Research article

A novel mechanism for the regulation of IFN-γ inducible protein-10 expression in rheumatoid arthritis

Ryosuke Hanaoka[1], Tsuyoshi Kasama[1], Mizuho Muramatsu[1], Nobuyuki Yajima[1], Fumitaka Shiozawa[1], Yusuke Miwa[1], Masao Negishi[1], Hirotsugu Ide[1], Hideyo Miyaoka[2], Hitoshi Uchida[3] and Mitsuru Adachi[1]

[1]Division of Rheumatology and Clinical Immunology, First Department of Internal Medicine, Showa University School of Medicine, Tokyo, Japan
[2]Department of Orthopedics, Showa University School of Medicine, Tokyo, Japan
[3]Department of Orthopedics, Furukawabashi Hospital, Tokyo, Japan

Corresponding author: Tsuyoshi Kasama (e-mail: tkasama@med.showa-u.ac.jp)

Abstract

Chemokines play an essential role in the progression of rheumatoid arthritis (RA). In the present study we examined the expression and regulatory mechanisms of IFN-γ inducible protein (IP)-10 in RA synovitis. RA synovial fluid contained greater amounts of IP-10 than did synovial fluid from patients with osteoarthritis. Immunolocalization analysis indicated that IP-10 was associated mainly with infiltrating macrophage-like cells, and fibroblast-like cells in the RA synovium. The interaction of activated leukocytes with fibroblast-like synoviocytes resulted in marked increases in IP-10 expression and secretion. Moreover, induction of IP-10 was mediated via specific adhesion molecules, as indicated by the finding that both anti-integrin (CD11b and CD18) and intercellular adhesion molecule-1 antibodies significantly inhibited IP-10 induction. These results suggest that IP-10 expression within inflamed joints appears to be regulated not only by inflammatory cytokines but also by the physical interaction of activated leukocytes with fibroblast-like synoviocytes, and that IP-10 may contribute to the recruitment of specific subpopulations of T cells (Th1 type) from the bloodstream into the synovial joints.

Keywords: adhesion molecule, fibroblast, IFN-γ inducible protein-10, rheumatoid arthritis

Introduction

The pathology of rheumatoid arthritis (RA) is characterized by the infiltration of several inflammatory cells into both the pannus and the joint fluid, and by subsequent tissue destruction. Chemokines, as well as other inflammatory mediators, appear to play key roles in the pathogenesis of RA, and the co-ordinated production of chemokines and proinflammatory cytokines is probably important in the orchestration of the inflammatory responses observed in patients with RA [1–4].

Chemokines belong to a gene superfamily of chemotactic cytokines that share substantial homology with four conserved cysteine amino acid residues [5–7]. The CXC family of chemokines (e.g. interleukin-8, growth-related oncogene, and epithelial cell-derived neutrophil attractant-78), in which the first two cysteines are separated by another amino acid residue, is chemotactic for neutrophils and T cells. The CC chemokine family (e.g. macrophage inflammatory protein-1, macrophage chemoattractant protein-1, and RANTES [regulated upon activation, normal T-cell expressed and secreted]), in which the first two cysteine residues are juxtaposed, is chemotactic for monocytes and subpopulations of T cells. IFN-γ inducible protein-10 (IP-10), a member of the CXC chemokine family, is expressed and secreted by monocytes, fibroblasts, and endothelial cells after stimulation with IFN-γ [5,8], and has important roles in the migration of

FLS = fibroblast-like synoviocyte; ICAM = intercellular adhesion molecule; IFN = interferon; IP-10 = IFN-γ inducible protein-10; OA = osteoarthritis; PMN = polymorphonuclear neutrophil; PCR = polymerase chain reaction; RA = rheumatoid arthritis; RT = reverse transcription; SF = synovial fluid; Th = T-helper (cell); TNF = tumor necrosis factor.

T cells into inflamed sites. It also furthers the regression of angiogenesis, in contrast with interleukin-8 [9–12].

A Th1/Th2 cytokine imbalance with a predominance of Th1 cytokines, including IFN-γ, is suggested to be of pathogenetic importance in RA [13–15]. The Th1 phenotype expresses certain chemokine receptors, including CXCR3 and CCR5 [16,17]. IP-10, a CXCR3 ligand, may be expressed in the inflamed synovium of RA, and appears to play an important role in the recruitment of Th1-type cells into the joint. Thus, the aim of the present study was to examine the regulatory mechanisms of IP-10 expression by synovial inflammatory cells and fibroblasts, especially by specific cell–cell interactions in rheumatoid synovitis.

Materials and methods
Reagent preparation
Completed medium consisted of Dulbecco's modified Eagle's medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 2 mmol/l L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% heat-inactivated fetal bovine serum (Gibco Laboratories, Grand Island, NY, USA). Monoclonal and biotinylated polyclonal antibodies against human IP-10 and recombinant human IP-10 were purchased from Genzyme/Techne (Cambridge, MA, USA). Monoclonal antibodies against human CD11b and CD18 were purchased from Ancell Corporation (Bayport, MN, USA), and those against intercellular adhesion molecule (ICAM)-1 were purchased from R & D Systems (Minneapolis, MN, USA).

Isolation and culture of peripheral blood and synovial fluid monocytes and polymorphonuclear neutrophils
RA or osteoarthritis (OA) synovial fluid (SF) was obtained from knee punctures in 32 RA patients and 10 OA patients. No patient received more than 5 mg oral prednisolone/day or intra-articular injections of glucocorticoids within 1 month of SF sample aspiration.

