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## Enhancing Effect of IL-1, IL-17, and TNF- $\alpha$ on Macrophage Inflammatory Protein-3 $\alpha$ Production in Rheumatoid Arthritis: Regulation by Soluble Receptors and Th2 Cytokines<sup>1</sup>

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# Enhancing Effect of IL-1, IL-17, and TNF- $\alpha$ on Macrophage Inflammatory Protein-3 $\alpha$ Production in Rheumatoid Arthritis: Regulation by Soluble Receptors and Th2 Cytokines<sup>1</sup>

Martine Chabaud, Guillaume Page, and Pierre Miossec<sup>2</sup>

Macrophage inflammatory protein (MIP)-3 $\alpha$  is a chemokine involved in the migration of T cells and immature dendritic cells. To study the contribution of proinflammatory cytokines and chemokines to the recruitment of these cells in rheumatoid arthritis (RA) synovium, we looked at the effects of the monocyte-derived cytokines IL-1 $\beta$  and TNF- $\alpha$  and the T cell-derived cytokine IL-17 on MIP-3 $\alpha$  production by RA synoviocytes. Addition of IL-1 $\beta$ , IL-17, and TNF- $\alpha$  induced MIP-3 $\alpha$  production in a dose-dependent manner. At optimal concentrations, IL-1 $\beta$  (100 pg/ml) was much more potent than IL-17 (100 ng/ml) and TNF- $\alpha$  (100 ng/ml). When combined at lower concentrations, a synergistic effect was observed. Conversely, the anti-inflammatory cytokines IL-4 and IL-13 inhibited MIP-3 $\alpha$  production by activated synoviocytes, but IL-10 had no effect. Synovium explants produced higher levels of MIP-3 $\alpha$  in RA than osteoarthritis synovium. MIP-3 $\alpha$ -producing cells were located in the lining layer and perivascular infiltrates in close association with CD1a immature dendritic cells. Addition of exogenous IL-17 or IL-1 $\beta$  to synovium explants increased MIP-3 $\alpha$  production. Conversely, specific soluble receptors for IL-1 $\beta$ , IL-17, and TNF- $\alpha$  inhibited MIP-3 $\alpha$  production to various degrees, but 95% inhibition was obtained only when the three receptors were combined. Similar optimal inhibition was also obtained with IL-4, but IL-13 and IL-10 were less active. These findings indicate that interactions between monocyte and Th1 cell-derived cytokines contribute to the recruitment of T cells and dendritic cells by enhancing the production of MIP-3 $\alpha$  by synoviocytes. The inhibitory effect observed with cytokine-specific inhibitors and Th2 cytokines may have therapeutic applications. *The Journal of Immunology*, 2001, 167: 6015–6020.

Rheumatoid arthritis (RA)<sup>3</sup> is characterized by chronic inflammation of the synovium with hyperplasia of synovial lining cells that interact with blood-derived mononuclear cells (1). Many of the features of the rheumatoid synovial environment, such as the selective accumulation of T cells, activated macrophages, and polymorphonuclear cells in the synovium and joint space, suggest a role for chemokines. Chemokines could be released by a number of cells present in the RA joint, including endothelial cells, fibroblasts, macrophages, and lymphocytes. Studies of RA synovium showed the presence of IL-8 and melanoma growth-stimulating activity, which attract neutrophils; macrophage inflammatory protein (MIP)-1 $\alpha$  and MIP-1 $\beta$ ; and monocyte chemoattractant protein-1 and RANTES, which recruit T cells and monocytes (2–8).

MIP-3 $\alpha$ , also called liver and activation-regulated chemokine (9), Exodus-1, or CCL20, is a CC chemokine with an in vivo distribution rather restricted to mucosal and inflamed tissues (10–13). Furthermore, MIP-3 $\alpha$  is inducible in vitro by mediators of inflammation and is expressed by both hemopoietic (dendritic cells (DC), monocytes, granulocytes, and T and B lymphocytes) and

nonhemopoietic cells (endothelial cells, tumor cells) (12, 14–16). MIP-3 $\alpha$  was found to be the most powerful chemokine in inducing the migration of CD34<sup>+</sup>-derived immature DC compared with MIP-1 $\alpha$  and RANTES (10, 12, 17–20). Because of the link between MIP-3 $\alpha$  expression and inflammation, we considered the contribution of MIP-3 $\alpha$  to RA pathogenesis. Indeed, the synovitis has been previously described as a lymphoid organ located in an ectopic position. These features suggest a role for chemotactic factors in the migration of lymphocytes and DC leading to the local organization that is characteristic of the follicular structure of RA synovium.

