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Modulation of Formyl Peptide Receptor Expression by IL-10 in Human Monocytes and Neutrophils

Maryse Thivierge, Jean-Luc Parent, Jana Stankova, and Marek Rola-Pleszczynski

IL-10, originally described as a cytokine synthesis inhibitory factor, is secreted by a number of cells of the immune system, including monocytes and T cells. Although IL-10 is being assigned as an immunosuppressive cytokine, our study showed that FMLP-R mRNA was rapidly up-regulated by exposure of monocytes to graded concentrations of this cytokine, with maximal (three- to fourfold) stimulation with 10 ng/ml. The effect was rapid, being observable as early as 1 h of treatment with IL-10, maximal between 2 and 4 h, and still evident after 24 h and was associated with an increase of receptor expression on the cell surface as assessed by flow cytometry analysis. Pretreatment of monocytes with actinomycin D completely abrogated the effect of IL-10, suggesting a transcriptional regulation. Moreover, IL-10-treated monocytes showed a significantly enhanced functional responsiveness to FMLP with enhanced (three- to fourfold) chemotaxis and augmented (twofold) intracellular calcium mobilization. In polymorphonuclear neutrophils (PMN), IL-10 also mediated a twofold augmentation of FMLP-R expression. In parallel experiments, we observed that IL-10 could differentially modulate other chemotactic receptors. Hence, we observed that IL-10 augmented two- to threefold platelet-activating factor receptor (PAF-R) expression, whereas it had no significant effect on the fifth component of complement (C5a) receptor (C5a-R) expression. Collectively, our results demonstrate that IL-10 may play an important role in inflammatory processes through modulation of chemotactic receptor expression. The Journal of Immunology, 1999, 162: 3590–3595.

The interaction of chemotactic factors and their corresponding receptors on leukocytes constitutes the basis for chemotaxis to inflammatory sites and cell activation. Thus, in addition to the production of chemotactic factors, the level of expression of their receptors on target cells will determine the magnitude of the response. Among the chemotactic ligand receptors on leukocytes are those for the synthetic bacterial analogue FMLP-R and the fifth component of complement C5a receptor (C5a-R), which may be instrumental in monocyte and neutrophil recruitment to inflammatory and/or infectious sites. These cells also express the receptor for platelet-activating factor (PAF)1, a potent lipid mediator implicated in inflammatory and immune responses. FMLP-R, C5a-R, and PAF-R cDNAs and genes have been cloned and shown to belong to a subclass of small seven-transmembrane-spanning G-protein-coupled receptors (1–7).

The functional potential of leukocytes in inflammation is diverse and depends heavily on the cytokine network that is activated after stimulation of these cells by various inflammatory agents encountered in the tissues. In this context, negative and positive regulation can result in part from the action of functionally distinct subsets of T helper cell-derived cytokines. IL-10, originally defined as a cytokine synthesis inhibitory factor, is produced mainly by Th2 lymphocytes, by B lymphocytes, and by monocytes/macrophages (8, 9). IL-10 is a pleiotropic cytokine that can exert either immunostimulatory or immunosuppressive effects on a variety of cell types. In vitro studies have shown that this cytokine exhibits immunosuppressive functions like down-regulation of T and NK cell activity and inhibition of Ag presentation (10–13). IL-10 is also a potent inhibitor of monocyte/macrophage activation and resultant cytotoxic effects. It can suppress the production of numerous cytokines, including TNF-α, IL-1, IL-6, IL-10, and IL-12, as well as the synthesis of reactive oxygen intermediates by activated monocyte/macrophages (14–16). Furthermore, evidence for the in vivo antiinflammatory role of IL-10 was demonstrated in different models (17–19). Although IL-10 is being billed as an immunosuppressive cytokine, numerous immunostimulatory effects can be ascribed to this cytokine. Hence, IL-10 is one of the primary stimulators of Ab production; it acts as a growth costimulator for thymocytes, mast cells, and B cells, as well as an enhancer of cytotoxic T cell development (20–22); it enhances monocyte-mediated Ab-dependent cellular cytotoxicity and T cell responses to IL-2 (23, 24); it has also been suggested as a potent recruitment signal for leukocyte migration in vivo (25, 26). Therefore, IL-10 has a variety of different activities, not all of which are immunosuppressives.