RA SF monocytes and polymorphonuclear neutrophils (PMNs) were obtained from knee punctures in 23 RA patients. Normal peripheral blood monocytes and PMNs were obtained from 10 age-matched and sex-matched healthy individuals. PMNs were isolated by centrifugation on a Ficoll-Hypaque (Pharmacia LKB Biotechnology Inc, Piscataway, NJ, USA) density gradient, after which they were separated from erythrocytes by lysing the erythrocytes in a solution of 0.15 mol/l NH_4Cl, 0.01 mol/l $NaHCO_3$, and 0.01 mol/l tetra EDTA. The recovered PMNs (purity 96–98%, viability 98%) were washed three times and resuspended at a density of 5×10^6 cells/ml in completed medium. The mononuclear cells, isolated by centrifugation on a Ficoll-Hypaque, were then separated by centrifugation on a density gradient (1.068 g/ml; Nycodenz, Nycomed AS Oslo, Norway), as described previously [18,19]. The isolated monocytes were washed, cytospun onto a glass slide, stained with Diff-Quik (Baxter, McGaw, IL, USA), and differentially counted using nonspecific esterase staining. The final cell preparations contained more than 75–80% monocytes, based on their morphology and nonspecific esterase staining; their viability was greater than 98%, as assessed by trypan blue exclusion. The recovered monocytes were washed three times and resuspended at a density of 1×10^6 cells/ml in completed medium.

All human experiments were performed in accordance with protocols approved by the Human Subjects Research Committee at our institution, and informed consent was obtained from all patients and volunteers.

Preparation of fibroblast-like synoviocytes
Synovial tissues were obtained from seven RA patients (five women and two men; mean age 63.5 years, range 48–72 years) with active synovitis, as determined by serum C-reactive protein levels (mean 3.3 mg/dl), who fulfilled the 1987 American College of Rheumatology criteria for RA [20], all of whom underwent joint replacement surgery. Synovial membrane cell suspension cultures were prepared by collagenase and DNase digestion of minced membranes, as described previously [21]. Isolated fibroblast-like synoviocytes (FLSs) were cultured in completed medium in 75-mm tissue culture flasks. The cells were used from passages 3 through to 10, when they morphologically resembled FLSs and were negative for Mo-1 and major histocompatibility complex class II, indicating the absence of type A or 'macrophage-like' synoviocytes.

Coculturing synovial fluid monocytes or polymorphonuclear neutrophils with fibroblast-like synoviocytes
SF monocytes or PMNs were layered onto unstimulated semiconfluent FLS monolayers in 48-well plates (Nalge-Nunc International, Tokyo, Japan), and culture supernatants were collected at selected times thereafter. In some experiments, a transwell membrane (pore size 0.45 µm; Becton Dickinson, Bedford, MA, USA) was used to separate the two cell groups, whereas in others anti-integrin antibodies or adhesion molecules were added to the cocultures.

Assay of cytokine levels using specific enzyme-linked immunosorbent assay
IP-10 was specifically quantified using the double-ligand enzyme-linked immunosorbent assay method, in a modification to a previously reported assay [22]. Monoclonal murine antihuman IP-10 (1 µg/ml) served as the primary antibody, and biotinylated polyclonal goat anti-IP-10 (0.1 µg/ml) served as the secondary antibody. The sensitivity limit for the IP-10 enzyme-linked immunosorbent assay was approximately 50 pg/ml.

Immunohistochemistry

Cell-associated IP-10 was visualized immunohistochemically in a modification to a previously reported assay [22]. Briefly, FLSs were grown to near confluence in an 8-well LabTeK chamber slide (Nalge Nunc International), and then incubated for 24 hours with or without either monocytes and PMNs. The slides were then incubated with polyclonal rabbit anti-IP-10 antibody (1:500 dilution; purchased from PeproTech EC, London, UK) or in preimmune rabbit IgG. Biotinylated goat antirabbit IgG (1:20; Biogenex Laboratories Inc, Burlingame, CA, USA) and peroxidase-conjugated streptavidin served as second and third reagents, respectively.

Isolation of total RNA and reverse transcription polymerase chain reaction

Total cellular RNA was isolated as previously described [22]. Briefly, samples were dispersed in a solution of 25 mmol/l Tris (pH 8.0) that also contained 4.2 mol/l guanidine isothiocyanate, 0.5% sarkosyl, and 0.1 mol/l 2-mercaptoethanol. The RNA was further extracted with chloroform-phenol and then alcohol precipitated.

Semiquantitative reverse transcription (RT)-PCR was performed as previously described [23]. Briefly, 2-µg samples of total RNA were reverse transcribed using M-MLV reverse transcriptase (GIBCO BRL). The primers used in the PCR reaction were 5′-TGA-CTC-TAA-GTG-GCA-TTC-AAG-G (sense) and 5′-GAT-TCA-GAC-ATC-TCT-TCT-CAC-CC (antisense) for IP-10 [24], and 5′-GTG-GGG-CGC-CCC-AGG-CAC-CA (sense) and 5′-CTC-CTT-AAT-GTC-ACG-CAC-GAT-TTC (antisense) for β-actin, which served as an internal control. The amplification buffer contained 50 mmol/l KCl, 10 mmol/l Tris-HCL (pH 8.3), and 1.5 mmol/l $MgCl_2$. Specific oligonucleotide primer was added (200 ng/sample) to the buffer, along with 1 µl of the reverse transcribed cDNA samples. The cDNA was amplified after determining the optimal number of cycles. The mixture was first incubated for 5 min at 94°C; it was then cycled 35 times at 95°C for 30 s and at 58°C for 60 s, and elongated at 72°C for 75 s. This format allowed optimal amplification with little or no nonspecific amplification of contaminating DNA. The amplified products were separated on 2% agarose gels containing 0.3 µg/ml ethidium bromide, and were visualized and photographed using ultraviolet transillumination.