To address this issue we analyzed the expression and cellular source of MIP-3 $\alpha$  in RA synoviocytes and synovium tissue. The data indicate that MIP-3 $\alpha$  production by RA synovium and synoviocytes is stimulated by proinflammatory cytokines and can be regulated by their specific inhibitors and Th2 cytokines.

## Materials and Methods

### Cytokines and reagents

Murine IL-17R (21), dimeric human TNF receptor p80/IgG1:Fc fusion protein (22), and human soluble type II IL-1R (23) were provided by Dr. K. Mohler (Immunex, Seattle, WA), and human rIL-4, rIL-10, rIL-13, and rIL-17 were provided by Dr. F. Fossiez (Schering-Plough Research Center, Dardilly, France). Human rIL-1 $\beta$  and human rTNF- $\alpha$  were purchased from Sigma-Aldrich (St. Louis, MO).

### Synovium explant and synoviocyte cultures

Rheumatoid synovium samples were obtained from 39 patients with RA, according to the revised criteria of the American College of Rheumatology (24), who were undergoing knee or wrist synovectomy or joint replacement. Osteoarthritis (OA) synovium samples were obtained during joint replacement from 29 patients. Synovium explant cultures were performed as previously described (25). Briefly, fat and fibrous tissues were removed, and synovium was cut into small explants with a volume of approximately

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<sup>3</sup> Abbreviations used in this paper: RA, rheumatoid arthritis; DC, dendritic cell; MIP, macrophage inflammatory protein; OA, osteoarthritis.

5 mm<sup>3</sup>. Explants of synovium were cultured in triplicate in complete medium consisting of  $\alpha$ MEM medium (Life Technologies, Grand Island, NY) with 2 mM L-glutamine, 100 U/ml penicillin, 50 mg/ml gentamicin, 20 mM HEPES buffer, and 10% FCS. Cultures were performed at 37°C in a 5% CO<sub>2</sub>/95% air-humidified environment.

To isolate synoviocytes, synovium explants were finely minced and digested with 4 mg/ml collagenase (Worthington, Freehold, NJ) in Dulbecco's PBS (Life Technologies) for 2–3 h at 37°C (26). After centrifugation, cells were suspended in complete medium and cultured in 100-mm culture petri dishes. After 48 h, nonadherent cells were removed. Adherent cells were cultured in complete medium and at confluence were trypsinized and passaged in 150-cm<sup>2</sup> culture flasks. Synoviocytes were used between passages three and eight. At this time they were a homogenous population of fibroblast-like cells, negative for the expression of CD1, CD3, CD19, CD14, and HLA-DR and positive for the expression of CD10, CD44, and CD54 as determined by FACS analysis.

To obtain culture supernatants, synoviocytes were plated in 96-well plates at 10<sup>4</sup> cells/well in 200  $\mu$ l of complete medium. The cytokines to be tested were added at the onset of culture. Supernatants were collected after 72 h of culture.

### Measurement of MIP-3 $\alpha$ levels

MIP-3 $\alpha$  levels were measured by a two-site sandwich ELISA. Briefly, supernatants or serial dilutions of MIP-3 $\alpha$  standards were incubated for 2 h and 30 min at 37°C in 96-microtiter plates (Nunc, Roskilde, Denmark) and coated overnight at 4°C with the mouse 319F6 anti-MIP-3 $\alpha$  mAb (1  $\mu$ g/ml). These two mAb were provided by Dr. C. Caux (Schering-Plough). After washing, a peroxidase-coupled mouse anti-MIP-3 $\alpha$  mAb (1  $\mu$ g/ml) was added and incubated for 2 h and 30 min at 37°C. After revelation with tetramethylbenzidine (Sigma-Aldrich), the plates were read at 450 nm.

