

## **Interleukin 10 Increases CCR5 Expression and HIV Infection in Human Monocytes**

By Silvano Sozzani,\* Silvia Ghezzi,<sup>‡</sup> Gioacchino Iannolo,\* Walter Luini,\* Alessandro Borsatti,\* Nadia Polentarutti,\* Antonio Sica,\* Massimo Locati,\* Charles Mackay,<sup>¶</sup> Timothy N.C. Wells,<sup>§</sup> Priscilla Biswas,<sup>‡</sup> Elisa Vicenzi,<sup>‡</sup> Guido Poli,<sup>‡</sup> and Alberto Mantovani\*<sup>||</sup>

From the \*Istituto di Ricerche Farmacologiche 'Mario Negri', 20157 Milan, Italy; <sup>‡</sup>the AIDS Immunopathogenesis Unit, San Raffaele Scientific Institute, 20132 Milan, Italy; <sup>§</sup>the Geneva Biomedical Research Institute, Glaxo Wellcome Research and Development, Geneva, Switzerland; <sup>¶</sup>the Section of Pathology and Immunology, Department of Biotechnology, University of Brescia, 25123 Brescia, Italy; and <sup>¶</sup>Millennium Biotherapeutics Inc., Cambridge, MA

### **Summary**

The immunosuppressive and antiinflammatory cytokine interleukin (IL) 10 selectively upregulates the expression of the CC chemokine receptors CCR5, 2, and 1 in human monocytes by prolonging their mRNA half-life. IL-10-stimulated monocytes display an increased number of cell surface receptors for, and better chemotactic responsiveness to, relevant agonists than do control cells. In addition, IL-10-stimulated monocytes are more efficiently infected by HIV BaL. This effect was associated to the enhancement of viral entry through CCR5. These data add support to an emerging paradigm in which pro- and antiinflammatory molecules exert reciprocal and opposing influence on chemokine agonist production and receptor expression.

Chemokines are a superfamily of proteins that play a crucial role in immune and inflammatory reactions and in viral infections (1–6). Chemokines can be grouped in two main subfamilies defined as CXC (or  $\alpha$ ) and CC (or  $\beta$ ) according to the spacing of the first two cysteine residues (1, 3). Recently, the new chemokines lymphotactin and fractalkine have been reported and define two additional classes of the chemokine superfamily (1, 3). Inflammatory cytokines (e.g., IL-1, TNF- $\alpha$ , and IL-6) and bacterial products are potent inducers of chemokine production both in vitro and in vivo (1–3). Contrary to this, molecules with immunosuppressive and antiinflammatory activity, such as IL-10 and glucocorticoid hormones, inhibit chemokine production (7, 8).

Chemokines bind to and activate seven-transmembrane domain receptors (1–3, 9–11). Four receptors for the CXC chemokines, named CXCR1–4, and eight for CC chemokines (CCR1–8) have been cloned and characterized in leukocyte populations. With only a few exceptions, chemokine receptors bind multiple chemokines and recently it was shown that some of them can function as entry/fusion cofactors for HIV-1 infection (4–6, 11).

The regulation of expression of chemokine receptors may play a central role in the tuning of the chemokine action, but to date it has been the object of limited attention (12–15). Here we report that the immunosuppressive and antiinflammatory cytokine IL-10 (16–18) selectively upregulates the expression of CC chemokine receptors in human mononuclear phagocytes by increasing the half-life of their mRNA. This unexpected action is functionally relevant for migration and HIV infection. These results are consistent with a novel paradigm of regulation of chemokines and their receptors by pro- and antiinflammatory signals.

### **Materials and Methods**

**Monocytes.** PBMCs were obtained from buffy coats of healthy blood donors. Monocytes were obtained by Ficoll (Biochrom, Berlin, Germany) and Percoll (Pharmacia Biotech AB, Uppsala, Sweden) gradients (19). Purity was >90% as assessed by immunofluorescence and FACS<sup>®</sup> analysis for cell surface expression of CD14.