Statistical analysis

Data were analyzed on a Power Macintosh computer using a statistical software package (Statview 4.5; Abacus Concept Inc, Berkeley, CA, USA) and expressed as mean±SEM. Groups of data were compared by analysis of variance; the means of groups with variances that were determined to be significantly different were then compared using Student's *t*-test for comparison of the means of multiple groups. $P < 0.05$ was considered statistically significant.

Results

### IP-10 expression in rheumatoid arthritis synovium

We first investigated the concentrations of IP-10 in SF from patients with RA (*n*=32) or OA (*n*=10) using enzyme-linked immunosorbent assay. As shown in Fig. 1, the IP-10 concentrations in the SF from patients with RA were significantly greater than those in the SF of patients with OA (RA 6.05±0.86 ng/ml versus OA 2.32±1.28 ng/ml), which is in agreement with previous findings [25]. We next examined the *in situ* expression of IP-10 in RA synovial tissue. Immunolocalization indicated that IP-10 was associated mainly with infiltrating macrophage-like and fibroblast-like cells of chronically inflamed synovial tissues (Fig. 2); there was little or no nonspecific staining in tissue sections incubated with control IgG (Fig. 2).

Production of IP-10 through the interaction of fibroblast-like synoviocytes and leukocytes

We next assessed the induction of IP-10 expression mediated by the interaction of FLSs and leukocytes (monocytes or PMNs). When plated alone, unstimulated FLSs and leukocytes derived from either RA SF or peripheral blood secreted very small amounts of IP-10 (Fig. 3). On the other hand, when unstimulated RA SF monocytes, and to a lesser extent RA SF PMNs, were cocultured with FLSs, significantly greater amounts of IP-10 (FLS monocytes 5698.0±865.0 pg/ml, FLS PMNs 417.0±48.5 pg/ml) were secreted into the supernatant (Fig. 3). In addition, in order to determine whether the augmented production of IP-10 was specific to leukocytes in the RA SF, we tested the capacity of FLSs and either peripheral blood monocytes or PMNs obtained from healthy individuals to produce IP-10. Although enhanced production of IP-10 was observed in RA FLS peripheral blood leukocyte cocultures, the enhancement was less pronounced than in RA FLS SF leukocyte cocultures (Fig. 3). In addition, IFN-γ and, to a lesser extent, tumor necrosis factor (TNF)-α are potent inducers of IP-10 [8,24,26,27]. Therefore, IFN-γ and TNF-α were neutralized with monoclonal antibodies (obtained from Chemicon International, Temecula, CA, USA, and from R & D Systems, respectively) in order to eliminate the effects of newly synthesized IFN-γ and TNF-α by *in situ* cell–cell interactions. IP-10 concentrations in the medium with FLS and leukocyte coculture in the presence or absence of either neutralizing antibody were measured, and no significant stimulatory or inhibitory effects were observed (Fig. 3).

Because FLS–lymphocyte interactions induce inflammatory mediators [28], it was important to rule out contaminating lymphocytes as a major source of IP-10 in the FLS–monocyte interactions. We examined the effect of mononuclear lymphocytes on IP-10 secretion in FLS lymphocytes. SF monocytes were depleted from mononuclear cell suspension by adhesion to a plastic dish for 2 hours. Although monocyte-depleted nonadherent lymphocytes

Figure 1
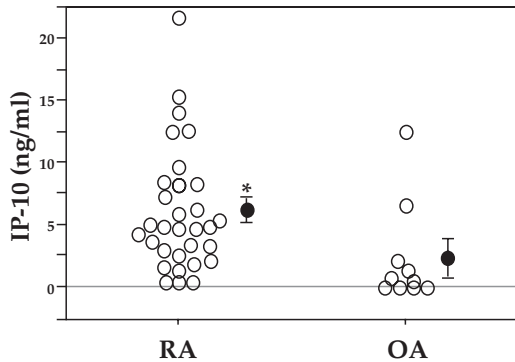
IFN-γ inducible protein-10 (IP-10) concentrations in synovial fluid (SF). SF was obtained from patients with rheumatoid arthritis (RA; $n = 32$) or osteoarthritis (OA; $n = 10$). IP-10 in SF was assayed using enzyme-linked immunosorbent assay. Each point represents an individual patient. Data are expressed as the mean (ng/ml) ± SEM. *$P < 0.01$, versus OA SF.

Figure 2
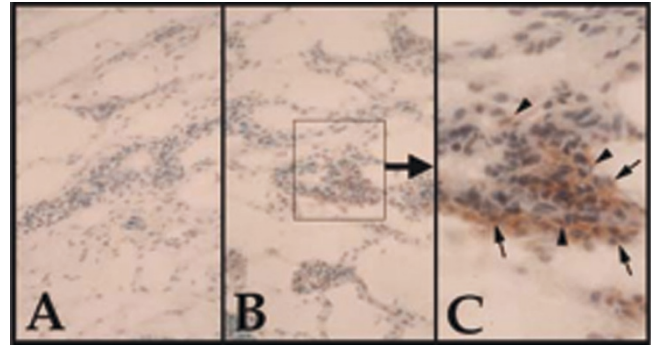
Immunohistochemical localization of IFN-γ inducible protein-10 (IP-10) in rheumatoid arthritis (RA) synovium. The sections were stained with **(A)** control IgG, and **(B and C)** with antibodies against IP-10. Panel C shows a magnification of the boxed area in panel B, demonstrating significant presence of cell-associated IP-10 antigen in macrophage-like cells (arrows) and in fibroblast-like cells (arrowheads). (Original magnifications: panels A and B 200×; panel C 400×.)