### MIP-3 $\alpha$ mRNA expression

RNA was extracted from synovium biopsies by the guanidinium isocyanate method (27). To synthesize cDNA, 1  $\mu$ g of total RNA was incubated with 2  $\mu$ g oligo(dT) primer (Boehringer Mannheim, Meylan, France), 0.5 mM dNTP, 10 mM DTT, 10  $\mu$ l of 10 $\times$  RT buffer, and 20 U of reverse transcriptase (Boehringer Mannheim). The PCR reaction was performed using 5  $\mu$ l of this cDNA in the presence of 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM MgCl<sub>2</sub>, 200  $\mu$ M dNTPs, 0.5  $\mu$ M of each primer, and 2.5 U of *Taq* polymerase (Boehringer Mannheim) in a total of 50  $\mu$ l and was incubated at 94°C for 3 min, 61°C for 1 min, and 72°C for 2 min. This cycle was repeated 40 times. PCR products (10  $\mu$ l) were separated by electrophoresis in a 1.5% agarose gel and visualized by ethidium bromide staining. After transfer to a nylon membrane (Boehringer Mannheim) and prehybridization, membranes were hybridized overnight at 68°C with an MIP-3 $\alpha$  oligonucleotide (5'-GAA-TCA-GAA-GCA-AGC-AA-3') and an actin cDNA probe labeled with digoxigenin. After stringency washes, membranes were incubated with an Ab conjugated to alkaline phosphatase and chemiluminescence substrate (Boehringer Mannheim). Blots were exposed to x-ray film (Kodak, Rochester, NY). Primer sequences were as follows: MIP-3 $\alpha$  sense, 5'-TTG-CTC-CTG-GCT-GCT-TTG-3'; MIP-3 $\alpha$  antisense, 5'-ACC-CTC-CAT-GAT-GTG-GAAC-3'; actin sense, 5'-GGG-TCA-GAA-GGA-TTC-CTA-TGG-3'; and actin antisense, 5'-CTC-CTT-AAT-GTC-ACG-CAC-GAT-TTC-3'.

### Immunohistochemistry

RA synovium samples were fixed in 4% phosphate-buffered paraformaldehyde and then embedded in paraffin. Four-micrometer sections were cut and mounted on glass slides. To block nonspecific activities, sections were pretreated with avidin and biotin solutions (blocking kit; Vector Laboratories, Burlingame, CA) for 10 min for each step and with 0.3% hydrogen peroxide (Sigma-Aldrich) for 15 min at room temperature. After brief washing in PBS, the sections were incubated with blocking serum (2% normal human serum) for at least 30 min before adding the primary Abs. Sections were stained with an anti-human MIP-3 $\alpha$  goat polyclonal Ab (IgG; R&D Systems, Minneapolis, MN) for 1 h at room temperature in a humid atmosphere. Binding of the anti-human MIP-3 $\alpha$  Ab was detected with a biotinylated rabbit anti-goat IgG followed by streptavidin-peroxidase. Peroxidase staining was revealed using 3-amino-9-ethylcarbazole as substrate. Negative controls were established by adding nonspecific isotype controls as primary Abs. For double staining with anti-MIP-3 $\alpha$  and anti-CD1a (IgG2b; BD Biosciences, Pont de Claix, France) Abs, after an initial blocking with rabbit serum and BSA, primary anti-MIP-3 $\alpha$  Ab was followed by a rabbit biotinylated anti-goat IgG and streptavidin-peroxidase.

Peroxidase was developed with 3-amino-9-ethylcarbazole. After a second blocking step for endogenous biotin with avidin-biotin, a mouse monoclonal CD1a (IgG2b) Ab was followed by a purified rat anti-mouse IgG2a/2b (BD Biosciences) and the mouse alkaline phosphatase-anti-alkaline phosphatase system (DAKO, Carpinteria, CA). Alkaline phosphatase was developed using Fast Blue as chromogen (blue color; Vector Laboratories). In control sections one of the two primary Abs was omitted.

### Statistical analysis

Results were expressed as the mean  $\pm$  SEM of *n* separate experiments. Differences between cytokine-treated groups and the control group were compared with the Wilcoxon test.

## Results

### Effects of IL-1 $\beta$ , IL-7, and TNF- $\alpha$ on MIP-3 $\alpha$ production by RA synoviocytes

To reproduce the inflammatory situation found in RA synovium, we investigated whether the monocyte-derived cytokines IL-1 $\beta$  and TNF- $\alpha$ , and the T cell-derived cytokine IL-17, could enhance MIP-3 $\alpha$  production by synoviocytes. These cells were incubated with increasing concentrations of IL-1 $\beta$ , TNF- $\alpha$ , and IL-17, used alone. After 72 h of culture, supernatants were collected and assayed for MIP-3 $\alpha$  production by ELISA. When IL-17 and TNF- $\alpha$  at concentrations ranging from 0.01 to 1000 ng/ml and IL-1 $\beta$  at concentrations ranging from 0.01 to 1000 pg/ml were used alone, MIP-3 $\alpha$  production increased in a dose-dependent manner (Fig. 1A). Unstimulated synoviocytes did not produce MIP-3 $\alpha$ . At optimal concentrations, IL-1 $\beta$  (100 pg/ml) induced 13 ng/ml MIP-3 $\alpha$  production, while TNF- $\alpha$  (100 ng/ml) and IL-17 (100 ng/ml) had a much lesser effect (4 and 1 ng/ml; Fig. 1A).

