Enhancing Effect of IL-1, IL-17, and TNF-α on Macrophage Inflammatory Protein-3 α Production in Rheumatoid Arthritis: Regulation by Soluble Receptors and Th2 Cytokines

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Enhancing Effect of IL-1, IL-17, and TNF-α on Macrophage Inflammatory Protein-3α Production in Rheumatoid Arthritis: Regulation by Soluble Receptors and Th2 Cytokines

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Macrophage inflammatory protein (MIP)-3α 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-1β and TNF-α and the T cell-derived cytokine IL-17 on MIP-3α production by RA synoviocytes. Addition of IL-1β, IL-17, and TNF-α induced MIP-3α production in a dose-dependent manner. At optimal concentrations, IL-1β (100 pg/ml) was much more potent than IL-17 (100 ng/ml) and TNF-α (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-3α production by activated synoviocytes, but IL-10 had no effect. Synovium explants produced higher levels of MIP-3α in RA than osteoarthritis synovium. MIP-3α-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-1β to synovium explants increased MIP-3α production. Conversely, specific soluble receptors for IL-1β, IL-17, and TNF-α inhibited MIP-3α 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-3α 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.
5 mm². Explants of synovium were cultured in triplicate in complete medium containing of 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% CO2/95% 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-cm² 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 maintain synoviocytes, explants were plated in 96-well plates at 10⁴ cells/well in 200 μl 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-3α levels

MIP-3α levels were measured by a two-site sandwich ELISA. Briefly, supernatants or serial dilutions of MIP-3α 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-3α mAb (1 μg/ml). These two mAb were provided by Dr. C. Caux (Schering-Plough). After washing, a peroxidase-coupled mouse anti-MIP-3α mAb (1 μg/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-3α mRNA expression

RNA was extracted from synovium biopsies by the guanidinium isocyanate method (27). To synthesize cDNA, 1 μg of total RNA was incubated with 2 μg oligo(dT) primer (Boehringer Mannheim, Meylan, France), 0.5 mM dNTP, 10 mM DTT, 10 μl of 10× RT buffer, and 20 μl of reverse transcriptase (Boehringer Mannheim). The PCR reaction was performed using 5 μl of this cDNA in the presence of 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM MgCl2, 200 μM dNTPs, 0.5 μM of each primer, and 2.5 μl of Taq polymerase (Boehringer Mannheim) in a total of 50 μl 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 μl) 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 presoakibilization, membranes were hybridized overnight at 68 °C with an MIP-3α oligonucleotide (5′-GAA-TCA-GAA-GGA-AAC-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-3α sense, 5′-ACC-CTT-CAT-GAT-GTA-GAC-3′; MIP-3α antisense, 5′-GTT-CTT-TTA-GAT-GGG-CAC-GAC-GG-3′.

Immunohistochemistry

RA synovium samples were fixed in 4% phosphate-buffered paraformaldehyde in 0.1% Triton X-100 in paraffin. Four-micron-thick paraffin sections were cut, deparaffinized, 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 then incubated with an anti-human MIP-3α 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-3α Ab was detected with a biotinylated rabbit anti-goat IgG followed by streptavidin-peroxidase. Peroxidase staining was revealed using 3-aminio-9-ethylcarbazole as substrate. Negative controls were established by adding nonspecific isotype controls as primary Abs. For double staining with anti-MIP-3α and anti-CD11a (IgG2b; BD Biosciences, Pont de Claiix, France) Abs, after an initial blocking with rabbit serum and BSA, primary anti-MIP-3α Ab was followed by a rabbit biotinylated anti-goat IgG and streptavidin-peroxidase. Peroxidase was developed with 3-aminio-9-ethylcarbazole. After a second blocking step for endogenous biotin with avidin-biotin, a mouse monoclonal CD11a (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 ± SEM of n separate experiments. Differences between cytokine-treated groups and the control group were compared with the Wilcoxon test.

Results

Effects of IL-1β, IL-7, and TNF-α on MIP-3α production by RA synoviocytes

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

When IL-1β, TNF-α, 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-1β (1 pg/ml) and TNF-α and IL-17 (1 ng/ml). With TNF-α combined with IL-17 or IL-16, synoviocytes produced much more MIP-3α than the sum of MIP-3α 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-1β.

Regulation by IL-4, IL-13, and IL-10 of MIP-3α production by RA synoviocytes induced by IL-1β, TNF-α, 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-3α by synoviocytes stimulated with optimal concentrations of IL-1β, TNF-α, 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-3α secretion. When combined with the proinflammatory cytokines, IL-4 and IL-13 reduced MIP-3α production. The effect was particularly pronounced with TNF-α-stimulated cells. In contrast, IL-10 had no effect on such MIP-3α production.

MIP-3α 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-1β, TNF-α, and IL-6 (25). Thus, we measured the spontaneous production of MIP-3α 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-3α were measured. Levels of MIP-3α produced by synovium explants were 12.8 ± 1.9 ng/ml.
MIP-3α-producing cells are present in RA synovium

To investigate the presence of MIP-3α-producing cells, immunostaining of RA synovium sections was performed with an anti-CD1a Ab, a marker of immature DC, followed by Southern blot analysis using specific labeled probes. MIP-3α mRNA could be detected in four of four separate experiments. Differences in MIP-3α mRNA expression by RA synovium explants (22, 26). After 7 days in culture, MIP-3α levels were measured by ELISA. Results were expressed as the mean ± SEM of four separate experiments. C, RA synoviocytes (10⁴) were cultured with IL-1β (50 pg/ml) or with IL-17 (50 ng/ml) or TNF-α (1 ng/ml) alone and in combination. After 72 h of culture, MIP-3α levels were measured by ELISA. Results were expressed as the mean ± SEM of four separate experiments. Differences in MIP-3α production between cytokine-treated groups and the control group were analyzed with the nonparametric paired Wilcoxon test (*, p < 0.05; #, p < 0.005).

MIP-3α polyclonal Ab. As shown in Fig. 3, cells stained positively for MIP-3α 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-1β, TNF-α, or IL-17 (data not shown). Double staining with anti-CD1a Ab, a marker of immature DC, showed a close association between MIP-3α-producing cells and CD1a-positive immature DC (Fig. 3C).

IL-1β, TNF-α, and IL-17 induce MIP-3α production by RA synovium

The effects of exogenous IL-1β, TNF-α, and IL-17 were analyzed on MIP-3α production by RA synovium explants. Samples from three patients were cultured in the presence of 50 ng/ml TNF-α and IL-17 or 100 pg/ml IL-1β. Addition of IL-1β increased MIP-3α 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 IL-1α (1 pg/ml) or IL-17 (1 ng/ml) used alone. After 72 h of culture, MIP-3α levels were measured by ELISA. Results were expressed as the mean ± SEM of four separate experiments. Differences in MIP-3α production between cytokine-treated groups and the control group were analyzed with the nonparametric paired Wilcoxon test (*, p < 0.05; #, p < 0.005).

MIP-3α 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 monocytic-derived cytokines IL-1β and TNF-α 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-3α. 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-3α. IL-4
and IL-13 inhibited RA synovium production of MIP-3α 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,
MIP-3α in RA synovium. Immunostaining of RA synovium with anti-MIP-3α 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-3α 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-3α (38). In vitro studies confirmed synoviocytes as a major source of MIP-3α. 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-α and IL-1β 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 the 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-3α plays a role in mediating inflammatory process by its ability to chemottract 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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**References**

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