Figure 3
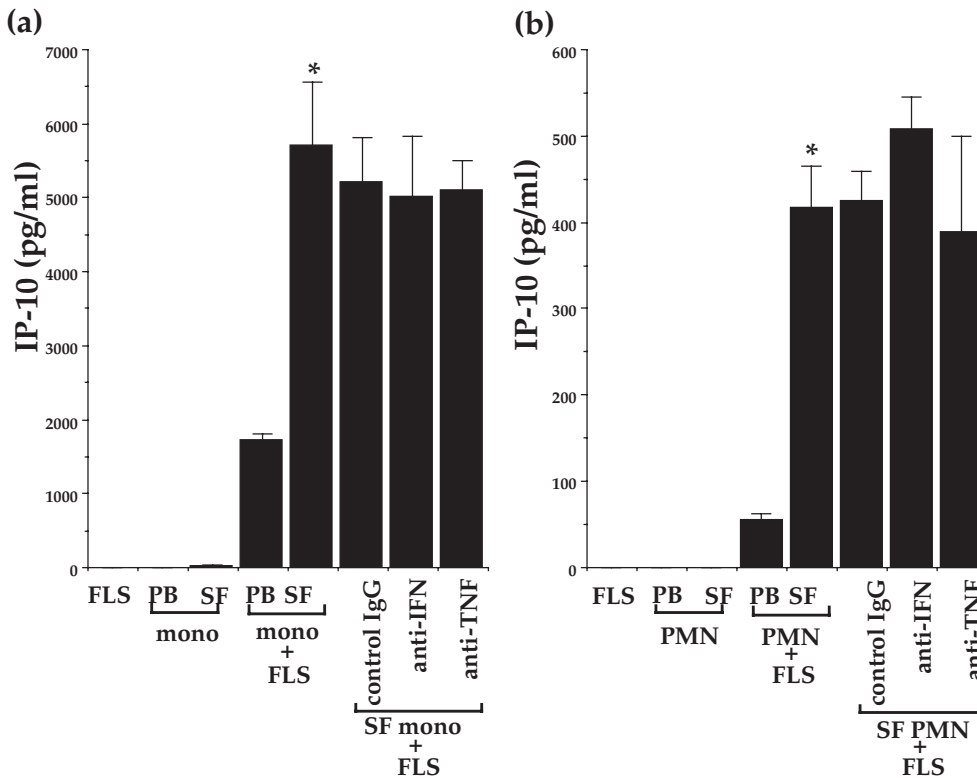
Secretion of IFN-γ inducible protein-10 (IP-10) mediated by the interaction of fibroblast-like synoviocytes (FLSs) and leukocytes. **(a)** Monocytes (mono; $5 \times 10^5/0.5$ ml per well) or **(b)** polymorhonuclear neutrophils (PMNs; $2.5 \times 10^6/0.5$ ml per well) obtained from either synovial fluid (SF) or peripheral blood (PB) were layered onto unstimulated semiconfluent rheumatoid arthritis (RA) FLS monolayers in 48-well plates, after which monoclonal antibodies (10 μg/ml) against IFN-γ or tumor necrosis factor (TNF)-α, and control mouse IgG (10 μg/ml) were added. Supernatants were collected at 24 hours after coculture, and then IP-10 was measured using enzyme-linked immunosorbent assay. Data represent the mean (pg/ml) ± SEM of seven independent experiments that were performed using three different RA fibroblasts and seven different RA SF leukocytes or normal PB leukocytes. *$P < 0.05$, versus cocultures with PB leukocytes.

Figure 4
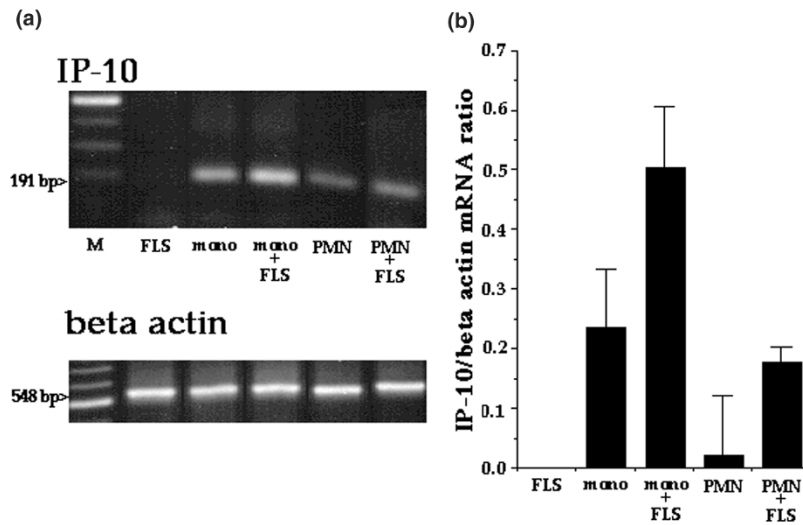
Reverse transcription (RT)-PCR analysis of IFN-γ inducible protein-10 (IP-10) mRNA expression induced by the interaction of synovial fluid (SF) leukocytes and rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLSs). SF monocytes (mono) or PMNs were layered onto RA FLS monolayers. Total RNA was isolated 12 hours later, after which RT-PCR was performed. **(a)** Representative expression of IP-10 mRNA; expression of β-actin mRNA served as an internal control. Lane M contains molecular weight markers (100 base pair [bp] ladder). **(b)** IP-10 mRNA expression was quantified and normalized to β-actin as the IP-10/β-actin ratio. Data are expressed as means ± SEM for three independent experiments that were performed using three different RA fibroblasts and three different RA SF leukocytes.