When IL-1 $\beta$ , TNF- $\alpha$ , and IL-17 were combined, an enhancing effect greater than that seen with one cytokine alone was observed. To remain in the concentration range that could reflect the *in vivo* situation, experiments were performed in the presence of 100-fold lower concentrations of IL-1 $\beta$  (1 pg/ml) and TNF- $\alpha$  and IL-17 (1 ng/ml). With TNF- $\alpha$  combined with IL-17 or IL-1 $\beta$ , synoviocytes produced much more MIP-3 $\alpha$  than the sum of MIP-3 $\alpha$  production with each cytokine alone, indicating a clear synergistic effect (Fig. 1B). This synergistic effect was further enhanced with the combination of three cytokines. In time-course studies this enhancing effect was seen as early as 12 h of culture (data not shown). This effect was not observed when IL-17 was combined with IL-1 $\beta$ .

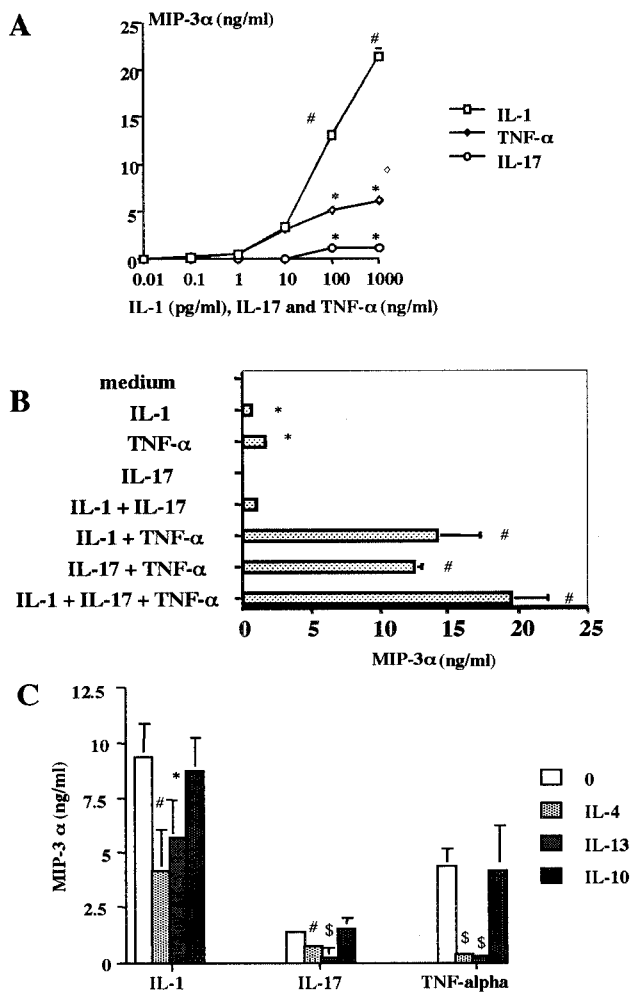
### Regulation by IL-4, IL-13, and IL-10 of MIP-3 $\alpha$ production by RA synoviocytes induced by IL-1 $\beta$ , TNF- $\alpha$ , and IL-17

As IL-4, IL-13, and IL-10 have been defined as anti-inflammatory cytokines (25, 28, 29), they were tested for their effects on the secretion of MIP-3 $\alpha$  by synoviocytes stimulated with optimal concentrations of IL-1 $\beta$ , TNF- $\alpha$ , and IL-17 alone. Levels measured after 72 h of culture are presented in Fig. 1C. Addition of IL-4, IL-13, and IL-10 alone at the onset of culture had no effect on spontaneous MIP-3 $\alpha$  secretion. When combined with the proinflammatory cytokines, IL-4 and IL-13 reduced MIP-3 $\alpha$  production. The effect was particularly pronounced with TNF- $\alpha$ -stimulated cells. In contrast, IL-10 had no effect on such MIP-3 $\alpha$  production.

### MIP-3 $\alpha$ is spontaneously secreted by RA synovium

We have previously shown that explants of whole RA synovial tissue in *ex vivo* cultures produce spontaneously high amounts of IL-1 $\beta$ , TNF- $\alpha$ , and IL-6 (25). Thus, we measured the spontaneous production of MIP-3 $\alpha$  in the same *ex vivo* model. As control, we used samples of OA synovium.

