IMMUNOLOGY ORIGINAL ARTICLE

# Interleukin-10 and interferon-$\gamma$ modulate surface expression of fractalkine-receptor (CX$_3$CR1) via PI3K in monocytes

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## Summary

The membrane-anchored form of the chemokine fractalkine (CX$_3$CL1) has been identified as a novel adhesion molecule that interacts with its specific receptor (CX$_3$CR1) expressed in monocytes, T cells and natural killer cells to induce adhesion. In addition, CX$_3$CL1 can be cleaved from the cell membrane to induce chemotaxis of CX$_3$CR1-expressing leucocytes. Recently, marked variations in CX$_3$CR1 monocyte expression have been observed during several pathological conditions. Regulation of CX$_3$CR1 in monocytes during basal or inflammatory/anti-inflammatory conditions is poorly understood. The aim of this study was therefore to examine CX$_3$CR1 expression during monocyte maturation and the effect of soluble mediators on this process. We found that basal expression of CX$_3$CR1 in fresh monocytes was reduced during culture, and that lipopolysacchairde accelerated this effect. In contrast, interleukin-10 and interferon-$\gamma$ treatment abrogated CX$_3$CR1 down-modulation, through a phosphatidylinositol 3 kinase-dependent pathway. Most importantly, CX$_3$CR1 membrane expression correlated with monocyte CX$_3$CL1-dependent function. Taken together, our data demonstrate that CX$_3$CR1 expression in monocytes can be modulated, and suggest that alterations in their environment are able to influence CX$_3$CL1-dependent functions, such as chemotaxis and adhesion, leading to changes in the kinetics, composition and/or functional status of the leucocyte infiltrate.

**Keywords:** chemokine receptors; cytokines; human macrophages/monocytes; innate immunity; signalling/signal transduction

## Introduction

Within the chemokine family, fractalkine (CX$_3$CL1) is expressed on neurons, macrophages and also on endothelial, epithelial and dendritic cells.[1,2] This chemokine has been identified as a transmembrane molecule that induces adhesion by interaction with its specific receptor (CX$_3$CR1) expressed in monocytes (Mo), T cells, mast cells and natural killer cells.[3] Adhesion mediated by CX$_3$CL1–CX$_3$CR1 does not require receptor signalling, is resistant to physiological shear flow and is independent of extracellular calcium.[4] Besides its activity as an adhesion molecule, CX$_3$CL1 can be cleaved from cell membrane to generate a soluble 80 000 molecular weight molecule, which induces chemotaxis of CX$_3$CR1-expressing leucocytes.[5]

Expression of CX$_3$CR1 in Mo is significantly modulated during different pathological conditions.[6–8] We have previously reported the drastic decrease of fractalkine receptor expression on both CD16$^-$ and CD16$^+$ Mo subpopulations,

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Abbreviations: CHX, cycloheximide; FITC, fluorescein isothiocyanate; GM-CSF, granulocyte–macrophage colony-stimulating factor; HUS, haemolytic uraemic syndrome; IFN, interferon; IgG, immunoglobulin G; IL, interleukin; JAK, Janus kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; Mo, monocytes; mRNA, messenger RNA; PBMC, peripheral blood mononuclear cells; PI, propidium iodide; PI3K, phosphatidylinositol 3 kinase; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethylsulphonyl fluoride; STAT, signal transducer and activator of transcription; Stx-1, Shiga toxin 1; TGF, transforming growth factor; Th2, T helper type 2; TNF, tumour necrosis factor.

in children with haemolytic uraemic syndrome (HUS).[9] This finding was especially striking because the loss of CX$_3$CR1 correlated with the severity of renal failure.[9] Similarly, in septic patients a higher down-expression was associated with poor evolution.[8] The reduction of CX$_3$CR1 expression in Mo could be related to different regulatory mechanisms at the cellular level, which implies down-regulation of either its membrane exposure or its synthesis. In an effort to address this issue, we have studied cellular and molecular mechanisms involved in the regulation of CX$_3$CR1 expression in Mo, under basal or stimulated conditions. The stimuli evaluated included lipopolysaccharide (LPS) and Shiga toxin-1 (Stx1) as the main pathogenic factors in sepsis and HUS,[10] and tumour necrosis factor-$\alpha$ (TNF-$\alpha$) as a common inflammatory mediator. On the other hand, as deactivating factors we studied interleukin-4 (IL-4), IL-10 and transforming growth factor-$\beta$ (TGF-$\beta$), all present at elevated concentration in HUS and sepsis.[11,12] Moreover, we analysed the interferon-$\gamma$ (IFN-$\gamma$) effect, which is constitutively expressed in Mo and plays a central role promoting its maturation and activation.[13]

