Uncoupling of inflammatory chemokine receptors by IL-10: generation of functional decoys

G . D'Amico[1], G. Frascaroli[1], G. Bianchi[1], P. Transidico[1], A. Doni[1], A. Vecchi[1], S. Sozzani[1], P. Allavena[1] and A. Mantovani[1,2]

As originally demonstrated for the interleukin 1 (IL-1) type II receptor, some primary proinflammatory cytokines from the IL-1 and tumor necrosis factor families are regulated by decoy receptors that are structurally incapable of signaling. Here we report that concomitant exposure to proinflammatory signals and IL-10 generates functional decoy receptors in the chemokine system. Inflammatory signals, which cause dendritic cell (DC) maturation and migration to lymphoid organs, induce a chemokine receptor switch, with down-regulation of inflammatory receptors (such as CCR1, CCR2, CCR5) and induction of CCR7. Concomitant exposure to lipopolysaccharide (LPS) and IL-10 blocks the chemokine receptor switch associated with DC maturation. LPS + IL-10–treated DCs showed low expression of CCR7 and high expression of CCR1, CCR2 and CCR5. These receptors were unable to elicit migration. We provide evidence that uncoupled receptors, expressed on LPS + IL-10–treated cells, sequester and scavenge inflammatory chemokines. Similar results were obtained for monocytes exposed to activating signals and IL-10. Thus, in an inflammatory environment, IL-10 generates functional decoy receptors on DC and monocytes, which act as molecular sinks and scavengers for inflammatory chemokines.

Decoy receptors are agonist-binding molecules that sequester inflammatory cytokines and signaling receptor components. The decoy receptor paradigm was originally formulated for the interleukin 1 (IL-1) type II receptor[1,2]. Decoy receptors that are structurally incapable of participating in signaling receptor complexes but are able to sequester ligands have subsequently been identified for IL-18, a member of the IL-1 family, and in the tumor necrosis factor receptor (TNFR) family (for example, osteoprotegerin)[3–8].

Chemokines are a superfamily of inflammatory cytokines that guide the recruitment and positioning of leukocytes in tissues by interacting with 7-transmembrane-domain receptors[9–11]. Chemokine receptors with defective signaling function have been identified[12,13] although their actual significance has not been ascertained. Pro- and anti-inflammatory signals regulate the levels and efficiency of inflammatory chemokine receptor coupling[14–17]. Here we report that concomitant exposure to proinflammatory signals and IL-10 generates functional chemokine decoy receptors in dendritic cells (DCs) and monocytes. Thus, structural and functional decoy receptors form a general strategy for regulating the action of proinflammatory cytokines and chemokines.

Results

IL-10 inhibits chemokine receptor switch

DCs are rapidly recruited at inflammatory sites and their correct trafficking through tissues and localization to lymphoid organs is essential for an optimal immune response[18–21]. DCs express receptors for, and respond to a defined set of, chemotactic agonists[22–24]. The current model for the role of chemokines in DC trafficking includes concomitant down-regulation of inflammatory chemokine receptors and induction

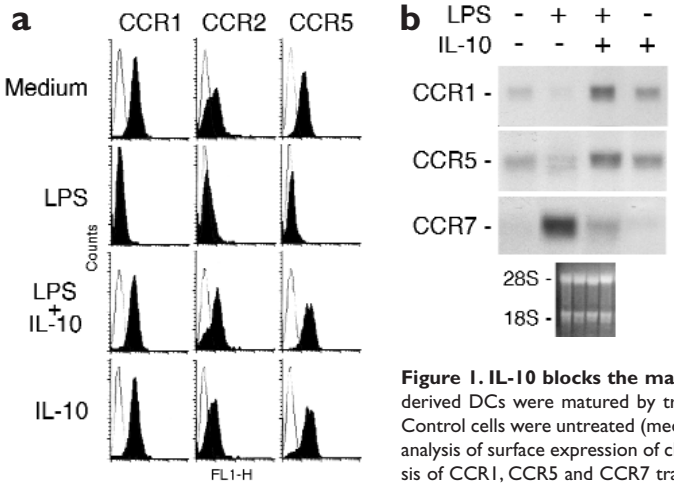

Figure 1. IL-10 blocks the maturation-induced switch of chemokine receptor expression in DCs. Monocyte-derived DCs were matured by treatment with LPS (10 ng/ml) in the presence or absence of IL-10 (20 ng/ml) for 24 h. Control cells were untreated (medium) or treated with IL-10 alone. Results are representative of five experiments. (**a**) FACS analysis of surface expression of chemokine receptors with anti-CCR1, anti-CCR2 and anti-CCR5. (**b**) Northern blot analysis of CCR1, CCR5 and CCR7 transcripts.

