, which was somewhat unexpected, since IL-10 inhibits both IL-6 and IL-8 production by LPS-activated human monocytes (25). A possible explanation may lie in the fact that in RA synovial membrane cultures another important source of these two cytokines are fibroblasts and endothelial cells (6, 37, 38). In our laboratory, IL-10 has failed to have any significant effect on synovial fibroblast IL-6, IL-8 or PGE$_2$ production (Butler, D., F. M. Brennan, and M. Feldmann, unpublished observation).

Exogenous addition of IL-10 to RA synovial membrane cultures resulted in a two- to threefold decrease of TNF-α and IL-1β production, noticeable by 24 h, the first assay point. However, IL-8 was only affected by day 5 in culture, and this may reflect an indirect effect of IL-10 on IL-8 expression subsequent to downregulating IL-1 and TNF. As mentioned above, IL-10 induces soluble TNF receptors and IL-1RA production by human monocytes, and thus the net effect of IL-10 on the biological activity of TNF-α and IL-1 in the synovial membranes would be expected to be even greater than that resulting exclusively from downmodulation of TNF-α and IL-1 protein levels. The above results also indicate that the IL-10/IL-10 receptor system is not saturated in these RA cultures. This implies that there is a "relative deficit" in IL-10 production in RA, which suggests the possibility of a novel therapeutic strategy for RA by augmenting this interaction.

TGF-β and IL-4 are both cytokines with potent antiinflammatory properties (39, 40), and therefore could possibility be antiinflammatory agents of use in RA. However in experimental animal models of arthritis, TGF-β treatment has yielded conflicting results (41, 42). In a report using RA synovial membrane explants cultured for 10 d, IL-4 was shown to inhibit IL-6, IL-1β, and TNF-α (43). However, the failure to characterize the cellular sources of cytokine production during the 10-d period of culture limits the interpretation of these results. In contrast to IL-10, TGF-β and IL-4 have both failed in our hands to inhibit inflammatory cytokine production in RA synovial membrane cell suspension cultures (12, 44). Suppression of LPS induced PBMC monokine production by TGF-β, and IL-4 depends on the pretreatment of these cells with these cytokines (12, 45). Thus, the inability of TGF-β and IL-4 to have any effect in RA cultures could be due to the chronic activation of monocytes. IL-10 is therefore the first cytokine shown to have an inhibitory effect on RA synovial membrane culture cytokine synthesis.

IL-1 and TNF-α were found to participate in the regulation of IL-10 production by the RA synovial membrane. This is in concordance with our recent data which indicate that IL-1, and to a lesser extent TNF-α, are both involved in the regulation of IL-10 production by LPS-activated human monocytes (Katsikis, P., unpublished observation). In the RA cultures it is possible that the anti-TNF-α-mediated downregulation of IL-10 may be the indirect result of its inhibition of IL-1 (46). This possibility is also supported by the lack of an additive or synergistic effect of anti-TNF-α with IL-1RA, when used in combination in the RA synovial membrane cultures.

An interesting paradox in the studies of the pathogenesis of RA which has been highlighted by Firestein et al. (13, 14), has been the failure to detect abundant T cell cytokines, although activated T cells as judged by cell surface markers are present in the synovium (15, 16). However, we have previously reported the detection of T cell cytokine mRNA (IL-2 and IFN-γ) in the RA synovium (17), suggesting that an inhibitor may be present and responsible for the low protein detection. Cloning of these T cells has revealed a Th1 cytokine profile (21, and Cohen, S., unpublished observations), making IL-10 a candidate for such an inhibitor, since IL-10 has been reported to be a potent inhibitor of Th1 cytokine production (23, 24, 27). Preliminary data shows that in two out of seven RA membranes studied, IFN-γ which was undetectable in control cultures, could be measured at significant concentrations after the neutralization of endogenous IL-10. Clearly, more rheumatoid membranes need to be studied, but the data already obtained suggest that IL-10 may be the (or one of the) factor responsible for the "elusiveness" of T cell–derived cytokines. T cell proliferative responses are also impaired in RA (18–20). TGF-β has been implicated as a major but not the sole immunosuppressive factor in RA synovial fluids (20). The direct inhibitory effect of IL-10 on T cell proliferation and IL-2 production (48, 49), and its indirect effect via APCs (24, 27, 50) raises the possibility that IL-10 also contributes to the low T cell proliferative responses in RA. This, however, remains to be established.

The data reported here indicate that in RA, IL-10 plays an important role in the cytokine network, by inhibiting the cytokine production of both monocytes and T cells. In the RA synovium, IL-10 regulates the production of TNF-α/IL-1 and vice-versa. In rheumatoid synovium, IL-10 and IFN-γ may be reciprocally regulated also, IL-10 possibly inhibiting IFN-γ production, and IFN-γ regulating IL-10 (51). Although most of the properties of IL-10 suggest that it has a negative regulatory role, recent observations indicate that IL-10 may not be a general inhibitor of immune responses, since it can stimulate monocyte expression of the Fc receptor for monomeric IgG, FcγRI (CD64), and enhance antibody-dependent cellular cytotoxicity (52). Another recent report (53) indicates that IL-10 might be a potent recruitment signal (or inducer of) for leukocyte migration in vivo. Thus, animal studies are needed to establish whether IL-10 treatment in vivo may modulate the arthritis. Recent reports of IL-10 treatment of experimental endotoxemia in mice have shown that in vivo administration of IL-10 significantly reduces TNF-α levels and mortality (54, 55), suggesting that IL-10 could be an effective antiinflammatory in vivo.

IL-10 was also detected in OA synovial membrane cultures and biopsies. Monokine-derived proinflammatory cytokines such as TNF-α, IL-1, and GM-CSF (9, 46) and antiinflammatory products such as soluble TNF receptors and IL-1RA (7, 8, 10, 11) are all produced by the OA synovial membrane macrophage. The role of proinflammatory cytokines in the pathogenesis of osteoarthritis, which is considered a nonautoimmune but inflammatory disease, has not yet been studied in detail. It seems that in OA as in RA, IL-10 may be produced alongside soluble TNF receptors and IL-1RA to limit the deleterious effect of proinflammatory cytokines.

In this study we have shown that both mRNA and pro-tein for IL-10 are present in the RA and OA synovium, and that IL-10 is an important participant in the cytokine network of the rheumatoid synovial membrane, playing an immunoregulatory role in inflammatory and possibly T cell cytokine production. Reciprocally, IL-10 itself is regulated by IL-1 and TNF-α, thus IL-10 appears to be an important component of the complex cytokine network of rheumatoid synovitis. Finally, exogenous IL-10 was shown to inhibit both TNF-α and IL-1β production by RA synovial membrane cultures. These findings raise the possibility of new therapeutic strategies for the treatment rheumatoid arthritis.

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