**FACS<sup>®</sup> Analysis.** Cell staining was performed using monoclonal antibodies followed by FITC-conjugated affinity-purified, isotype-specific goat anti-mouse antibody (Techno-Genetics Turin, Italy). Anti-CD14 (IgG2a; gift of Dr. P. Beverly, Jemmer Institute, London, UK) and LS87 5C7 (anti-CCR5; IgG2a) (20) were used. Mouse IgG2a, kappa (UPC10) (Sigma Chemical Co., St. Louis, MO) was used as irrelevant control antibody. In some cases

S. Sozzani and S. Ghezzi contributed equally to this work.

results are expressed as relative fluorescence intensity (RFI), calculated according to the formula:  $RFI = \frac{\text{mean fluorescence (sample)} - \text{mean fluorescence (control)}}{\text{mean fluorescence (control)}}$ .

**Chemotaxis.** Monocyte migration was evaluated using a chemotaxis microchamber technique (NeuroProbe, Pleasanton, CA) using polycarbonate filters (5  $\mu\text{m}$  pore size; NeuroProbe), as previously described (19). Human recombinant monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1 $\beta$  and RANTES (regulated on activation, normal T cell expressed and secreted) were from PeproTech Inc. (Rocky Hill, NJ). Human rMIP-1 $\alpha$ /LD78 was from Dr. L. Czaplewski (British Bio-technology Limited, Cowley, UK). Aminooxypentane (AOP)-RANTES (gift of Dr. A.E.I. Proudfoot, Glaxo, Geneva, Switzerland) was prepared as previously described (21). The chamber was incubated at 37°C in air with 5% CO<sub>2</sub> for 90 min. At the end of the incubation, filters were removed and stained with Diff-Quik (Baxter, Rome, Italy). Five high power oil-immersion fields were counted.

**Northern Blot and Runoff Analysis.** RNA was extracted by the guanidium thiocyanate method, and blotted and hybridized as previously described (15). Probes were labeled by Megaprime DNA labeling system (Amersham International, Buckinghamshire, UK) with  $\alpha^{32}\text{P}$ -dCTP (3000 Ci/mmol; Amersham). Membranes were prehybridized at 42°C in Hybrisol (Oncor, Inc., Gaithersburg, MD) and hybridized overnight with 10<sup>6</sup> cpm/ml of <sup>32</sup>P-labeled probe. Membranes were then washed three times at room temperature for 10 min in 0.2 $\times$  SSC (1 $\times$  SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 0.1% SDS, and then washed twice at 60°C for 20 min in 0.2 $\times$  SSC, 0.1% SDS before being autoradiographed using Kodak XAR-5 films and intensifier screens (Eastman Kodak Co., Rochester, NY) at -80°C. cDNA probes were obtained as previously described (15). For CCR2 and CCR5, fragments of the open reading frame including a portion of the 3'-untranslated regions were generated by reverse transcriptase PCR from NK cell total RNA (14). The specificity of the two probes was confirmed in Northern blot experiments using CCR2 and CCR5 single cell transfectants (data not shown).

**HIV Assays.** Monocytes were plated in 24-well plates (Falcon, Becton-Dickinson Labware, Lincoln Park, NJ) at 0.5  $\times$  10<sup>6</sup> cells/ml in RPMI 1640 (Bio Whittaker, Verviers, Belgium) supplemented with 10% FCS (Hyclone Europe, Oud-Beijerland, The Netherlands). Monocytes were stimulated with IL-10 (0.1–10 ng/ml) for 6 h and then incubated in the presence and absence of 200 ng/ml of CC chemokines including RANTES, MIP-1 $\alpha$ , MIP-1 $\beta$ , and MCP-1, or of AOP-RANTES for 30 min before HIV infection. The macrophage-tropic BaL strain of HIV-1, known to infect CD4<sup>+</sup> cells with the cooperation of CCR5 (22, 23), was treated with 2  $\mu\text{g}/\text{ml}$  of RQ1 RNase-free DNase (Promega, Madison, WI) for 30 min at room temperature, and added to the cultures at the multiplicity of infection of 0.1. Culture supernatants were collected at fixed intervals, stored at -80°C until tested for a conventional analysis of their Mg<sup>2+</sup>-dependent reverse transcriptase activity (24). Aliquots (0.5  $\times$  10<sup>6</sup> cells) of either untreated or IL-10-treated cells were harvested 1, 16, and 40 h after infection, and were then centrifuged; cell pellets were stored at -80°C until they were tested for proviral DNA synthesis. Cell pellets were lysed by incubation at 56°C for 1 h with 250  $\mu\text{l}$  of a buffer of SDS-NaCl EDTA-Tris containing 1,200  $\mu\text{g}/\text{ml}$  of proteinase K. PCR amplification of HIV-1 DNA was based on published methods (25, 26). In brief, 50  $\mu\text{l}$  of lysed suspension were diluted to 100  $\mu\text{l}$  in a buffer containing 50 mM Tris (pH 8.5), 15 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2.5 mM MgCl<sub>2</sub>, 10  $\mu\text{g}$  BSA, 0.2 mM deoxynucleoside triphosphates, 0.5  $\mu\text{M}$  of each oligonucleotide primer, and Taq DNA polymerase (2 U; Perkin-Elmer Corp., Nor-