[1]*Department of Immunology and Cell Biology, Istituto di Ricerche Farmacologiche 'Mario Negri', Via Eritrea 62, 20157 Milano, Italy.* [2]*Institute of General Pathology, University of Milano, Via Mangiagalli, 31, 20100, Milano, Italy. Correspondence should be addressed to A. M. (mantovani@irfmn.mnegri.it).*

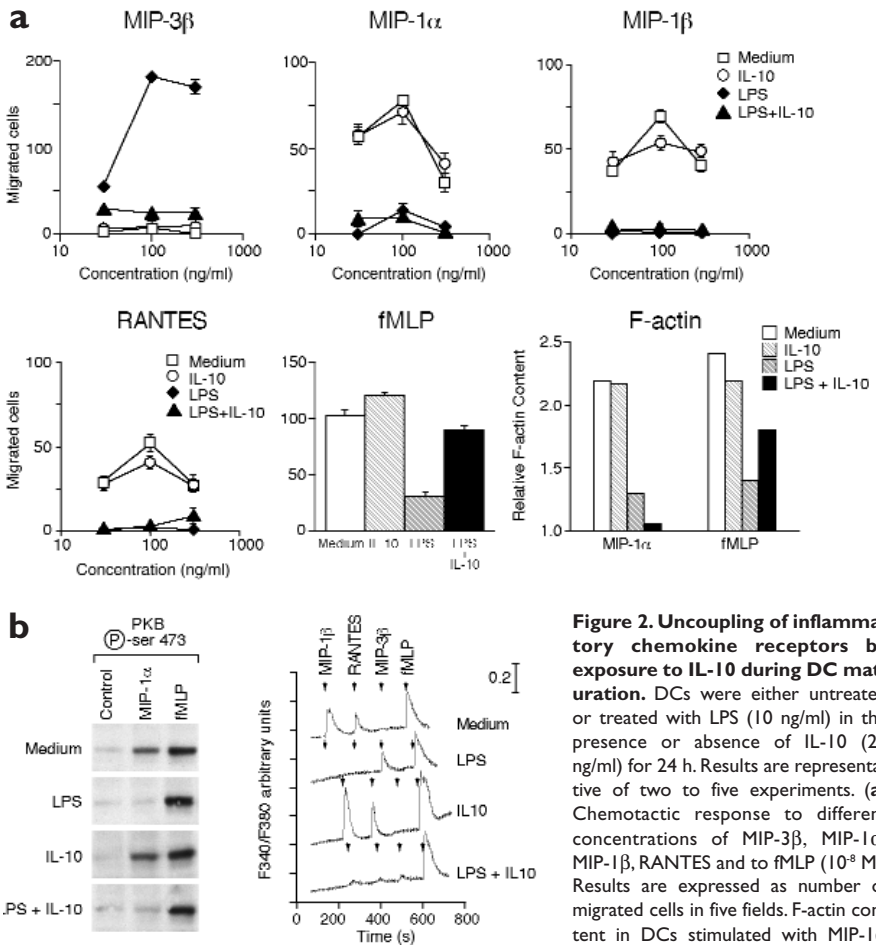

Figure 2. Uncoupling of inflammatory chemokine receptors by exposure to IL-10 during DC maturation. DCs were either untreated or treated with LPS (10 ng/ml) in the presence or absence of IL-10 (20 ng/ml) for 24 h. Results are representative of two to five experiments. (**a**) Chemotactic response to different concentrations of MIP-3β, MIP-1α, MIP-1β, RANTES and to fMLP (10^{-8} M). Results are expressed as number of migrated cells in five fields. F-actin content in DCs stimulated with MIP-1α (50 ng/ml) or fMLP (10^{-8} M) for 3 min. (**b**) Phosphorylation of PKB in DCs stimulated for 3 min with MIP-1α (100 ng/ml) or fMLP (10^{-8} M). Measurement of intracellular calcium in DCs stimulated with MIP-1α or MIP-3β (100 ng/ml) or fMLP (10^{-8} M).

of CCR7. These two temporally coordinated events are induced by pathogen recognition or engagement of inflammatory cytokine receptors[25–27]. It is well established that the anti-inflammatory cytokine IL-10[28] potently inhibits DC differentiation[29,30] and therefore we investigated the effect of IL-10 on the chemokine receptor switch in DCs.