Explants of synovium were cultured for 7 days, supernatants were collected, and levels of MIP-3 $\alpha$  were measured. Levels of MIP-3 $\alpha$  produced by synovium explants were 12.8  $\pm$  1.9 ng/ml



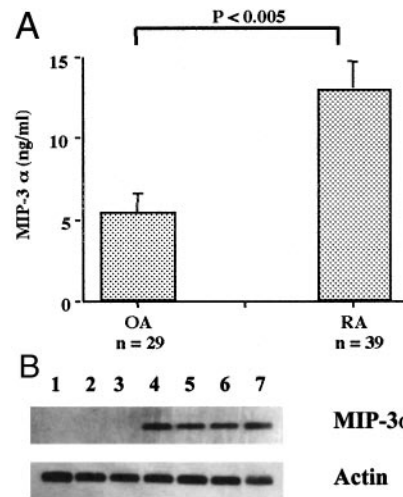
**FIGURE 1.** Effects of IL-1 $\beta$ , TNF- $\alpha$ , and IL-17 on MIP-3 $\alpha$  production by synoviocytes and regulation by Th2 cytokines. *A*, RA synoviocytes ( $10^4$ ) were cultured with increasing concentrations of IL-1 $\beta$  (picograms per milliliter) or IL-17 or TNF- $\alpha$  (nanograms per milliliter) used alone. After 72 h of culture, MIP-3 $\alpha$  levels were measured by ELISA. Results were expressed as the mean  $\pm$  SEM of four separate experiments. *B*, RA synoviocytes ( $10^4$ ) were cultured with IL-1 $\beta$  (1 pg/ml) or with IL-17 (1 ng/ml) or TNF- $\alpha$  (1 ng/ml) alone and in combination. After 72 h of culture, MIP-3 $\alpha$  levels were measured by ELISA. Results were expressed as the mean  $\pm$  SEM of four separate experiments. *C*, RA synoviocytes ( $10^4$ ) were cultured with IL-1 $\beta$  (50 pg/ml) or with IL-17 (50 ng/ml) or TNF- $\alpha$  (50 ng/ml) in the presence of 50 ng/ml IL-4, IL-10, or IL-13. After 72 h of culture, MIP-3 $\alpha$  levels were measured by ELISA. Results were expressed as the mean  $\pm$  SEM of four separate experiments. Differences in MIP-3 $\alpha$  production between cytokine-treated groups and the control group were analyzed with the nonparametric paired Wilcoxon test (\*,  $p < 0.05$ ; #,  $p < 0.01$ ; \$,  $p < 0.005$ ).

(range, 0–20;  $n = 39$ ) for RA and much lower for OA ( $5.3 \pm 1.3$  ng/ml; range, 0–42;  $n = 29$ ;  $p < 0.005$ ; Fig. 2A).

To confirm and extend these results, the expression of mRNA specific for MIP-3 $\alpha$  was studied in five RA and two OA synovium samples using RT-PCR techniques followed by Southern blot analysis with specific probes. MIP-3 $\alpha$  mRNA could be detected in four of five RA samples (Fig. 2B, lanes 3–7) and in none of the OA samples (Fig. 2B, lanes 1 and 2). Actin was used as an internal control for gene expression.

*MIP-3 $\alpha$ -producing cells are present in RA synovium*

To investigate the presence of MIP-3 $\alpha$ -producing cells, immunostaining of RA synovium sections was performed with an anti-



**FIGURE 2.** MIP-3 $\alpha$  production and MIP-3 $\alpha$  mRNA expression by RA synovium explants. *A*, RA and OA synovium explants (six explants per point) were cultured for 7 days in medium alone. MIP-3 $\alpha$  levels were measured by ELISA in culture supernatants. *B*, Total mRNA was extracted from synovial tissue of two OA (lanes 1 and 2) and five RA (lanes 3–7) patients. Southern blot analysis using specific labeled probes is shown for MIP-3 $\alpha$  (top) and actin (bottom).

MIP-3 $\alpha$  polyclonal Ab. As shown in Fig. 3, cells stained positively for MIP-3 $\alpha$  were synoviocytes and were localized in the lining layer (A) and, to a lesser extent, in the lymphocyte aggregates (B). A similar staining pattern was observed in synoviocytes stimulated with IL-1 $\beta$ , TNF- $\alpha$ , or IL-17 (data not shown). Double staining with anti-CD1a Ab, a marker of immature DC, showed a close association between MIP-3 $\alpha$ -producing cells and CD1a-positive immature DC (Fig. 3C).

*IL-1 $\beta$ , TNF- $\alpha$ , and IL-17 induce MIP-3 $\alpha$  production by RA synovium*