Regulation of FMLP-R expression has not been extensively investigated but was previously shown to be modulated by cAMP-elevating agents on HL-60 cells and U937 (27, 28). Furthermore, recent studies demonstrated that the inflammatory cytokines IFN-γ and TNF-α could regulate this receptor, but cytokine modulation of FMLP-R has been reported for granulocytes and granulocytic cells but not for monocytes. Hence, in human neutrophils, TNF-α was shown to promote expression of FMLP-R (29), whereas IFN-γ was shown to induce the expression of these receptors in HL-60 cells differentiated into neutrophils (30). Considering the important immunomodulatory effects of IL-10 and in particular its capacity to antagonize the effects of IFN-γ and TNF-α, we were interested...
to study the effect of this cytokine on chemotactic receptors, particularly FMLP-R. Here, evidence is provided that IL-10 can also be implicated in controlling inflammatory processes through modulation of chemotactic receptor expression.

Materials and Methods

Chemical reagents

IL-10 was obtained from Pepro Tech (Rocky Hill, NJ); FMLP and histrionic were from Sigma (St. Louis, MO); PAF was from Biomol (Plymouth Meeting, PA); actinomycin D was from Merck Sharp & Dohme International (Rahway, NJ).

Preparation of monocytes

Human venous blood from healthy medication-free volunteers was collected on citrate/dextrose/adenine. The peripheral blood mononuclear leukocytes were enriched by dextran sedimentation, layered over a Ficoll-Hypaque cushion, and centrifuged at 400 × g for 20 min. Mononuclear leukocytes were collected at the interface and washed twice with PBS and resuspended in RPMI 1640 medium with 10% heat-inactivated FBS. Monocytes were then purified by adherence (60 min, 37°C) to the surface of plastic petri dishes coated with defbrinized autologous serum and removed with EDTA (0.01 M) in RPMI 1640, 10% FBS. This was effective in enriching the cell population to greater than 90% monocytes, with a viability greater than 98%, as assessed by Wright-Giemsa staining and trypan blue exclusion, respectively. Cells were resuspended in RPMI 1640, 10% FBS, at a final concentration of 2 × 10^6 cells/ml. Monocytes were allowed to rest overnight in polypropylene tubes to allow them to return to baseline status following initial activation by adherence. The medium was then removed by pipetting and replaced by fresh RPMI 1640, 10% FBS, before stimulation with the appropriate stimuli.

mRNA studies

After appropriate treatment, cells were pelleted in 15-ml polypropylene tubes, and total cellular RNA was isolated by acid guanidium thiocyanate-phenol-chloroform extraction according to Chomczynski and Sacchi (31). RNA (10 μg) was separated by electrophoresis on 1% agarose and transferred onto a Hybond-N (Amersham, Arlington Heights, IL) for 20 min at 10% was added. The membranes were then washed in PBS and resuspended in PBS before flow cytometry analysis. The acquisition was done with 2000 events per sample.

Calcium fluorometry

For Ca^{2+} mobilization assays, 3 × 10^6 cells were loaded in HBSS (Life Technologies, Gaithersburg, MD) containing 350 mg/L NaHCO_3 and 10 mM HEPES, pH 7.0, with the calcium indicator fura 2-AM (Molecular Probes) for 30 min at room temperature. Loaded cells were washed twice, suspended in fresh loading buffer, and added to a constantly stirred cuvette in a SLM/Aminco spectrophotofluorometer (SLM Instruments, Urbana IL). The concentration of Ca^{2+} was brought to 1.5 mM by adding a solution of CaCl_2 into the cuvette 10 min before recordings. Maximal fluorescence (F_max) was determined by adding Triton X-100 to a final concentration of 0.5%. Minimal fluorescence (F_min) was determined by subsequent addition of EGTA, in Tris-HCl buffer (100 mM, pH 9.0) to 125 mM. Stimuli consisted of FMLP, PAF, and histamine.