Immature monocyte-derived DCs were cultured with lipopolysaccharide (LPS), which acted as a prototypic maturation signal. In agreement with previous studies[25–27], LPS-induced maturation was associated with down-regulation of CCR1, CCR2 and CCR5 and induction of CCR7, as assessed by surface expression, analysis by northern blotting and responsiveness to appropriate agonists. The LPS-induced chemokine receptor switch was completely blocked by IL-10. Maturing DC exposed to IL-10 retained the receptor repertoire of immature DC, with high CCR1, CCR2, CCR5 and low CCR7 (**Fig. 1**).

Uncoupling of chemokine receptors in DC

The functionality of chemokine receptors in IL-10–treated DCs was examined next. As previously described[25–27], immature DCs migrated in response to the inflammatory chemokine macrophage-inflammatory protein 1α (MIP-1α, also known as CCL3) [11], MIP-1β (also known as CCL4) and RANTES (also known as CCL5), whereas the CCR7 ligand MIP-3β (also known as CCL19) showed no appreciable activity (**Fig 2a**). Treatment with IL-10 alone did not qualitatively modify the migratory activity of the DCs. Upon LPS-induced maturation, the DCs became highly responsive to MIP-3β and lost their responsiveness to the inflammatory chemokines MIP-1α, MIP-1β and RANTES. IL-10 completely blocked the acquisition of MIP-3β responsiveness that was associated with LPS-induced maturation. In addition, despite high surface expression of CCR1 and CCR5 (**Fig. 1**), DCs treated with LPS + IL-10 showed little or no migration in response to MIP-1α, MIP-1β and RANTES (**Fig. 2a**). In a series of five experiments the response to MIP-1α by DCs treated with LPS or LPS + IL-10 was only 19±6% and 28±5% (mean±s.e.) of control untreated cells, respectively. However, DCs treated with LPS + IL-10 responded chemotactically to *N*-formyl-methionleucyl-phenylalanine (fMLP) (**Fig. 2a**). This indicated that the failure of LPS + IL-10–treated DCs to migrate in response to inflammatory chemokines did not reflect a general impairment of cell locomotion.

The influence of IL-10 treatment on receptor signaling was examined next. LPS + IL-10–treated DC also showed defective elevation of intracellular free Ca^{2+} (**Fig. 2b**) and defective polymerization of F-actin (**Fig. 2a**) in response to inflammatory chemokines, but not fMLP. It was recently shown that the γ isoform of phosphoinositide 3-kinase (PI3Kγ) plays an essential role in chemokine-induced chemotaxis[31–33]. We therefore assessed protein kinase B (PKB) phosphorylation (a major target of PI3Kγ) in DCs treated with LPS + IL-10. As shown in **Fig. 2b**, defective activation of PKB by MIP-1α, but not fMLP, was observed. Similar results were obtained when ERK1, ERK2 and p38 kinase were studied (data not shown).

These results indicated that IL-10 had blocked the chemokine receptor switch associated with LPS maturation of DCs. Further, inflammatory chemokine receptors retained by IL-10 on the cell membrane recognized ligands (see below) but showed a defective capacity for signaling and eliciting chemotaxis. As expected, concomitant addition of IL-10 completely inhibited LPS-induced production of MIP-1β, MIP-1α and monocyte chemotactic protein 1 (MCP-1, also known as CCL2), as assessed by measuring protein in supernatants and by northern analysis (data not shown). For instance, in one of four typical experiments performed, immunoreactive MIP-1β concentrations in 24-h supernatants were 0.16, 0.10, 447 and 0.23 ng/ml for control cells and cells treated with IL-10, LPS or LPS + IL-10, respectively.