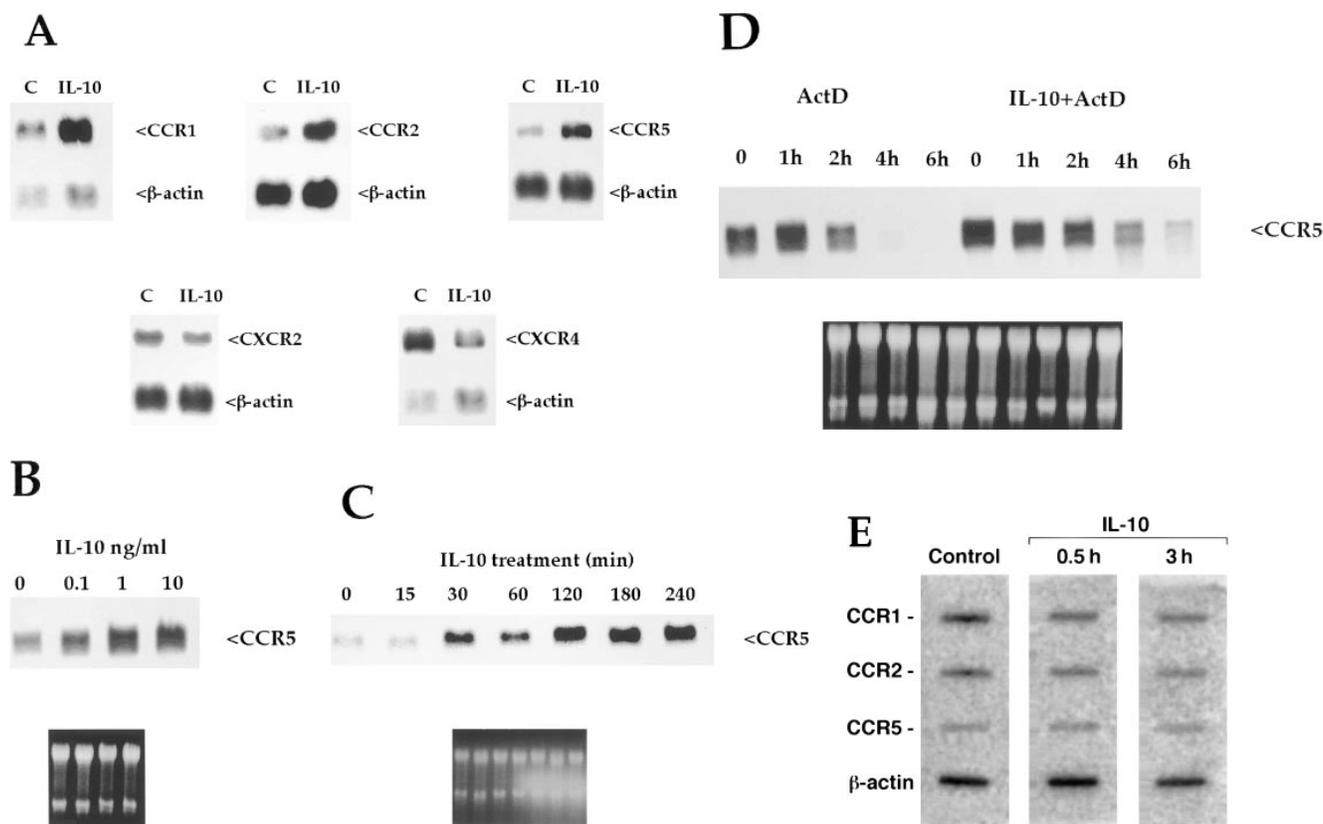
walk, CT). The region spanning U5 and leader sequence was detected by using primers 5'-CTCTAGCAGTGGCGCCCC AACAA-3' and 5'-TCTCCTTCTAGCCTCCGCTAGTC-3' (26). The intersample variation was monitored by parallel PCRs measuring total cellular DNA with primers specific for HLA (25) after 25 cycles of amplification.