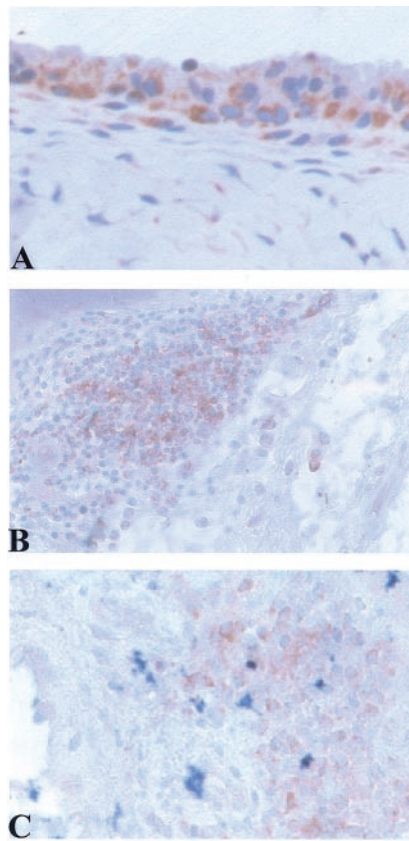
The effects of exogenous IL-1 $\beta$ , TNF- $\alpha$ , and IL-17 were analyzed on MIP-3 $\alpha$  production by RA synovium explants. Samples from three patients were cultured in the presence of 50 ng/ml TNF- $\alpha$  and IL-17 or 100 pg/ml IL-1 $\beta$ . Addition of IL-1 $\beta$  increased MIP-3 $\alpha$  production by 70% and was more potent than IL-17 (15%; Fig. 4).

Conversely, the bioactivities of these three endogenous cytokines were estimated by incubating the samples with their respective soluble receptor. In preliminary experiments we selected 1  $\mu$ g/ml as an optimal concentration (30). After 7 days in culture, such blocking resulted in a decrease in MIP-3 $\alpha$  production by a mean of 60%. With some samples, IL-17 soluble receptor showed a particularly striking inhibitory effect. The maximal effect was observed when the three receptors were combined, which reduced MIP-3 $\alpha$  levels by a mean of 95% (Fig. 5).

*MIP-3 $\alpha$  production by RA synovium is inhibited by IL-4 and IL-13*

The anti-inflammatory cytokines IL-4, IL-13, and IL-10 have been found to inhibit the production of the monocyte-derived cytokines IL-1 $\beta$  and TNF- $\alpha$  and the T cell-derived cytokine IL-17 by RA synovium explants (25, 31). Using the same assay system, we investigated whether the same effect was observed for MIP-3 $\alpha$ . Accordingly, synovium explants from four RA patients were cultured with or without 50 ng/ml IL-4, IL-10, or IL-13. Such concentrations were previously found to be optimal to inhibit proinflammatory cytokine production by synovium samples (22, 26). After 7 days the supernatants were collected and assayed for MIP-3 $\alpha$ . IL-4



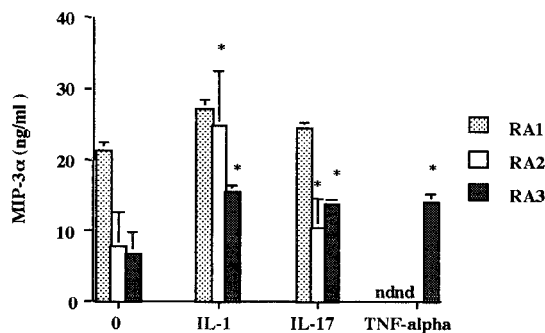


**FIGURE 3.** Immunostaining of MIP-3 $\alpha$ -producing cells in RA synovium. Sections of RA synovial tissue were incubated with anti-MIP-3 $\alpha$  and stained with immunoperoxidase. *A*, Positive staining in the lining layer (magnification,  $\times 250$ ). *B*, Positive staining in a lymphocytic infiltrate (magnification,  $\times 250$ ). *C*, Double staining with an anti-MIP-3 $\alpha$  Ab and an anti-CD1a Ab, showing the close association between MIP-3 $\alpha$ -producing cells and CD1a-positive immature DC (magnification,  $\times 250$ ).

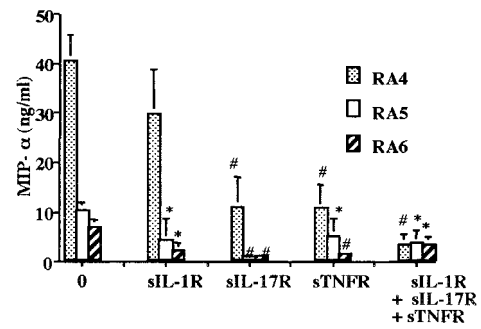
and IL-13 inhibited RA synovium production of MIP-3 $\alpha$  by 53 and 48%, respectively (Fig. 6). In contrast, IL-10 had a very modest or no effect on such production, whereas it was able to reduce IL-6 production in the same supernatants (data not shown).