Uncoupling of chemokine receptors in monocytes

We next needed to ascertain whether chemokine receptor uncoupling was restricted to the human DC–LPS + IL-10 combination under investigation, so the study was extended to mouse bone marrow–derived DCs (see below) and to human monocytes. Previous studies had shown that primary proinflammatory signals (LPS, IL-1, TNF and IFN-γ) and anti-inflammatory agents (IL-10, glucocorticoid hormones) have reciprocal and divergent effects on the expression of certain inflammatory chemokine receptors, including CCR2 in monocytes[14–17]. Particular attention was focused on CCR2, in addition to CCR1 and CCR5,

because this receptor and its agonist MCP-1 play an essential, nonredundant role in monocyte recruitment[9,10]. A combination of LPS and IFN-γ was used as the activation signal[17]. Down-regulation of CCR1, CCR2 and CCR5 by LPS–IFN-γ treatment was completely blocked by IL-10 (**Fig. 3a**). Despite high surface expression of chemokine receptors, monocytes exposed to LPS–IFN-γ and IL-10 were totally unresponsive to MCP-1, MIP-1α, MIP-1β and RANTES but were responsive to fMLP (**Fig. 3b** and data not shown), and did not increase intracellular free Ca^{2+} when stimulated with these agonists (**Fig. 3c**). In a series of four chemotaxis experiments, the response by LPS–IFN-γ + IL-10-treated monocytes to MCP-1 and MIP-1α was 6±2% and 4±1.5% (mean±s.e.) of control untreated cells, respectively. Thus, both in monocytes and in DC, it is the combination of a primary proinflammatory signal (such as LPS and IFN-γ) with IL-10 that generates the high surface CCR2 (or CCR1 and CCR5) phenotype associated with receptor uncoupling.

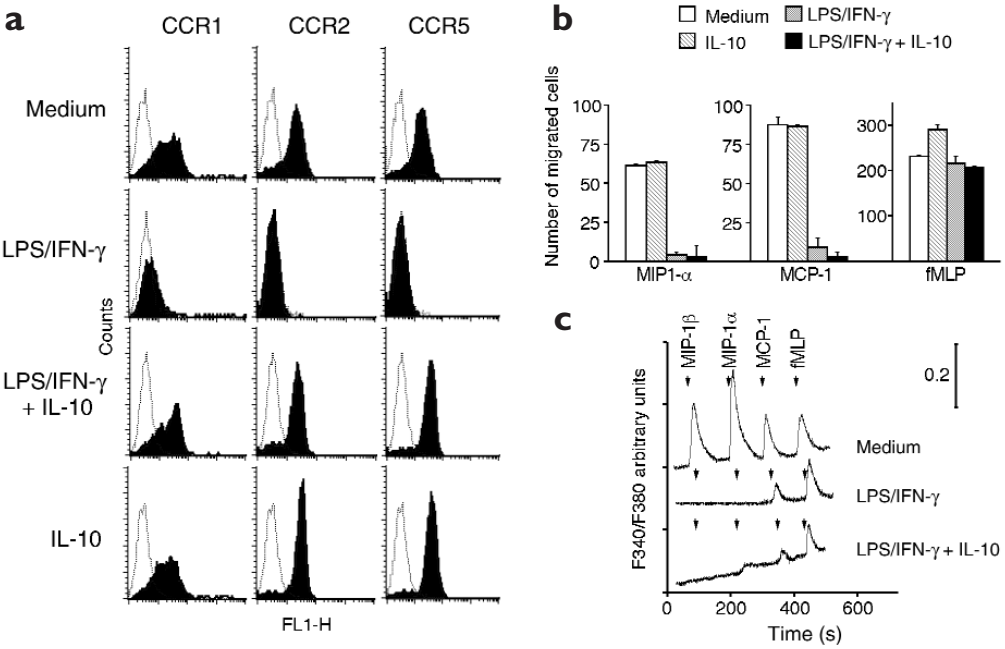

Figure 3. Uncoupling of inflammatory chemokine receptors in monocytes exposed to IL-10 and LPS–IFN-γ. Purified monocytes were either untreated (medium) or treated with LPS (100 ng/ml) and IFN-γ (500 U/ml), in the presence or absence of 20 ng/ml of IL-10 for 24 h. Results are representative of three or four experiments. (**a**) FACS analysis of surface expression of chemokine receptors with anti-CCR1, anti-CCR2 and anti-CCR5. (**b**) Chemotactic responsiveness to MIP-1α, MCP-1 (100 ng/ml) and fMLP (10^{-8} M). Results are expressed as number of migrated cells in five fields. (**c**) Measurement of intracellular calcium after stimulation with MIP-1β, MIP-1α, MCP-1 (100 ng/ml) and fMLP (10^{-8} M).