## Results

Incubation of human monocytes with an optimal concentration of IL-10 (10 ng/ml) for 4 h increased the expression of CCR1, 2, and 5 as evaluated by Northern blot analysis (Fig. 1 A). CCR3 and CCR4 are expressed at very low levels in human monocytes (15) and their expression was not induced by IL-10 (data not shown). No major variations in the expression of CXCR2 were detectable in 3 different donors (Fig. 1 A). In one experiment a partial reduction of CXCR4 mRNA levels was detected (Fig. 1 A). Because of the peculiarity of CCR5 in terms of ligands and its relevance as HIV fusion cofactor, subsequent studies were conducted on this receptor. The effect of IL-10 was concentration dependent (effective concentration [EC]<sub>50</sub> = 0.3  $\pm$  0.1 ng/ml; 0.015 nM) and fast, already detectable after 30 min and reaching a plateau after 2 h of stimulation, with a maximal increase observed at 10 ng/ml (0.5 nM) IL-10 (Fig. 1, B and C). The estimated half-life of CCR5 mRNA was 165 min and was augmented to 260 min ( $n = 2$ ) after exposure to IL-10 (Fig. 1 D). In contrast, the rate of nuclear transcription of the gene, as investigated by nuclear runoff analysis, was not affected (Fig. 1 E).

Having observed that IL-10 selectively upregulated expression of the CC chemokine receptors CCR1, 2, and 5, it was important to investigate the functional relevance of this enhancement. As shown in Fig. 2, IL-10-treated monocytes responded better to CC chemokines in terms of chemotactic migration (Fig. 2 A) and intracellular calcium transients (data not shown). The effect was best observed when suboptimal agonist concentrations were used (e.g., 1 and 10 ng/ml for MCP-1 and MIP-1 $\beta$ , respectively). At the concentration of 10 ng/ml, IL-10-treated monocytes showed an increase of 237 and 189% in chemotaxis above control values for MCP-1 and MIP-1 $\beta$ , respectively. It is noteworthy that IL-10 pretreatment did not appreciably affect the spontaneous migration of monocytes. In agreement with these results, IL-10 substantially increased the expression of CCR5 evaluated by both cytofluorimetric analysis (Fig. 2, B and C) and by ligand binding assays with radiolabeled MIP-1 $\beta$  (13,864  $\pm$  3,257 and 22,925  $\pm$  3,804 receptors per cell,  $P < 0.01$ ; with identical affinity 2.6  $\pm$  0.9 and 3.4  $\pm$  0.9 nM for control and treated cells, 20 ng/ml for 20 h, respectively). The effect of IL-10 on CCR5 surface expression was also observed when monocytes were exposed to HIV BaL (Fig. 2 C).

The macrophage-tropic HIV-1 strain BaL (27) was used to investigate whether IL-10-induced upregulation of CC chemokine receptors affected the susceptibility of monocytes to infection. A productive HIV infection was observed in human peripheral blood monocytes that were incubated



**Figure 1.** Selective upregulation of CC chemokine receptors by IL-10. (A) Monocytes were stimulated with 10 ng/ml IL-10 for 4 h before Northern blot analysis. (B) Monocytes were stimulated with different concentrations of IL-10 for 4 h. (C) Monocytes were stimulated with 10 ng/ml IL-10 for different times. (D) Monocytes were incubated in the presence or absence of IL-10 (10 ng/ml) for 3 h and then Actinomycin-D (1  $\mu$ g/ml; ActD; Sigma) was added for the indicated times. (E) Nuclear runoff analysis of CCR1, 2, and 5 genes. Human monocytes were incubated with 10 ng/ml IL-10 for different periods as indicated.