Chemotaxis assay

Monocyte chemotactic activity was performed with Boyden chambers using a modified Boyden chamber chemotaxis assay. FMLP or control medium were added to the lower chamber and 200 μl of monocytes (6 × 10^5) in Geo’s BSS (Life Technologies) supplemented with 2% BSA were added to the upper chamber. The two chambers were separated by a 5-μm pore size polycarbonate filter (Neuroprobe, Cabin John, MD). After incubation for 2 h, the filter was disassembled, and the upper side of the filter was scraped free of cells. Cells on the lower side were removed with EDTA (5 mM) and centrifuged before counting by FACScan (Becton Dickinson) analysis with scatter-gating on monocytes. The results were then converted to a chemotaxis index (mean number of cells migrating to a specific stimulus/mean number of cells migrating to control medium). The statistical significance of the chemotactic indices of cells migrating in response to FMLP vs medium control was evaluated using the Student’s t test.

Flow cytometry

The expression of FMLP-R, PAF-R, and C5a-R on the surface of monocytes was monitored with a fluorescent analogue of FMLP, N-formyl-norleucyl-leucyl-phenylalaninyleucyl-tirosyl-lysine-fluorescein (FLPPEP) (Molecular Probes, Eugene, OR), our monoclonal anti-hPAF-R Ab, or the monoclonal anti-CD88 Ab (Serotec, Raleigh, NC). In brief, 3 × 10^5 treated cells were washed twice with PBS and labeled 30 min at 4°C with FLPEP (2 μM), anti-CD88 (0.5 μg), or anti-PAF-R (dilution 1:50). Alternatively, cells incubated with anti-PAF-R or anti-CD88 were then washed with cold PBS and incubated 30 min at 4°C with FITC-conjugated goat anti-mouse IgG (Bio/Can Scientific, Mississauga, Ontario, Canada). Finally, cells were washed in PBS and resuspended in PBS before flow cytometry analysis with a FACScan flow cytometer (Becton Dickinson, San Jose, CA). The acquisition was done with 2000 events per sample.
IL-10 resulted in a gradual augmentation in the levels of FMLP-R transcripts. Whereas 1 ng/ml of IL-10 was effective to induce a significant augmentation in the levels of FMLP-R expression, concentrations from 5 to 20 ng/ml resulted in a 2.5- to more than threefold augmentation of the transcripts. The maximal effect was observed with 10 ng/ml of IL-10, and this concentration was used for subsequent experiments. In additional experiments, we tested whether IL-10 could modulate other chemotactic receptors. In these experiments, monocytes were cultured in the presence or absence of IL-10 (10 ng/ml) for up to 24 h. At selected time points (1, 2, 4, 8, and 24 h poststimulation), RNA was isolated, and the levels of FMLP-R, PAF-R, and C5a-R mRNA were determined. As shown in Fig. 2, IL-10 treatment differentially modulated the levels of expression of these genes. Hence, as for FMLP-R, PAF-R gene expression was shown to be up-regulated by IL-10. Kinetic studies revealed that IL-10-mediated up-regulation for both genes was rapid, observed as early as 1 h, peaked in less than 4 h poststimulation, and was still noticeable at 24 h. In contrast to FMLP-R and PAF-R, the expression of C5a-R was unaffected by IL-10.

Since polymorphonuclear neutrophils (PMN) constitutively express the IL-10 receptor (33) and represent a cellular target of IL-10 (Refs. 34–37, and therein), we investigated the effect of this cytokine on FMLP-R expression on human PMN. Freshly isolated PMN were incubated 2 h and 4 h with IL-10 (10 ng/ml) and examined for their FMLP-R mRNA levels. As illustrated in Fig. 3, PMN treated with IL-10 showed an augmented expression of surface FMLP-R protein, which was significant (IL-10-treated, 21.2 ± 1.8, vs control cells, 12.7 ± 1.7; p < 0.0139) 24 h after their initial exposure to this stimulus. Monocytes treated with IL-10 also showed a significant increase in the peak channel fluorescence, as compared with untreated cells (Fig. 6). IL-10-induced up-regulation of FMLP-R protein expression was maximal by 24 h (IL-10-treated, 60.5 ±