Ligand binding and scavenging

We next needed to assess whether IL-10–"frozen" chemokine receptors retained the ability to recognize and internalize ligands. Using ^{125}I–MIP-1β and ^{125}I–MIP-1α or biotinylated MIP-1α, we found surface binding capacity consistent with flow cytometric analysis (data not shown). ^{125}I–MIP-1α and ^{125}I–MIP-1β were then used to investigate the rate of chemokine scavenging in IL-10–treated DCs over a period of 3 h. Untreated DCs bound and internalized exogenous ^{125}I–MIP-1β, with 28±1.5×10^5 molecules per cell scavenged after 1 h (**Fig. 4a**). IL-10 alone slightly increased the rate of MIP-1β scavenging (36×10^5 molecules per cell). In contrast, LPS inhibited ^{125}I–MIP-1β sequestration (8×10^5 molecules per cell) by 72%, as expected on the basis of inhibition of CCR5 receptor expression (**Fig. 1**) and macropinocytosis[34]. IL-10 completely reversed the LPS-mediated block of chemokine scavenging, with a fourfold

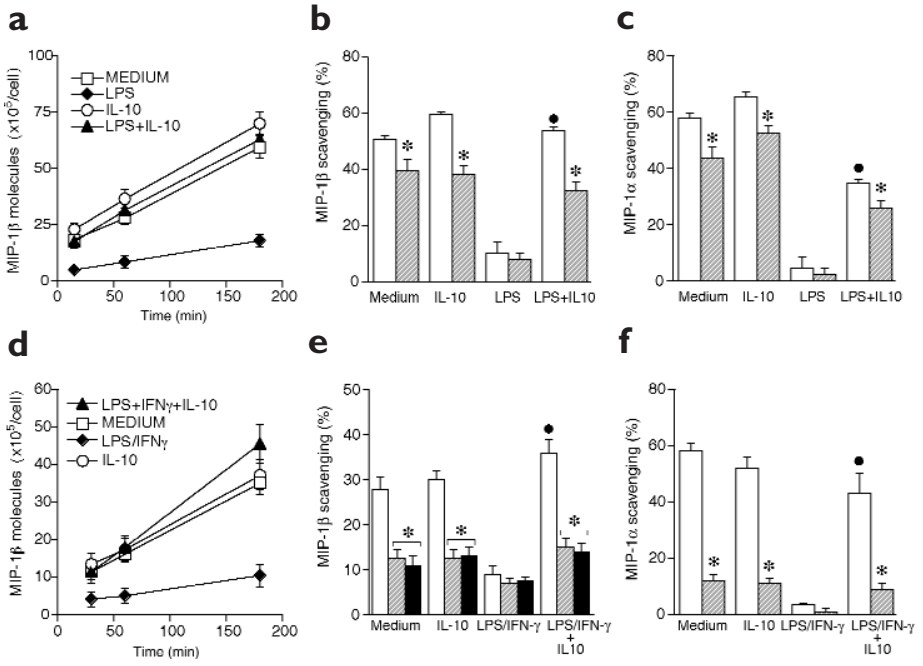

Figure 4. Chemokine scavenging by uncoupled receptors in IL-10–treated cells. Results are representative of two experiments for MIP-1β and of four experiments for MIP-1α. (**a–c**) DCs and (**d–f**) monocytes were treated as described in Figs 2 and 3. (**a,d**) Internalization of ^{125}I–MIP-1β (100 nM) over time. Results are expressed as number of scavenged ^{125}I–MIP-1β molecules × 10^5 per cell. (**b,e**) CCR5-mediated scavenging of MIP-1β: internalization of ^{125}I–MIP-1β was evaluated after 1 h at 37 °C. Results are expressed as the percentage of ^{125}I–MIP-1β internalization over total radioactivity added in cells treated with medium (control), AOP-RANTES (**b,e**) or anti-CCR5 (**e**). (**c,f**) CCR5-mediated scavenging of MIP-1α: internalization of ^{125}I–MIP-1α (2.75 nM) was evaluated after 1 h at 37 °C. Results are expressed as the percentage of ^{125}I–MIP-1α internalization over total radioactivity added, in cells pretreated with medium (control) or AOP-RANTES. (Open bars, control; hatched bars, AOP-RANTES; filled bars, anti-CCR5. *P<0.01, significantly different from cells without AOP-RANTES or without anti-CCR5; •P<0.01, significantly different from cells treated with LPS of LPS–IFN-γ, both determined by Dunnett's test.)