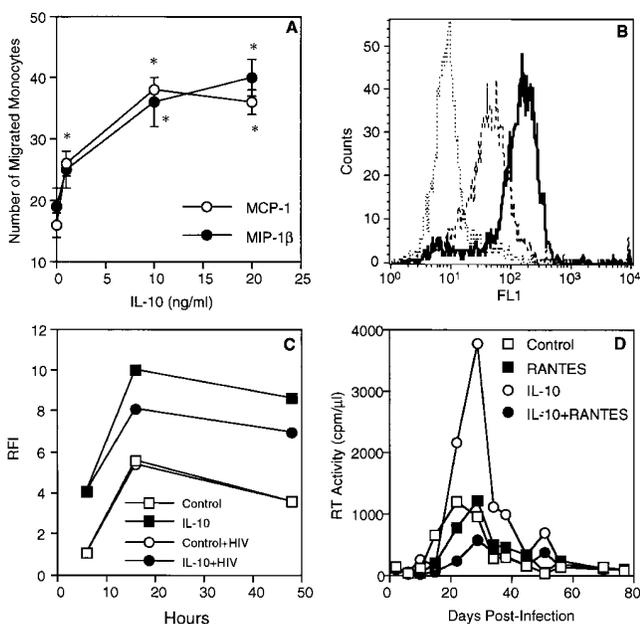
with the virus shortly after isolation. IL-10 caused a clear enhancement of virus multiplication, as previously reported using monocyte-derived macrophages (24). A panel of CC chemokines, including MIP-1 $\alpha$ , MIP-1 $\beta$ , and MCP-1 was tested in parallel to RANTES for their capacity to interfere with HIV replication in control and in IL-10-stimulated monocytes. RANTES caused a detectable, although modest, delay in the onset of virus production in untreated monocytes and completely inhibited IL-10-induced upregulation of viral replication (Fig. 2 D). In comparison, the other tested chemokines showed a very modest effect on viral replication (not shown). In this regard, the higher potency of RANTES as HIV inhibitor compared to other chemokines has been recently reported (28). In addition, AOP-RANTES, a RANTES mutein with antagonistic activity (21), completely abolished HIV replication in control as well as IL-10-treated monocytes (data not shown).

To validate this hypothesis, we analyzed the kinetics of proviral HIV DNA accumulation in control versus IL-10-stimulated monocytes. Proviral HIV DNA was readily demonstrated 16 h after infection (a time frame compatible with a single round of HIV replication) in IL-10-stimulated, but not control, monocytes, whereas similar signals

were observed in control and IL-10-treated cells 40 h after infection (Fig. 3). AOP-RANTES substantially suppressed HIV DNA accumulation in both control and IL-10-stimulated cells (Fig. 3), as a result of interference with viral entry. CCR5 membrane expression, which was already upregulated after 6 h of incubation with IL-10, remained elevated during the subsequent 40 h. HIV infection resulted only in a minor decrease of IL-10-induced CCR5 expression (Fig. 2 C).

## Discussion

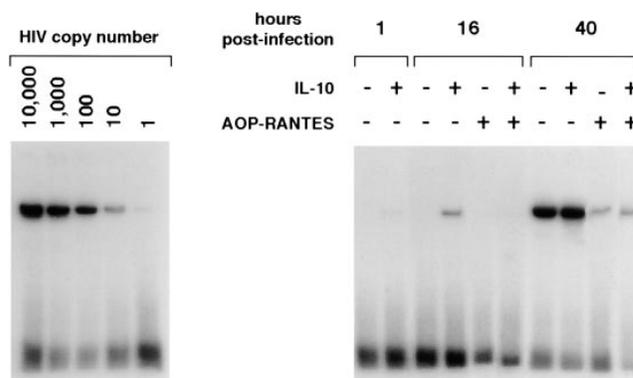
The results presented here show that the potent anti-inflammatory and immunosuppressive cytokine IL-10 can upregulate expression of functional CCR1, 2, and 5 receptors in human monocytes. The effect of IL-10 was selective in that CCR3 and 4, which are normally expressed at very low levels, were not induced, and, in one experiment, CXCR4, which is present and functional in monocytes, was slightly decreased. The modulatory action of IL-10 was mediated by prolongation of mRNA half-life. This observation, together with recent findings with prototypic pro- and anti-inflammatory molecules (15 and our unpublished