(5×10^5/ml; monocyte contamination ≤5%) were added to unstimulated FLS cultures, stimulatory effects of lymphocytes on IP-10 secretion were not observed (FLS <50 pg/ml, monocyte-depleted lymphocyte <50 pg/ml, FLS plus monocyte-depleted lymphocyte 146.0 ± 66.2 pg/ml), suggesting that contaminated lymphocytes were not involved in the increased IP-10 secretion.

Steady-state expression of IP-10 mRNA in the cocultures was assessed using RT-PCR. Consistent with the expression of IP-10 protein, RT-PCR revealed that substantial steady-state expression of IP-10 mRNA was significantly upregulated in either monocytes or PMNs cocultured with FLSs (Fig. 4). Immunohistochemical analysis confirmed that IP-10 was upregulated in leukocytes when cocultured with FLSs (Fig. 5). Although small amounts of IP-10 antigen were present in both unstimulated leukocytes and FLSs, markedly greater amounts were observed in leukocytes cocultured with FLSs, indicating that the major cellular sources of IP-10 are probably either monocytes or PMNs during coculture.

Involvement of integrin–ICAM-1 ligand interactions in the upregulation of IP-10 secretion by cocultures

In order to gain a better understanding of the mechanism whereby the interaction between leukocytes and FLSs induces IP-10 expression in the leukocytes, the two cell groups were cultured together in a chamber in which they were separated by a transwell membrane (pore size 0.45 μm) that allowed passage of soluble factors but pre-

Figure 5
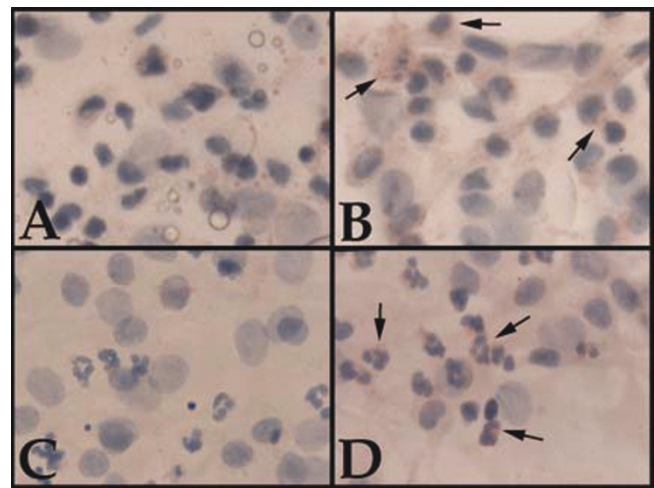
Representative photomicrographs showing the immunohistochemical localization of antigenic IFN-γ inducible protein-10 (IP-10) within interacting leukocytes and fibroblast-like synoviocytes (FLSs). After 24 hours of incubation, the cells were labeled with anti-IP-10 antibodies. Rheumatoid arthritis FLSs plus **(A and B)** monocytes or **(C and D)** polymorphonuclear neutrophils. Panels A and C, stained by control IgG; panels B and D, stained by anti-IP-10 antibody. Panels B and D demonstrate a significant presence of cell-associated IP-10 antigen (arrows) in leukocytes. (Original magnification: 500×.)

vented physical contact between the cell groups. As shown in Table 1, augmentation of IP-10 secretion was

Table 1

**Effects of a transwell membrane filter on IFN-γ inducible protein-10 secretion**

Conditions	IP-10	% Inhibition
FLS	11.0 ± 7.0	ND
Monocyte	16.6 ± 5.7	ND
FLS + monocyte	5698.1 ± 865.9	–
FLS + monocyte (FLS-sup)	16.7 ± 6.7**	99.7
FLS + monocyte (monocyte-sup)	49.0 ± 13.0**	99.1
PMN	0	ND
FLS + PMN	417.4 ± 48.5	–
FLS + PMN (FLS-sup)	9.8 ± 4.1**	97.7
FLS + PMN (PMN-sup)	26.3 ± 16.0**	93.7

Synovial fluid monocytes or polymorphonuclear neutrophils (PMNs) were layered onto fibroblast-like synoviocyte (FLS) monolayers in the presence or absence of a transwell membrane (pore size 0.45 μm). After 24 hours of incubation, the supernatants were collected from the cocultures and from the FLS monolayer (FLS-sup) and leukocyte suspension (monocyte-sup or PMN-sup) sides of the transwell membrane, and assayed using enzyme-linked immunosorbent assay. Values represent the mean (pg/ml) ± SEM of three independent experiments, which were performed using two different rheumatoid arthritis fibroblasts and three different rheumatoid arthritis synovial fluid leukocytes. Percentage inhibition was calculated by subtracting the IFN-γ inducible protein-10 (IP-10) contents obtained with either FLS-sup or monocyte-sup/PMN-sup from those with cocultures and dividing by the IP-10 contents obtained with cocultures (as 100%). **$P < 0.01$, versus the respective coculture. ND, not done.

completely blocked by the transwell membrane, suggesting that direct cell–cell contact is important for this process. Fibroblasts interact with monocytes or PMNs via pathways that are mediated by adhesion molecules, including the ICAM-1–integrin pathway [19,29]. The potential role of these molecules in FLS–leukocyte interactions was investigated by assessing the capacity of specific antibodies to inhibit IP-10 production by cocultured leukocytes. Although control mouse IgG had no significant effects on IP-10 secretion, the addition of anti-CD11b, anti-CD18, or anti-ICAM-1 monoclonal antibody (5 μg/ml) to either FLS–monocyte or FLS–PMN coculture reduced production of IP-10 by 59.1%, 56.5%, and 53.0%, and by 79.0%, 87.4%, and 54.0%, respectively (Fig. 6). Addition of the antibodies to the cells when cultured individually had little effect on IP-10 production (Fig. 6).