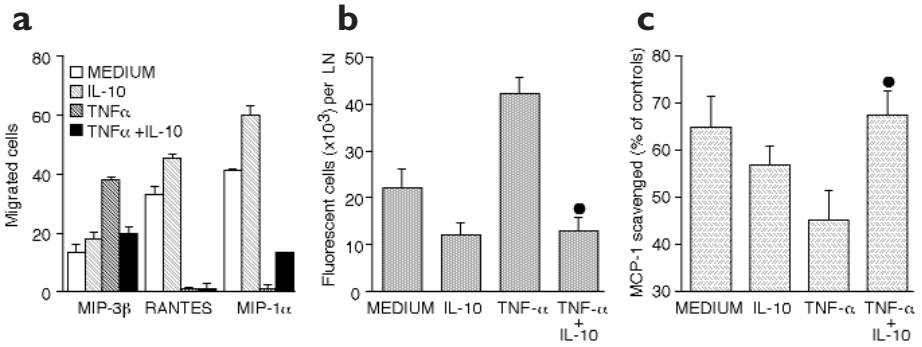

Figure 5. Trafficking to lymph nodes and MCP-1 scavenging by mouse DCs exposed to IL-10 and TNF. (**a**) *In vitro* chemotactic migration of mouse DCs exposed to IL-10 and TNF. Bone marrow–derived DCs were either untreated (medium) or treated with TNF (20 ng/ml) in the presence or absence of IL-10 (10 ng/ml) for 24 h. Results (mean±s.d.) are representative of five experiments. (**b**) Trafficking of mouse DCs exposed to IL-10 and TNF to lymph nodes. Results (mean±s.d.) of five mice individually tested are representative of two experiments. (**c**) *In vivo* scavenging of human MCP-1 by mouse DCs exposed to IL-10 and TNF. Results (mean±s.d.) of seven mice individually tested are representative of two experiments.

increase in scavenging compared to cells exposed to LPS alone (32×10^5 molecules per cell in 1 h, $P<0.01$, **Fig. 4a**).

Aminooxypentane-RANTES (AOP-RANTES)[35,36] was used to assess the contribution of receptor-mediated internalization, relative to other scavenging mechanisms (for example, fluid phase macropinocytosis). AOP-RANTES recognizes CCR5 and blocks receptor recycling[35–37]. AOP-RANTES did not interfere with uptake of Lucifer yellow and fluorescein isothiocyanate (FITC)-dextran, suggesting that it does not interfere with fluid phase macropinocytosis and mannose receptor endocytosis. Addition of a monoclonal antibody that blocked the anti-mannose receptor did not affect ^{125}I–MIP-1β scavenging (data not shown). The CCR5-mediated uptake of MIP-1β, identified by the degree to which ^{125}I–MIP-1β scavenging was blocked by AOP-RANTES, accounted for 25% in immature untreated DCs, and for 35%, 10% and 38% in IL-10–treated, LPS-treated and LPS + IL-10–treated DCs, respectively (**Fig. 4b**). When ^{125}I–MIP-1α was used, similar results were obtained, with dramatic stimulation of chemokine scavenging by the IL-10 + LPS combination relative to LPS alone (**Fig. 4c**).

The analysis of chemokine scavenging was then extended to monocytes. Monocytes were less efficient than DCs at chemokine scavenging (~50% less in the experiment shown in **Fig. 4d** compared to **Fig. 4a**). LPS–IFN-γ addition reduced chemokine sequestration by monocytes, but was fully restored by concomitant exposure to IL-10 (**Fig. 4d–f**). A higher fraction of scavenging, ranging from 50% to 80% in different experiments, was chemokine receptor–mediated in monocytes, compared to DCs, as assessed by AOP-RANTES and/or anti-CCR5 blocking (**Fig. 4b**). This finding is consistent with the lower macropinocytic activity of monocytes compared to DCs.

In an effort to confirm the scavenging of inflammatory chemokines by IL-10 + LPS–treated cells using a different approach, exogenous MCP-1 (100 ng/ml) was added to differently treated cell cultures and its disappearance monitored by enzyme-linked immunosorbent assay (ELISA). Results obtained in monocytes and DCs were comparable to those obtained with ^{125}I-labeled agonists (data not shown).