**Figure 2.** Augmented expression of functional CC chemokine receptors and upregulation of HIV infection by IL-10. (A) Effect on chemotaxis. Monocytes were cultured for 16 h with medium or IL-10 (10 ng/ml) and tested for chemotaxis to a suboptimal concentration of MCP-1 (1 ng/ml) or MIP-1 $\beta$  (10 ng/ml). Values are mean ( $\pm$  SE, three replicates) number of migrated cells after subtraction of spontaneous migration that was not affected by IL-10 (data not shown). (B) Surface expression of CCR5. Monocytes were exposed to IL-10 (10 ng/ml) for 16 h and surface expression was determined by flow cytometry using the 5C7-18 anti-CCR5 mAb. Dots, irrelevant mAb; broken line, control monocytes stained with anti-CCR5; continuous line, IL-10-treated monocytes stained with anti-CCR5. (C) Kinetics of CCR5 expression in control and HIV-infected monocytes cultured for the time indicated with 10 ng/ml IL-10. Results from a single donor representative of two to four tested are expressed as relative fluorescence index as described in Materials and Methods. (D) Effect of IL-10 on HIV infection of monocytes. Cells were incubated in 48-well tissue culture plastic plates in the presence or absence of IL-10 (0.1 ng/ml) for 6 h before incubation for 30 min with RANTES (200 ng/ml) and infection with the macrophage-tropic BaL strain of HIV-1. Mg<sup>2+</sup>-dependent reverse transcriptase activity was measured in the culture supernatants (24).

observations, see also below), indicates that receptor mRNA stability is a crucial set point for the action of chemokines.

IL-10 has been shown to have divergent effects on HIV replication in macrophages *in vitro*, depending on experimental conditions such as cytokine concentrations (24, 29–31). In this study, we found that IL-10 promoted a productive infection of monocytes by the macrophage-tropic HIV BaL strain, an effect that was associated with an increase of viral entry. Since the IL-10-mediated enhancement was inhibited by RANTES and completely abolished by AOP-RANTES, we infer that upregulation of CCR5 plays a major role in IL-10 enhancement of HIV replication, at least under these experimental conditions. A.S. Fauci has recently observed a transient decrease of circulating HIV virions (viremia) in HIV-infected individuals who were injected intravenously with IL-10 (Fauci, A.S., personal communication). Our results suggest a potential mechanism perhaps contributing to this *in vivo* effect, i.e., the



**Figure 3.** Kinetics of proviral HIV DNA accumulation. Monocytes were stimulated with IL-10 (10 ng/ml) for 6 h in the presence and absence of 200 ng/ml of AOP-RANTES for 30 min before infection with HIV-1 BaL. Aliquots were harvested 1, 16, and 40 h after infection and tested for proviral DNA synthesis.

enhancement of cell surface expression of CCR5 and other chemokine receptors by IL-10 may favor the sequestration and, eventually, the entry of free circulating virions. However, it should be stressed that IL-10 may exert multiple effects on HIV infection, such as the inhibition of HIV replication dependent upon release of proinflammatory cytokines, as previously reported (29), in addition to the effect observed in this study.

The *in vivo* relevance of IL-10-mediated upregulation of CC chemokine receptors/HIV fusion coreceptors is a matter of speculation. Subjects homozygous for the  $\Delta$ 32 mutation do not express functional CCR5 and are resistant to infection after multiple exposure to HIV (32–34), whereas heterozygous for this mutation tend to have a decrease rate of disease progression (35, 36). IL-10 production in mucosal tissues plays a key role in the control of inflammation, as indicated by the inflammatory bowel disease observed in IL-10<sup>-/-</sup> mice (18). We suggest that this tonic production of IL-10 may maintain CCR5 expression in mucosal tissues, contributing to the dominant role of this fusion coreceptor in primary HIV infection.

In addition to IL-10, we recently found that other molecules with antiinflammatory activity, such as glucocorticoid hormones, upregulate certain CC chemokine receptors (e.g., CCR2; data not shown). These agents concomitantly inhibit chemokine (e.g., MCP-1) production in monocytes (7, 8). Reciprocally, at least certain prototypic primary proinflammatory agents (endotoxin, TNF) induce chemokine production and inhibit receptor expression (references 12, 15 and our unpublished observations). Hence, an emerging paradigm indicates that at least some pro- and antiinflammatory molecules exert reciprocal and opposing influences on chemokine ligand production and receptor expression. This interplay may serve as a negative feedback mechanism and as a means to regulate the efflux of mononuclear phagocytes from sites of inflammation. The regulation of chemokine receptor expression mediated at the level of transcript stability may represent a novel target for pharmacological intervention in inflammatory diseases and viral infections.

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Address correspondence to Dr. Alberto Mantovani, Istituto di Ricerche Farmacologiche 'Mario Negri', via Eritrea 62, 20157 Milan, Italy. Phone: 39-2-3901-4493; Fax: 39-2-354-6277; E-mail: mantovani@irfmn.mnegri.it

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