Discussion

In the present study, RA SF contained greater amounts of IP-10 as compared with OA SF. Immunolocalization analysis indicated that IP-10 was associated mainly with infiltrating macrophage-like cells, and fibroblast-like cells in the RA synovium, as described previously [25]. In addition, substantial amounts of IP-10 were also secreted from RA SF monocytes *in vitro* and, to a lesser extent, from RA SF PMNs cocultured with FLSs. The present study clearly demonstrates that cell–cell interactions that occur in the RA joint tissues are important for induction of IP-10 expression.

Figure 6
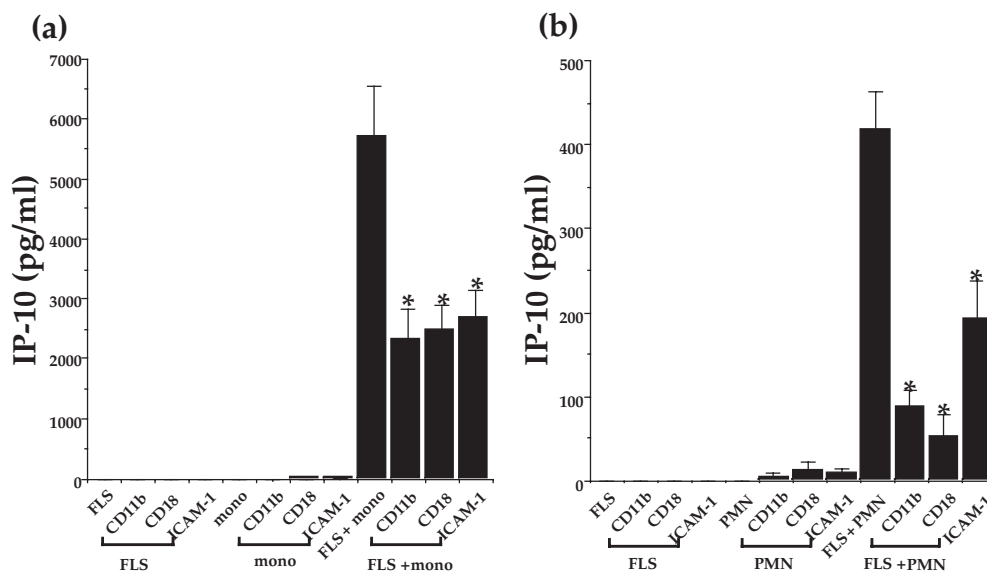
Effects of anti-integrin and antiadhesion molecule neutralizing monoclonal antibodies on IFN-γ inducible protein-10 (IP-10) secretion. **(a)** Synovial fluid monocytes (mono) or **(b)** polymorphonuclear neutriphils (PMNs) were layered onto rheumatoid arthritis fibroblast-like synoviocyte (FLS) monolayers in 48-well plates, after which monoclonal antibodies (5 μg/ml) against CD11b, CD18 or intercellular adhesion molecule (ICAM)-1 were added. After incubating for 24 hours, the supernatants were harvested and assayed using enzyme-linked immunosorbent assay. Each bar represents the mean (pg/ml) ± SEM of four independent experiments, which were performed using two different rheumatoid arthritis fibroblasts and four different rheumatoid arthritis synovial fluid leukocytes. *$P < 0.05$, versus the respective coculture in the absence of monoclonal antibody.

The augmentation of IP-10 production was dependent on an interaction between synovial FLSs and leukocytes; individually, none of the cell populations tested produced substantial amounts of IP-10. Indeed, the necessity for physical contact between the cells was apparent from the finding that IP-10 production was completely blocked by a transwell membrane that separated FLSs from the leukocytes, but was permeable to soluble factors.

The pathway governing IP-10 expression was further examined by determining the role of adhesion molecules in the regulation of IP-10 production mediated by FLS–leukocyte interactions. Application of neutralizing anti-CD11b, CD18, or anti-ICAM-1 monoclonal antibodies to FLS–leukocyte cocultures significantly inhibited IP-10 production (Fig. 6). This implies that upregulation of IP-10 production by cell–cell contact was, in large part, promoted through a β_2-integrin/ICAM-1-mediated mechanism, although it remains to be tested whether other adhesion molecules are involved in the induction of IP-10 mediated by the interaction of RA FLSs and leukocytes. This pathway cannot solely account for the response, however, because monoclonal antibodies against either β_2-integrin or ICAM-1 inhibited IP-10 secretion by, at most, 53–59% in FLS–monocyte coculture and by 54–87% in FLS–PMN coculture.

In addition, the findings presented here reveal that IP-10-inducible soluble factors, such as IFN-γ and TNF-α, which may be induced by cell–cell interactions, were not involved in IP-10 induction in this system, because we failed to detect significant inhibitory effects of anti-IFN-γ or anti-TNF-α antibodies on IP-10 secretion. Furthermore, we recently demonstrated that the secretion of a potent angiogenic factor, namely vascular endothelial growth factor, was markedly induced by the interaction of FLS with synovial leukocytes via the integrin/ICAM-1 pathway [19]. Taken together, these data support the notion that the physical contact between either SF monocytes or neutrophils and FLSs might be important for producing inflammatory mediators, such as IP-10 or vascular endothelial growth factor, as is observed in the synovium of RA, and is further implicated in the progression of RA.

Additionally, IP-10 was originally found to be expressed and secreted by monocytes, fibroblasts, and endothelial cells after stimulation with IFN-γ [5,8]. The present data clearly demonstrate that activated PMNs interacting with fibroblasts are an important cellular source of IP-10 in RA synovitis, because most of the leukocytes infiltrating the SF of rheumatoid joints are PMNs. PMNs in the RA SF are in an activated state, and produce a variety of other inflammatory mediators [22,30–33]. Furthermore, neutrophils are recognized as an important cellular source of IP-10 [34]. This biosynthetically active leukocyte population almost certainly contributes significantly to the disease process during active RA.