To assess the *in vivo* relevance of these *in vitro* observations, an adoptive transfer approach was used. First, the *in vitro* analysis was extended to mouse DCs. As previously reported[38], mouse DCs had a very similar pattern of migratory response to chemokines as compared to human DCs. Exposure to TNF suppressed the migration of mouse DCs to inflammatory chemokines (RANTES, MIP-1α and not shown, MIP-1β) and rendered them responsive to MIP-3β (**Fig. 5a**). Treatment with IL-10 alone did not qualitatively modify the migratory activity of DCs compared to untreated cells. In contrast, cells exposed to a combination of IL-10 and TNF did not respond to inflammatory chemokines, and IL-10 blocked the TNF-induced responsiveness to MIP-3β (**Fig. 5a**).

As expected on the basis of these *in vitro* results, using an adoptive transfer approach IL-10 completely blocked the traffick of TNF-stimulated mouse DCs to lymph nodes (**Fig. 5b**). Scavenging was assessed by monitoring the disappearance *in vivo* of human MCP-1 at sites of adoptive transfer of mouse DCs. As shown in **Fig. 5c**, TNF-treated DCs showed reduced scavenging of MCP-1, which was restored by IL-10, consistent with *in vitro* findings.

Discussion

Our results show that IL-10 inhibits the down-regulation of inflammatory chemokine receptors (CCR1, CCR2, CCR5) in DCs and monocytes exposed to microbial agents or to primary proinflammatory cytokines. The "frozen" chemokine receptors, retained on the surface of IL-10–treated cells, are uncoupled and unable to elicit migration. However, they do trap inflammatory chemokines, removing them from the inflammatory site. Thus, in an inflammatory microenvironment, IL-10 generates functional decoy receptors that act as molecular sinks and scavengers for inflammatory chemokines.

IL-10 completely blocked the production of inflammatory chemokines in DCs and monocytes, as assessed by measurement of MIP-1β, MIP-1α and MCP-1 in supernatants and by northern analysis (data not shown). Therefore, IL-10–mediated freezing and uncoupling of receptors in an inflammatory environment, concomitant with inhibition of agonist production, set DCs and monocytes in an anti-inflammatory, chemokine-scavenging mode.

The first pure decoy identified was the IL-1 type II receptor[1,2]. This molecule has a short cytoplasmic tail, is unable to participate in signal transducing receptor complexes and (in membrane-bound or soluble form) traps IL-1β and the signaling IL-1 receptor accessory protein. Structural decoys have subsequently been identified for IL-18 and for members of the TNF family[3–8] (for reviews see[39,40]). Two chemokine receptors, Duffy antigen–related chemokine receptor (DARC) and D6, both apparently unable to signal, have been described[12,13] and may act as chemokine sinks. It has also been suggested that US28, a chemokine receptor encoded by the human cytomegalovirus, if it is able to signal[41], acts as a chemokine scavenger[42]. The results presented here demonstrate that generation of functional chemokine decoys occurs in IL-10–dominated inflammatory reactions, as a strategy to block excessive leukocyte recruitment and activation. Therefore, structural decoys such as the IL-1 type II receptor and osteoprotegerin and functional decoys such as chemokine receptors in an IL-10–dominated microenvironment represent a general strategy for blocking and scavenging inflammatory cytokines and chemokines.

Methods

Cell preparation. Human monocytes were obtained by Percoll gradient separation and cultured with 50 ng/ml of granulocyte–macrophage colony-stimulating factor (GM-CSF, Novartis, Basel) and 20 ng/ml of IL-13 (Sanofi Elf Bio Research, Labege) for 7 days to generate DCs[25]. DCs were matured by 24-h stimulation with 10 ng/ml of LPS (Sigma, St. Louis, MO) in the presence or absence of 20 ng/ml of IL-10 (a gift of S. Narula, Schering Plough,

NJ). Monocytes were stimulated with 100 ng/ml of LPS and 500 U/ml of IFN-γ (Roussel Uclaf, Paris) in the presence or absence of 20 ng/ml of IL-10, for 24 h.