We found that CX$_3$CR1 membrane expression decreased in Mo during culture. Moreover, while LPS accelerated this process, IL-10 and IFN-$\gamma$ prevented it through a phosphatidylinositol 3-kinase (PI3K) -dependent pathway. Protein synthesis inhibitors impaired the down-modulation during culture, but this effect was not additive with the inhibiting effect induced by IL-10. Most importantly, CX$_3$CR1 membrane expression correlated with Mo CX$_3$CL1-dependent function. In addition, phorbol 12-myristate 13-acetate (PMA) maturation of THP-1 monocytic cells reproduced the CX$_3$CR1-down-regulation and allowed us to study the signal transduction pathways involved in cytokine-mediated effects.

This study demonstrates that locally produced cytokines or bacterial products are able to regulate CX$_3$CR1 expression in Mo. In addition, it suggests that environment can influence the kinetics, composition and functional status of the leucocyte infiltrate by affecting both CX$_3$CL1 expression on endothelial cells and CX$_3$CR1 expression in Mo.

## Materials and methods

### Reagents and antibodies

Propidium iodide (PI), fluorescein isothiocyanate (FITC) -conjugated annexin V, cycloheximide (CHX), actinomycin D (Act D), aprotinin, leupeptin, pepstatin A, sodium orthovanadate (NaVO$_4$), sodium fluoride (NaF), phenylmethylsulphonyl fluoride (PMSF), TNF-$\alpha$, LPS, TGF-$\beta$ and IFN-$\gamma$ were obtained from Sigma (St Louis, MO). The mitogen-activated protein kinase (MAPK) inhibitors SB203580 and PD98059, the PI3K inhibitor LY294002, PMA, anisomycin and, Wortmannin were purchased from

Calbiochem-Novabiochem (La Jolla, CA). Purified Stx1 holotoxin was purchased from Denka Seiken Co., Ltd. (Chuo-Ku, Tokyo, Japan). Human IL-4, IL-10, granulocyte–macrophage colony-stimulating factor (GM-CSF) and soluble CX3CL1 (sCX3CL1) sCX$_3$CL1 were from Preprotech (Preprotech Mexico, DF, Mexico). Unless stated otherwise, all other reagents were obtained from Sigma Chemical Co. All tissue culture flasks, dishes and multiwell plates were Falcon (Orange Scientific, Graignette Business Park, Belgium).

### Purification of monocytes

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood collected from adult normal volunteers by centrifugation over a Ficoll–Hypaque (Ficoll Pharmacia, Uppsala; Hypaque, Wintthrop Products, Buenos Aires, Argentina) gradient. Monocytes were further isolated from PBMC using Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden) gradient centrifugation as previously described.[14] Viability of Mo was > 96% as determined by trypan blue exclusion test and CD14 staining of Mo revealed that their purity was > 90%. Finally, Mo were suspended and cultured at $10^6$/ml in RPMI-1640 (Hyclone Laboratories Inc., Logan, Utah) supplemented with 10% heat-inactivated fetal calf serum (Natocor, Córdoba, Argentina), and Antibiotic–Antimycotic liquid (Gibco, Invitrogen, San Diego, CA).

### Cell cultures

The THP-1 human monocytic cell line (American Type Culture Collection, Manassas, VA) was cultured at 37° and 5% CO$_2$ in RPMI-1640 medium containing 10% fetal calf serum and antibiotics and were matured with 5 ng/ml PMA (Sigma).