## Discussion

The RA synovitis is characterized by cell interactions between blood-derived cells, such as monocytes, T cells, B cells, and DC,



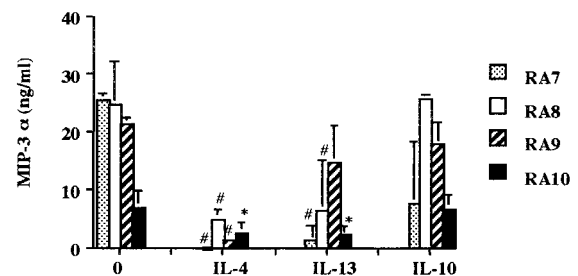
**FIGURE 4.** Effects of exogenous IL-17, IL-1 $\beta$ , and TNF- $\alpha$  on MIP-3 $\alpha$  production by RA synovium. RA synovium samples (six explants per point) from three patients were incubated for 7 days in the presence of IL-17 (50 ng/ml), IL-1 (100 pg/ml), or TNF- $\alpha$  (50 ng/ml). MIP-3 $\alpha$  levels were measured by ELISA in culture supernatants. nd, not done. Differences in MIP-3 $\alpha$  production between cytokine-treated groups and the control group were analyzed with the nonparametric paired Wilcoxon test (\*,  $p < 0.05$ ).



**FIGURE 5.** Effects of IL-17, IL-1 $\beta$ , and TNF- $\alpha$  soluble receptors on MIP-3 $\alpha$  production by RA synovium explants. Synovium samples (eight explants per point) from RA patients ( $n = 3$ ) were incubated for 7 days in the presence of 1  $\mu$ g/ml soluble IL-17R, IL-1R type II, and TNF receptor alone or in combination. Normal IgG, used as a control for the fusion IgGfc part, had no effect (data not shown). MIP-3 $\alpha$  levels were measured by ELISA in culture supernatants. Differences in MIP-3 $\alpha$  production between soluble receptor-treated groups and the untreated group were analyzed with the nonparametric paired Wilcoxon test (\*,  $p < 0.05$ ; #,  $p < 0.01$ ).

and resident mesenchymal cells, namely synoviocytes (32). The interaction between and activation of these cells perpetuate chronic inflammation and accelerate joint damage. T cells and B cells in RA synovium frequently accumulate underneath the synovial surface, defined as the lining layer, and organize into lymphocyte aggregates with some features of germinal centers (33, 34). The development of these structures appears to contribute to the pathogenesis of the disease, with local production of cytokines, chemokines, and autoantibodies resulting in increased severity. Recently, several lymphoid chemokines, which are mainly expressed in secondary lymphoid tissues, have been described and implicated in such organization typical of secondary lymphoid tissue (35). The synovium microenvironment is able to support the formation of functional germinal centers (34). Accordingly, chemokine expression might be important for the generation and maintenance of germinal centers by recruiting cells to the RA synovium. As the presence of germinal centers appears to be linked to severity, factors contributing to their formation may represent targets for treatment.

MIP-3 $\alpha$  was found to induce migration of immature DC, NK, and T cells, but not that of monocytes (10, 12, 36, 37). In this study we demonstrate that RA synovium was able to produce significant quantities of MIP-3 $\alpha$  in ex vivo cultures, while OA synovium produced much lower levels. Next we investigated the localization of



**FIGURE 6.** Effects of Th2 cytokines on MIP-3 $\alpha$  production by RA synovium. RA synovium samples (eight explants per point) from four patients were incubated for 7 days in the presence of IL-4 (50 ng/ml) or IL-13 (50 ng/ml) and IL-10 (50 ng/ml). Analysis of MIP-3 $\alpha$  production was performed using specific ELISA. Differences in MIP-3 $\alpha$  production between IL-4-, IL-13-, or IL-10-treated groups and the control group were analyzed with the nonparametric paired Wilcoxon test (\*,  $p < 0.05$ ; #,  $p < 0.001$ ).

MIP-3 $\alpha$  in RA synovium. Immunostaining of RA synovium with anti-MIP-3 $\alpha$  localized the positive cells in the lining layer and to a lesser extent in the lymphocyte aggregates. In addition, immature DC, defined by CD1a expression, were found at the same sites of MIP-3 $\alpha$  expression. Furthermore, our preliminary results indicate an accumulation of immature DC over mature DC in RA synovium in association with the local expression of MIP-3 $\alpha$  (38). In vitro studies confirmed synoviocytes as a major source of MIP-3 $\alpha$ . Such production could be involved in the trafficking of T cells and immature DC from blood to the inflammatory site, leading to proper cell positioning and cell-cell interactions.

Lymphoid follicle formation is also observed in chronic inflammatory sites, considered ectopic lymphoid organs. Indeed, RA synovium has been described as such a structure. Such an organization could contribute to Ag presentation in situ, to clonal expansion of Ag-specific B and T cells, and to inflammation chronicity (39). Although the synovial inflammatory microenvironment is associated with the recruitment of immature DC, it is unclear whether full DC maturation can be achieved. Lindhout et al. (40) reported that synoviocytes stimulated with TNF- $\alpha$  and IL-1 $\beta$  had intrinsic properties of follicular DC, which are characteristic of germinal centers.