Mouse DCs were generated from $CD34^+$ bone marrow cells as described[38]. Immunomagnetically selected $CD34^+$ cells were cultured in RPMI 1640 medium with 10% fetal calf serum, 2×10^{-5} M of 2–mercaptoethanol, 40 ng/ml of mouse GM-CSF (Sandoz, Basel) and 100 ng/ml of Flt3 ligand (Immunex, Seattle, WA) for 9 days. DCs were matured by 24-h stimulation with 20 ng/ml of murine TNF-α (BASF, Knoll) in the presence or absence of 10 ng/ml of IL-10.

Animals. DBA/2CrlBR male mice of 8–12 weeks of age were used. Procedures involving animals and their care were conducted in conformity with national and international laws and policies.

Chemokines and chemokine receptors. MIP-1α, MIP-1β, RANTES, MIP-3β and MCP-1 were purchased from PeproTech (Rocky Hill, NJ). AOP-RANTES was a gift of R. E. Offord (University of Geneva) and A. Proudfoot (Serono, Geneva). Surface expression of chemokine receptors was studied by flow cytometry using the following monoclonal antibodies: anti-CCR1 (clone 53504.111, R&D System, Minneapolis, MN), anti-CCR2 (clone L5132.1D9, a gift G. La Rosa, Leukosite, Cambridge, MA), anti-CCR5 (2D7, PharMingen, San Diego, CA).

Northern blot analysis. Total RNA was extracted by the guanidynium thiocyanate method, blotted and hybridized as previously described[43].

Chemotaxis assay. Cell migration was evaluated using a chemotaxis microchamber technique as previously described[43]. Migration through polycarbonate filters (5 μm pore size; Neuroprobe, Bethesda, MD) was evaluated after incubation for 90 min. Results are expressed as the mean number of migrated cells counted in five microscope high power fields (magnification: ×1000). Each experiment was performed in triplicate.

Actin polimerization. F-actin content was evaluated in DCs stimulated with MIP-1α or fMLP, for 3 min. Cells were fixed and stained with nitrobenzoxadiazole-phallacidin (Molecular Probe, Eugene, OR). Fluorescence was evaluated by flow cytometry.

Measurement of intracellular calcium. Fura-2/AM–loaded DCs (10^7 cells per ml, 2 μM per 30 min at 37 °C) were stimulated with chemokines or fMLP. Results are expressed as the ratio of fluorescence at two excitation wavelengths (340 and 380 nm) and emission at 487 nm.

PKB phosphorylation. PKB phosphorylation was assessed as described[31].

Chemokine scavenging. For internalization studies, cells were incubated at 37 ¡C with 2.75 nM MIP-1α or 100 nM MIP-1β in the presence of trace amounts (0.2 μCi/sample) of ^{125}I–MIP-1α or ^{125}I–MIP-1β (Amersham, specific activity 2000 Ci/mmol) for 15–180 min before assessment of cell-associated radioactivity. In selected samples, cells were pretreated for 30 min at 37 °C with AOP-RANTES (100 nM) or anti-CCR5 (PharMingen) (100 μg/ml) before the addition of the radiolabeled ligand. For scavenging of MCP-1, the chemokine was added to cell cultures (100 ng per 5×10^6 cells/ml), over a period of 1–7 h at 37 °C. The amount of chemokine remaining in the supernatant was assessed by ELISA.

For *in vivo* studies, the air pouch model was used. The dorsal pouch was induced as described[44], injecting air subcutaneously on days 0 and 3. On day 6, 1 ml of 0.3% carboxymethycellulose in saline was injected in the pouches. On day 7, 5×10^6 cultured mouse DCs in 0.5 ml of saline were injected into the pouches, 15 min later 500 ng of human MCP-1 in 0.5 ml of saline was injected. Mice were killed 3.5 h after MCP-1 injection, the exudates recovered, centrifuged and the amount of human MCP-1 in the supernatants assessed by ELISA.

***In vivo* migration of murine DC.** DCs from day 9 of culture were labeled with the vital dye carboxyfluorescein diacetate succinimidyl diester (CFSE) as described[45] and 2×10^6 labeled cells in 30 μl of saline were injected subcutaneously in the hind leg footpad. Popliteal lymph nodes were recovered 24 h later and cell suspensions prepared by enzymatic treatment[46]. Cells were counted using a microscope and the presence of fluorescent CFSE-labeled DCs was evaluated by FACS analysis of at least 100,000 cells/sample. Results are presented as number of fluorescent DC per lymph node.

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