### Flow cytometry

Measurement of CD14, and CX$_3$CR1 surface expression on Mo or THP-1 cells ($3 \times 10^5$) was performed by direct immunofluorescence flow cytometry on purified cells using the following conjugated anti-human monoclonal antibodies: CD14-phycoerythrin cyanine-5 (PE-Cy5) [mouse immunoglobulin G2a (IgG2a); Immunotech, Marseille, France], and CX$_3$CR1-FITC (rat IgG2b; Medical & Biological Laboratories Co, Woburn, MA). In all cases, isotype-matched antibodies were assayed in parallel. Monocytes were analysed for membrane and total CX$_3$CR1 expression by indirect immunofluorescence using rabbit anti-human CX$_3$CR1 IgG (Torrey Pines Biolabs, Inc, East Orange, NJ) followed by the FITC-conjugated secondary antibody (Sigma-Aldrich) as previously reported.[8] Briefly, total expression was determined with paraformaldehyde-fixed cells after permeabilization with

Permeabilizing Solution#2 (Becton Dickinson, San Jose, CA) for 10 min at room temperature. Membrane expression was assayed on fixed intact cells without permeabilization. Intracellular expression was determined by the difference between total and surface expression. The mean $CX_3CR1$ specific fluorescence was corrected for background, determined with non-specific rabbit IgG as the primary antibody. Fluorescence was measured with a Becton Dickinson FACScan. The analysis was made on 10 000 events on each sample by using the CELL QUEST program (Becton Dickinson). The Mo were identified and gated according to their forward and side scattering (FSC/SSC) dot-plot profiles and positivity for CD14.

### Apoptosis assay

The proportion of apoptotic Mo was determined by using PI (50 µg/ml) and Annexin V-FITC. The percentage of Annexin V-positive cells, which includes early apoptotic cells (single positive) and late apoptotic cells (double positive), was quantified using a flow cytometer (Becton Dickinson FACScan).

### Calcium mobilization

For the detection of intracellular calcium, the PBMC ($5 \times 10^6$ cells/ml) were suspended in incubation buffer (RPMI-1640 + 1 mM $CaCl_2$, pH 7·4) containing the $Ca^{2+}$ indicator fluo3-AM (4 µM; Sigma). The cells were incubated for 30 min at 37°, washed twice with fluo3-AM-free incubation buffer and analysed by flow cytometry.

### Measurement of surface expression of CD11b

CD11b expression was measured as an indicator of $CX_3CL1$-dependent signalling in a manner similar to that previously described.[8] Briefly, Mo ($1 \times 10^5/0·1$ ml) under different culture conditions were incubated with $sCX_3CL1$ (0·2 and 2 ng/ml) for 15 min at 37°. The incubation was terminated by the addition of ice-cold phosphate-buffered saline and centrifugation. The cells were stained with anti-CD11b PE-conjugated (mouse IgG1; DAKO, Carpinteria, CA) and anti-CD14-PECy5. After cell washing, CD11b expression was analysed by flow cytometry on $CD14^+$ Mo.

### Western blot of THP-1 cell lysates.

The PMA-maturated THP-1 cells ($1 \times 10^6$ cells/ml) under different treatments were lysed by incubation on ice for 15 min in 0·4 ml 100 mM Tris–HCl (pH 8·0), 100 mM NaCl, 2 mM ethylenediaminetetraacetic acid, 1% Nonidet P-40 (RIPA buffer), 1 mM $Na_3VO_4$, 50 mM NaF, 0·3 U/ml aprotinin, 2 mM PMSF and 1 µg/ml each of leupeptin and pepstatin A. Lysates were centrifuged for 15 min at 14 000 *g*. Protein concentration was determined using a micro Bradford assay (Pierce, Rockford, IL). The supernatants were prepared for sodium dodecyl sulphate–polyacrylamide gel electrophoresis under reducing conditions.

The sodium dodecyl sulphate–polyacrylamide gele electrophoresis was run on 10% minigels using standard Tris–glycine buffers. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (BioRad, Hercules, CA) for 1.5 hr at 300 mA and blocked with phosphate-buffered saline 3% non-fat dried milk for 30 min. The membrane was probed with primary rabbit antibody anti-Phospho-Akt (Cell Signaling Technology, Beverly, MA) or anti $\beta$-actin (Cell Signaling Technology) overnight. After washing, blots were incubated for 2 hr with a horseradish peroxidase-conjugated goat anti-rabbit IgG (Caltag, Burlingame, CA). Immunoreactivity was detected using the ECL Western blotting detection reagent (Pierce Biotechnology, Rockford, IL).