The maintenance of an inflammatory infiltrate requires a distorted balance among leukocyte recruitment, retention, proliferation, and death. The failure of synovial T cell apoptosis coupled to an enhanced recruitment would lead to an accumulation of these cells within the rheumatoid synovium. The active retention of T cells, particularly the Th1 subset, by chemokine-driven mechanisms further contributes to their accumulation within the synovial compartment. Thus, inflammation in RA appears to persist as a direct result of sustained recruitment, retention, and survival of T cells mediated by stromal-derived factors associated with the inflamed synovial joint itself.

Recently, numerous studies have suggested a major involvement of T cells in RA pathogenesis, in particular with the demonstration of the presence of Th1 cells expressing IL-17. IL-17 shares mainly properties with IL-1 $\beta$  and TNF- $\alpha$ . The three cytokines activate the common transcription factor NF- $\kappa$ B in a variety of cell types. They all stimulate stromal cells such as dermal and synovial fibroblasts, endothelial cells, and epithelial cells to secrete IL-6, IL-8, and prostaglandin E<sub>2</sub> (41, 42). The present results extend these concepts to chemokines. Interactions among these three cytokines further amplify these effects. Indeed, combination of IL-17 with IL-1 $\beta$  or TNF- $\alpha$  often leads to synergistic or additive effects (41, 42). The present study demonstrates the potency of IL-17, IL-1 $\beta$ , and TNF- $\alpha$  alone and, moreover, in combination to induce MIP-3 $\alpha$  synthesis by synoviocytes and synovium. IL-1 $\beta$  was the most powerful inducer of MIP-3 $\alpha$ , and addition of IL-17 to TNF- $\alpha$  was synergistic. Furthermore, the combination of these cytokines significantly increases its production. It is of interest to link IL-17, a T cell cytokine that induces MIP-3 $\alpha$ , a chemokine that recruits CD4<sup>+</sup> memory T cells. This interaction could sustain inflammatory processes within the joint and amplify the involvement of T cells in the pathogenesis of RA. Conversely, blocking of IL-17 was associated with a profound inhibition of MIP-3 $\alpha$  production, contrasting with the poor inducing effect of IL-17 used alone on synoviocytes. Such a degree of inhibition may result from the additive or synergistic interactions commonly observed between IL-1, TNF, and IL-17 (25).

Regarding the respective contributions of IL-17, IL-1 $\beta$ , and TNF- $\alpha$  to RA pathogenesis, their control with specific or nonspecific inhibitors may lead to a therapeutic anti-inflammatory effect. The single addition of a soluble receptor reduced MIP-3 $\alpha$  release. A better effect was observed with the combination of three recep-

tors, confirming the interactions among these factors. The Th2 cytokines, IL-4, IL-13, and IL-10, have been classified as anti-inflammatory on the basis of their inhibitory effect on the production of IL-1 $\beta$ , TNF- $\alpha$ , IL-6, and IL-8 by monocytes and, moreover, synovium targets (25, 28, 29). We studied their effects on the production of MIP-3 $\alpha$  by RA synoviocytes stimulated with IL-1 $\beta$ , TNF- $\alpha$ , and IL-17. Both IL-4 and IL-13 were also able to inhibit the production of MIP-3 $\alpha$  by RA synovium and cytokine-stimulated synoviocyte. Not all cytokines classified as anti-inflammatory were able to block MIP-3 $\alpha$  production; in particular, addition of IL-10 had no effect on the production of MIP-3 $\alpha$  by RA synovium and RA synoviocytes stimulated with cytokines. This finding could be related to the elevated levels of endogenous IL-10 locally produced by synovium monocytes (29, 43). In keeping with this, IL-10 was found previously to have no effect on LIF or IL-6 production by isolated synoviocytes (26).

The present study shows that blocking the effects of IL-1 $\beta$ , TNF- $\alpha$ , and IL-17 at a specific level with a soluble receptor as well as at a broader level with Th2 cytokines inhibited not only the proinflammatory properties of synovium cells, but also the potential recruitment pattern induced by these cells. Such blocking reduced the production of a chemokine known to attract both DC and T cells involved in inflammation. Our observations suggest that MIP-3 $\alpha$  plays a role in mediating inflammatory process by its ability to chemoattract immature DC and T cells. Accordingly, this chemokine may represent a target for RA treatment, using cytokine-specific or nonspecific inhibition. Conversely, induction of full DC differentiation may favor better control of the inflammatory process and the causative agent.

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