**Effect of IL-10 on FMLP-R gene transcription**

The drastic up-regulation of FMLP-R mRNA accumulation in monocytes secondary to IL-10 treatment could result from a transcriptional activation of the FMLP-R gene. To assess this issue, we performed experiments in which monocytes were pretreated with the transcriptional inhibitor actinomycin D for 15 min, and then stimulated with IL-10 (10 ng/ml) for 2 h before RNA extraction and Northern blot analysis. As shown in Fig. 4, this pretreatment completely abolished the IL-10-induced accumulation of human FMLP-R mRNA and suggested a transcriptional effect on the FMLP-R gene.
6.2, vs control cells, 26.8 ± 3.6; p < 0.0001). By 48 h, the difference had vanished (IL-10-treated, 51.0 ± 5.2, vs control cells, 40.2 ± 4.6, p < 0.01; data not illustrated). Parallel cytometry experiments were performed to evaluate the effect of IL-10 on PAF-R and C5a-R expression. As illustrated in Figs. 5 and 6, IL-10 induced a significant increase in PAF-R expression (IL-10-treated PMN, 7.3 ± 0.8, vs control cells, 4.5 ± 0.5; p < 0.0384, and IL-10-treated monocytes, 29.1 ± 4.2, vs control cells, 13.8 ± 1.8; p < 0.0009), whereas it had no effect on C5a-R expression (IL-10-treated PMN, 5.9 ± 0.7, vs control cells, 7.5 ± 1.2; p < 0.1682.
1.5 mM CaCl2 10 min before stimulation with FMLP (10 nM) and loaded with fura 2-AM. After loading, cells were supplemented with medium or IL-10 (10 ng/ml), washed, and incubated 24 h with medium or IL-10 (10 ng/ml), washed; chemotactic activity was measured in response to FMLP using a modified Boyden-chamber chemotaxis assay. The figure illustrates the means ± SEM of three independent experiments. *, p < 0.04, IL-10-treated vs untreated cells.

and IL-10-treated monocytes, 14.5 ± 2.2, vs control cells, 14.8 ± 6.5; p < 0.9262).

**Effect of IL-10 on monocyte functional responsiveness to FMLP**

We next investigated whether the IL-10-induced increase in FMLP-R expression was associated with an augmentation of biologic responsiveness to FMLP. The biological activity of the receptor on monocytes was evaluated for chemotactic response and calcium mobilization at 24 h after exposure to IL-10 (10 ng/ml). As illustrated in Fig. 7, IL-10-treated monocytes showed a significantly (p < 0.04) increased migration to FMLP when compared with medium-treated cells. Similarly, PMN pretreated with IL-10 showed an increased migration to FMLP when compared with medium-treated cells (data not illustrated). Furthermore, as shown in Fig. 8, monocytes pretreated for 24 h with IL-10 showed an increase in their response to FMLP in terms of intracellular Ca²⁺ mobilization (Δ[Ca²⁺]) = 329 nM ± 45.8 for control cells vs 543 nM ± 89.4 for IL-10-treated cells, p < 0.045). Similarly, the [Ca²⁺]i rise induced by PAF was also enhanced by a prior stimulation with IL-10. In counterpart, IL-10 had no effect on the response of monocytes to a different stimulus, namely histamine. Collectively, these data show that elevation of FMLP-R expression following stimulation with IL-10 is accompanied by an enhanced functional activity of these receptors.

**Discussion**

Monocytes are believed to play a pivotal role at sites of inflammation, since they have the potential to activate other cell types through production of stimulatory cytokines. Functionally, monocytes are among the most sensitive cells to the effects of IL-10. The release of reactive oxygen metabolites, in addition to cytokines such as IL-1, IL-6, TNF-α, and IL-10, is markedly inhibited by IL-10 (14–16). Moreover, PMN, often regarded as terminally differentiated cells devoid of transcriptional activity, were recently shown to release, like monocytes, proinflammatory cytokines such as TNF-α, IL-1β, and IL-8 that can be suppressed by IL-10 (Ref. 37 and therein). Other effector functions of PMN were also shown to be modulated by IL-10, such as PAF production, phagocytic and bactericidal activities, and oxidative metabolism (34–36). Data presented here provide evidence that, in addition to its immunosuppressive potential, IL-10 may regulate leukocyte trafficking through modulation of FMLP-R expression on these cells. In fact, we have shown that FMLP-R mRNA is rapidly induced by IL-10, within 2 h, and that this induction is primarily due to transcriptional activation. According to our data, the IL-10-induced accumulation of FMLP-R mRNA level was accompanied by an augmentation of FMLP-R expression at the cell surface. The up-regulation was evident at 24 h of stimulation with IL-10 and declined thereafter. We demonstrated that these newly induced receptors were functional since pretreatment with IL-10 was associated with an augmentation of biologic responsiveness to FMLP in terms of chemotaxis and calcium mobilization. Whereas IL-10 concentrations in the range of 10 ng/ml may not be quite physiological, pathophysiological concentrations, especially at the local level, such as those that may be expected to modulate chemotactic receptor expression in inflammatory sites, could well be quite elevated. Moreover, concentrations in the ng/ml level have been used in various studies to demonstrate the regulatory potential of IL-10 in a number of immune and inflammatory events.