### Statistics

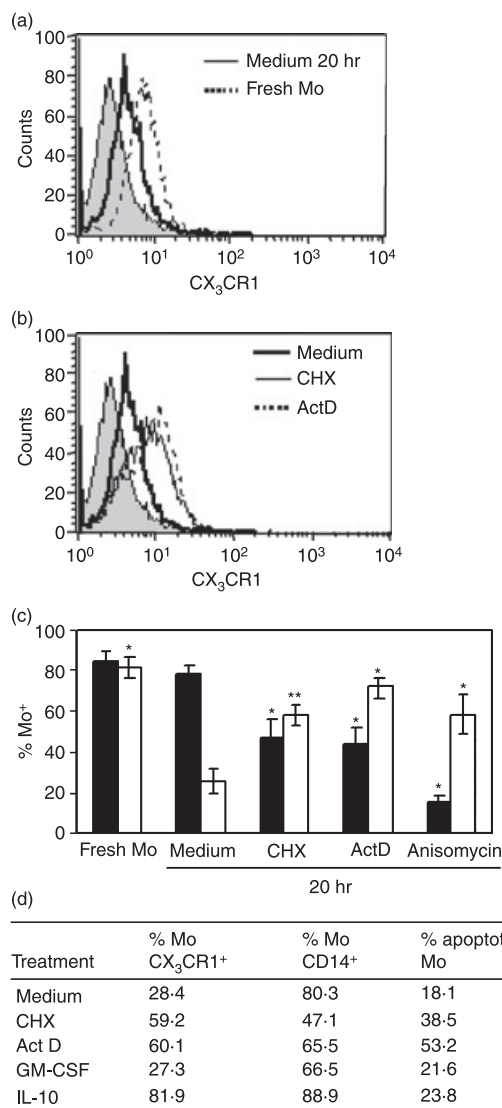When required, the significance of differences between groups were evaluated using Student's paired *t*-test.

## Results

### Monocytes down-modulated the expression of $CX_3CR1$ but not that of CD14 after culture

Human Mo were purified as described in the Materials and methods (purity > 95%; 88–92% with $CD14^+$ $CD16^-$ $CX_3CR1^+$ phenotype) and incubated in complete medium for 20 hr. Although expression of CD14 was not modified during this time, $CX_3CR1$ expression was significantly down-modulated (Fig. 1a,c).

To test the involvement of protein synthesis in the regulation of $CX_3CR1$ membrane expression, Mo were incubated in the presence of CHX (1 µg/ml), ActD (0·5 µg/ml) or anisomycin (0·1 µg/ml). We found that while all inhibitors decreased CD14 expression, they significantly prevented the $CX_3CR1$ down-modulation from Mo membrane upon overnight incubation (Fig. 1b,c). The sustained expression of $CX_3CR1$ after incubation in the presence of protein inhibitors could be interpreted as the inhibition of some active mechanism involved in the down-modulation of $CX_3CR1$ dependent on protein synthesis (Fig. 1c).

To rule out that $CX_3CR1$ down-modulation is induced by cellular death signals, Mo were cultured overnight in the presence of GM-CSF (50 ng/ml). This survival factor did not modify CD14 or $CX_3CR1$ expression, as compared with Mo cultured without this factor (data not shown). In addition, apoptosis was evaluated in parallel with receptor expression. We found that Mo cultured in medium either with or without GM-CSF presented

**Figure 1.** Monocytes down-regulated CX₃CR1 but not CD14 during culture. Purified Mo ($1 \times 10^6$/ml) were cultured for 20 hr with medium or with the following protein synthesis inhibitors: cycloheximide (CHX; 1 μg/ml), actinomycin D (ActD; 0·5 μg/ml) or anisomycin (0·1 μg/ml). After washing, cells were stained with fluorescein isothiocyanate-conjugated anti-CX₃CR1, phycoerythrin-Cy5-conjugated anti-CD14 monoclonal antibodies, or isotype-matched control antibody and analysed by flow cytometry. (a) Representative histograms of surface CX₃CR1-expression in fresh Mo (broken line) or after culture 20 hr (solid line), filled histogram represents the isotype control. (b) Representative histograms of surface CX₃CR1 expression upon incubation for 20 hr with medium (black line), or with CHX (thin line) or with ActD (broken line). (c) The results are expressed as the percentage of CX₃CR1-positive (white bars) or CD14-positive (black bars) cells into the gate for Mo defined by forward (FSC) and side (SSC) scatter parameters. Each bar represents the mean ± SEM of 17 healthy donors for culture in medium, or six donors for experiments with protein synthesis inhibitors. *$P < 0.05$; **$P < 0.01$ statistically different compared with the same parameter of Mo cultured in medium for 20 hr. (d) Purified Mo ($1 \times 10^6$/ml) were cultured for 20 hr with medium or with the following agents: CHX (1 μg/ml), ActD (0·5 μg/ml), granulocyte–macrophage colony-stimulating factor (GM-CSF; 50 ng/ml), interleukin-10 (IL-10; 10 ng/ml). The table shows the percentage of CX₃CR1, CD14 and AnnexinV positive Mo calculated by flow cytometry, of one experiment representative of three.