Th1 cells and Th1-type cytokines play an important role in the development of progressive synovitis in RA [13,35]. CXCR3, a specific IP-10 receptor, is expressed preferentially in Th1 as compared with Th2 cells, and Th1 but not Th2 cells respond to IP-10 [36–38]. Indeed, there are CXCR3-positive cells in RA synovium [25,39]. Findings from those studies, together with the present data, support the hypothesis that IP-10 secreted by activated SF leukocytes interacting with fibroblasts might contribute to migration of Th1 cells through CXCR3 in the development of RA.

Conclusion
IP-10 expression within inflamed joints appears to be regulated not only by inflammatory cytokines but also by the physical interaction of activated leukocytes with FLSs. Once expressed, IP-10 probably plays a crucial role in the migration of Th1 cells during the synovial inflammation that occurs in RA.

Competing interests
None declared.

Acknowledgments

References
1. Kunkel SL, Lukacs NW, Kasama T, Strieter RM: **The role of chemokines in inflammatory joint disease.** *J Leukoc Biol* 1996, **58**:6-12.
2. Choy EHS, Panayi GS: **Cytokine pathways and joint inflammation in rheumatoid arthritis.** *N Engl J Med* 2001, **344**:907-916.
3. Gerard C, Rollins BJ: **Chemokines and disease.** *Nat Immunol* 2001, **2**:108-115.
4. Lee DM, Weinblatt ME: **Rheumatoid arthritis.** *Lancet* 2001, **358**: 903-911.
5. Luster AD: **Chemokines: chemotactic cytokines that mediate inflammation.** *N Engl J Med* 1998, **338**:436-445.
6. Zlotnik A, Morales J, Hedrick JA: **Recent advances in chemokines and chemokines and chemokine receptors.** *Crit Rev Immunol* 1999, **19**:1-47.
7. Mackay CR: **Chemokines: immunology's high impact factors.** *Nature Immunol* 2001, **2**:95-101.
8. Luster AD, Ravetch JV: **Biochemical characterization of a γ interferon-inducible cytokine (IP-10).** *J Exp Med* 1987, **166**: 1084-1097.
9. Angiolillo AL, Sgadari C, Taub DD, Liao F, Farber JM, Maheshwari S, Kleinman HK, Reaman GH, Tosato G: **Human interferon-inducible protein 10 is a potent inhibitor of angiogenesis in vivo.** *J Exp Med* 1995, **182**:155-162.
10. Strieter RM, Kunkel SL, Arenberg DA, Burdick MD, Polverini PJ: **Interferon gamma-inducible protein 10 (IP-10), a member of the C-X-C chemokine family, is an inhibitor of angiogenesis.** *Biochem Biophys Res Commun* 1995, **210**:51-57.
11. Arenberg DA, Kunkel SL, Polverini PJ, Morris SB, Burdick MD, Glass M, Taub DT, Iannetoni MD, Whyte RI, Strieter RM: **Interferon-gamma-inducible protein 10 (IP-10) is an angiostatic factor that inhibits human non-small cell lung cancer (NSCLC) tumorigenesis and spontaneous metastases.** *J Exp Med* 1996, **184**:981-992.
12. Tannenbaum CS, Tubbs R, Armstrong D, Finke JH, Bukowski RM, Hamilton TA: **The CXC chemokines IP-10 and Mig are necessary for IL-12-mediated regression of the mouse RENCA tumor.** *J Immunol* 1998, **161**:927-932.

13. Miossec P, van den Berg W: **Th1/Th2 cytokine balance in arthritis.** *Arthritis Rheum* 1997, **40**:2105-2115.

14. Yin Z, Siegert S, Neure L, Grolms M, Liu L, Eggens U, Radbruch A, Braun J, Sieper J: **The elevated ratio of interferon gamma-/interleukin-4-positive T cells found in synovial fluid and synovial membrane of rheumatoid arthritis patients can be changed by interleukin-4 but not by interleukin-10 or transforming growth factor beta.** *Rheumatology (Oxford)* 1999, **38**: 1058-1067.

15. Canete JD, Martinez SE, Farres J, Sanmarti R, Blay M, Gomez A, Salvador G, Munoz-Gomez J: **Differential Th1/Th2 cytokine patterns in chronic arthritis: interferon gamma is highly expressed in synovium of rheumatoid arthritis compared with seronegative spondyloarthropathies.** *Ann Rheum Dis* 2000, **59**:263-268.

16. Sallusto F, Lenig D, Mackay CR, Lanzavecchia A: **Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes.** *J Exp Med* 1998, **187**:875-883.

17. Rossi D, Zlotnik A: **The biology of chemokines and their receptors.** *Annu Rev Immunol* 2000, **18**:217-242.

18. Lukacs NW, Strieter RM, Elner VM, Evanoff HL, Burdick MD, Kunkel SL: **Intercellular adhesion molecule-1 mediates the expression of monocyte-derived MIP-1α during monocyte–endothelial cell interactions.** *Blood* 1994, **83**:1174-1178.

19. Kasama T, Shiozawa F, Kobayashi K, Yajima N, Hanyuda M, Takeuchi TT, Mori Y, Negishi M, Ide H, Adachi M: **Vascular endothelial growth factor expression by activated synovial leukocytes in rheumatoid arthritis. Critical involvement of the interaction with synovial fibroblasts.** *Arthritis Rheum* 2001, **44**: 2512-2524.

20. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, Healey LA, Kaplan SR, Liang MH, Luthra HS, Medsger TA, Mitchell DM, Neustadt DH, Pinals RS, Schaller JS, Sharp JT, Wilder RL, Hunder GG: **The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis.** *Arthritis Rheum* 1988, **31**:315-324.

21. Iwabuchi H, Kasama T, Hanaoka R, Miwa Y, Hatano Y, Kobayashi K, Mori Y, Negishi M, Ide H, Adachi M: **Down-regulation of intercellular adhesion molecule-1 expression on human synovial fibroblasts by endothelin-1.** *J Rheumatol* 1999, **26**:522-531.

22. Kasama T, Kobayashi K, Yajima N, Shiozawa F, Yoda Y, Takeuchi TT, Mori Y, Negishi M, Ide H, Adachi M: **Expression of vascular endothelial growth factor by synovial fluid neutrophils in rheumatoid arthritis.** *Clin Exp Immunol* 2000, **121**:533-538.

23. Kasama T, Yamazaki J, Hatano Y, Kobayashi K, Negishi M, Ide H, Adachi M: **Biphasic regulation by interleukin 12 of the development of murine type II collagen-induced arthritis: possible involvement of endogenous interleukin 10 and tumor necrosis factor alpha.** *Arthritis Rheum* 1999, **42**:100-109.

24. Luster AD, Unkeless JC, Ravetch JV: **Gamma-interferon transcriptionally regulates an early-response gene containing homology to platelet proteins.** *Nature* 1985, **315**:672-676.

25. Patel DD, Zachariah JP, Whichard LP: **CXCR3 and CCR5 ligands in rheumatoid arthritis synovium.** *Clin Immunol* 2001, **98**:39-45.

26. Bedard PA, Golds EE: **Cytokine-induced expression of mRNAs for chemotactic factors in human synovial cells and fibroblasts.** *J Cell Physiol* 1993, **154**:433-441.

27. Ohmori Y, Wyner L, Narumi S, Armstrong D, Stoler M, Hamilton TA: **Tumor necrosis factor-alpha induces cell type and tissue-specific expression of chemoattractant cytokines in vivo.** *Am J Pathol* 1993, **142**:861-870.

28. Burger D, Rezzonico R, Li J-M, Modoux C, Pierce RA, Welgus HG, Dayer J-M: **Imbalance between interstitial collagenase and tissue inhibitor of metalloproteinases 1 in synoviocytes and fibroblasts upon direct contact with stimulated T lymphocytes: involvement of membrane-associated cytokines.** *Arthritis Rheum* 1998, **41**:1748-1759.

29. Springer TA: **Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm.** *Cell* 1994, **76**: 301-314.

30. De Clerck LS, de Gendt CM, Bridts CH, van Osselaer N, Stevens WJ: **Expression of neutrophil activation markers and neutrophil adhesion to chondrocytes in rheumatoid arthritis patients: relationship with disease activity.** *Res Immunol* 1995, **146**:81-87.

31. Lloyd AR, Oppenheim JJ: **Poly's lament: the neglected role of the polymorphonuclear neutrophil in the afferent limb of the immune response.** *Immunol Today* 1992, **13**:169-172.

32. Kasama T, Strieter RM, Standiford TJ, Burdick MD, Kunkel SL: **Expression and regulation of human neutrophil-derived macrophage inflammatory protein 1α.** *J Exp Med* 1993, **178**: 63-72.

33. Hatano Y, Kasama T, Iwabuchi H, Hanaoka R, Takeuchi TT, Lu J, Mori Y, Kobayashi K, Negishi M, Ide H, Adachi M: **Macrophage inflammatory protein 1 alpha expression by synovial fluid neutrophils in rheumatoid arthritis.** *Ann Rheum Dis* 1999, **58**: 297-302.

34. Gasperini S, Marchi M, Calzetti F, Laudanna C, Vicentini L, Olsen H, Murphy M, liao F, Farber J, Cassatella MA: **Gene expression and production of the monokine induced by IFN-γ (MIG), IFN-inducible T cell a chemoattractant (I-TAC), and IFN-γ-inducible protein-10 (IP-10) chemokines by human neutrophils.** *J Immunol* 1999, **162**:4928-4937.

35. Aarvak T, Chabaud M, Källberg E, Miossec P, Natvig JB: **Change in the Th1/Th2 phenotype of memory T-cell clones from rheumatoid arthritis synovium.** *Scand J Immunol* 1999, **50**:1-9.

36. Sallusto F, Lanzavecchia A, Mackay CR: **Chemokines and chemokine receptors in T-cell priming and Th1/Th2-mediated responses.** *Immunol Today* 1998, **19**:568-574.

37. Proudfoot AEI, Power CA, Wells TNC: **The strategy of blocking the chemokine system to combat disease.** *Immunol Rev* 2000, **177**:246-256.

38. Arimilli S, Ferlin W, Solvason N, Deshpande S, Howard M, Mocci S: **Chemokines in autoimmune diseases.** *Immunol Rev* 2000, **177**:43-51.

39. Katschke KJ Jr, Rottman JB, Ruth JH, Qin S, Wu L, LaRosa G, Ponath P, Park CC, Pope RM, Koch AE: **Differential expression of chemokine receptors on peripheral blood, synovial fluid, and synovial tissue monocytes/macrophages in rheumatoid arthritis.** *Arthritis Rheum* 2001, **44**:1022-1032.

Correspondence

Tsuyoshi Kasama, Division of Rheumatology and Clinical Immunology, First Department of Internal Medicine, Showa University School of Medicine, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8666, Japan. Tel: +81 33784 8532; fax: +81 33784 8742; e-mail: tkasama@med.showa-u.ac.jp