Moreover, we found that IL-10 up-regulation was not restricted to FMLP-R and could also be observed for PAF-R expression. In contrast to FMLP-R and PAF-R, the expression of C5a-R was shown to be unaffected by IL-10. During the course of this work and in concordance with our observations, another family of chemotactic receptors, the CC chemokine receptors CCR5, CCR2, and CCR1, were recently shown to be up-regulated by IL-10 on human monocytes (Ref. 38, and our unpublished results).

Whereas coupling and desensitization processes for FMLP-R have been extensively investigated (39), only a few studies describing the regulation of FMLP-R expression have been reported. Augmentation of FMLP-R expression in HL-60 cells was observed following elevation of cAMP levels (27, 28). Cytokine modulation of FMLP-R has been reported for granulocytic cells but not for monocytes. Hence in human neutrophils, TNF-α was shown to promote expression of FMLP-R (30), whereas IFN-γ was shown to induce the expression of these receptors by HL-60 cells differentiated into neutrophils (29). The mechanisms involved in IL-10-mediated up-regulation of FMLP-R expression remain to be elucidated.

The mechanisms underlying the various actions of IL-10 are partially understood. In monocytes, it was shown that IL-10 signaling pathway promotes the activation of the Janus kinase (JAK) 1 and TYK2 tyrosine kinases, leading to the tyrosine phosphorylation of the signal transducers and activators STAT1α and STAT3

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**FIGURE 7.** Effect of IL-10 on monocyte chemotaxis. Monocytes were incubated 24 h with medium or IL-10 (10 ng/ml) and washed; chemotactic activity was measured in response to FMLP using a modified Boyden-chamber chemotaxis assay. The figure illustrates the means ± SEM of three independent experiments. *, p < 0.04, IL-10-treated vs untreated cells.

**FIGURE 8.** Effect of IL-10 on ligand-induced monocyte [Ca²⁺]i flux. Monocytes were incubated 24 h with medium or IL-10 (10 ng/ml), washed, and loaded with fura 2-AM. After loading, cells were supplemented with medium or IL-10 (10 ng/ml), washed; chemotactic activity was measured in response to FMLP using a modified Boyden-chamber chemotaxis assay. The figure illustrates the means ± SEM of three independent experiments. *, p < 0.04, IL-10-treated vs untreated cells.
(40); the resulting STAT proteins form DNA binding complexes that bind as homo- and heterodimers to GAS (IFN-γ activation sequence) in the regulatory regions of target genes (41, 42). Recently, detectable amounts of transcription factors such as STAT1 and STAT3 were demonstrated in human neutrophils, which further emphasized the potential ability of these cells to be functionally regulated at the level of gene transcription (33). According to our data, IL-10 mediated the up-regulation of FMLP-R expression through a transcriptional mechanism. Whether the signaling events triggered by IL-10 involve GAS-related elements in the promoter region of the FMLP-R gene remains to be demonstrated.

In summary, our results suggest that IL-10, a known immunosuppressive cytokine, could contribute to proinflammatory processes through up-regulation of FMLP-R. In which physiological or pathophysiological circumstances such events may play a significant role remains to defined. This paradoxical situation suggests that IL-10 can sequentially combine proinflammatory and immunosuppressive actions on target cells. In a context of an inflammatory reaction, N-formyl peptides released by invading bacteria and lysed cells could promote migration of leukocytes such as monocytes and PMN, the principal targets of formyl peptides, and generally considered to be the central players in the process of inflammation. Once in the inflamed tissue, they can release inflammatory mediators and cytokines. In such a context, IL-10 may provide both a mechanism for accumulation of mononuclear and polymorphonuclear cells within the inflammatory lesion and a feedback mechanism to counteract an overwhelming response by controlling their oxidative metabolism and their production of proinflammatory cytokines.

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