less than 25% of Annexin V-positive cells. When Mo apoptotic rate was increased by protein inhibitors the decrease in the CX₃CR1 membrane expression was significantly blocked, suggesting that the down-regulation of CX₃CR1 membrane expression is not associated with apoptosis (Fig. 1d).

### Effect of bacterial factors and cytokines on CX₃CR1 membrane expression

Next, we examined whether bacterial factors and cytokines were able to modify the basal CX₃CR1 expression and modulation in culture. Both LPS and Stx1 slightly reduced CX₃CR1 expression after 4 hr in culture, although only LPS, either alone or together with Stx1 had a significant effect (medium: 100%; LPS = 72·6 ± 5·3%*; Stx1 = 82·2 ± 7·4; LPS + Stx1 = 77·4 ± 7·0*; TNF-α = 92·1 ± 7·5; n = 7, *$P < 0.01$). However, LPS only acceler-

ated CX₃CR1 down-modulation because the expression of CX₃CR1 at 20 hr was similar in the presence or absence of this agonist (data not shown).

On the other hand, neither inflammatory (TNF-α) nor anti-inflammatory (IL-4 and TGF-β) mediators modified CX₃CR1 surface expression, at least at the time and experimental conditions assayed. In contrast, IL-10 significantly prevented CX₃CR1 down-modulation at 20 hr of incubation (medium = 48·1 ± 5·1%; IL-10 = 78·3 ± 5·8%*; n = 17, *$P < 0.0001$). This effect was specific for CX₃CR1 because IL-10 simultaneously enhanced CD14 membrane expression at 20 hr, as previously reported.[15,16]

These data suggest that the CX₃CR1 down-modulation observed in Mo after overnight culture is a consequence of an active process, which could be modulated by IL-10.

### CX₃CR1 modulation by IL-10 was dose- and time-dependent

We further investigated the IL-10 effect on the modulation of CX₃CR1 expression in Mo. We found that IL-10 prevented CX₃CR1 down-modulation in a dose-dependent manner (Fig. 2a,b). As a consistent effect on CX₃CR1 membrane expression was obtained with 10 ng/ml IL-10, subsequent experiments were carried out at this concentration. When IL-10 was added for increasing periods of time and then washed and incubated in medium up to 20 hr, CX₃CR1 expression increased in a time-dependent manner (Fig. 2c).
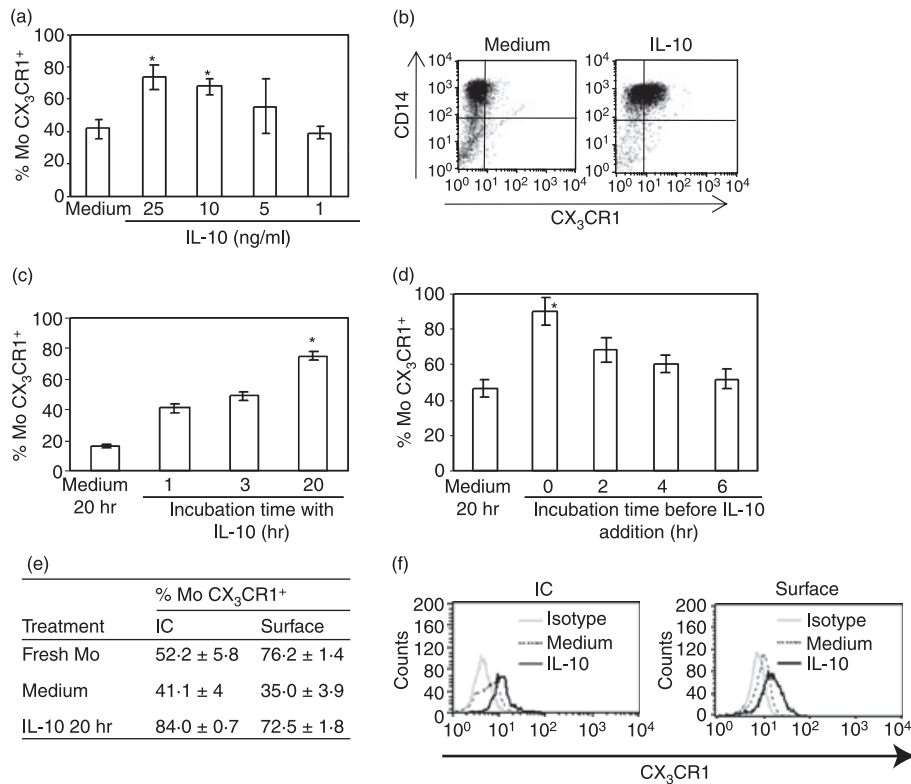
**Figure 2.** Interleukin-10 (IL-10) avoided down-regulation of $CX_3CR1$ during culture. Purified monocytes (Mo; $1 \times 10^6$/ml) were incubated for 20 hr with medium or with IL-10 at different doses and times, followed by flow cytometry analysis of $CX_3CR1$ expression. (a) Purified Mo ($1 \times 10^6$/ml) were incubated for 20 hr with IL-10 at the concentrations indicated below each bar. (b) Representative dot-plots for double CD14/$CX_3CR1$-staining showing the up-regulation of $CX_3CR1$ expression in Mo upon 20 hr culture with IL-10 (10 ng/ml). (c) Purified Mo ($1 \times 10^6$/ml) were incubated with medium or IL-10 (10 ng/ml) added during different periods, washed and then cultured in medium for up to 20 hr (or IL-10 over all 20 hr). (d) Purified Mo ($1 \times 10^6$/ml) were incubated with medium for different periods before adding IL-10 (time 0 represents IL-10 over all 20 hr). Each bar represents the mean ± SEM of 16 healthy donors for culture in medium or IL-10 (10 ng/ml), and three donors for each point on the time and dose curves. *$P < 0.05$ statistically different compared with the same parameter of Mo in medium. (e) Table showing the $CX_3CR1$-surface expression and the intracellular content (IC) after Mo permeabilization as detailed in the Materials and methods. Data are expressed as the mean ± SEM of three independent experiments. (f) Representative histograms showing $CX_3CR1$-surface and intracellular (IC) expression in Mo only upon 20 hr culture with medium or IL-10 (10 ng/ml).

On the other hand, when Mo were incubated with medium and IL-10 was added at different times and the incubation was continued up to 20 hr, the restraining effect of IL-10 on $CX_3CR1$ expression was time dependent (Fig. 2d). These results suggest that the $CX_3CR1$ down-modulation upon culture is not reversible and that the IL-10 modulatory effect is proportional to the incubation time.

## Cellular mechanisms involved in $CX_3CR1$ modulation by IL-10

To understand the cellular mechanism involved in the regulation of $CX_3CR1$ by culture or IL-10, we examined the intracellular receptor pool by analysing total and surface expression by flow cytometry in permeabilized and intact cells, respectively. We observed that after overnight culture there was a significant decrease in both $CX_3CR1$ membrane expression and intracellular content compared with fresh Mo. In contrast, after 20 hr of culture in the presence of IL-10, the membrane expression was preserved and the intracellular $CX_3CR1$ pool was markedly increased as compared with fresh Mo (Fig. 2e,f). In addition, we evaluated the effect of this cytokine in the presence of the protein synthesis inhibitor CHX. The simultaneous treatment of Mo with IL-10 and CHX did not modify the pattern of $CX_3CR1$ down-modulation obtained with each agent when individually assayed (%$CX_3CR1^+$ Mo: Medium = 24·1 ± 7·5; IL-10 = 57·6 ± 3·1*; Medium + CHX = 49·6 ± 8·4*; IL-10 + CHX = 52·1 ± 1·5*; *$P < 0.05$). In contrast, CHX decreased the expression of CD14 and completely counteracted the IL-10-induced enhancement (data not shown).
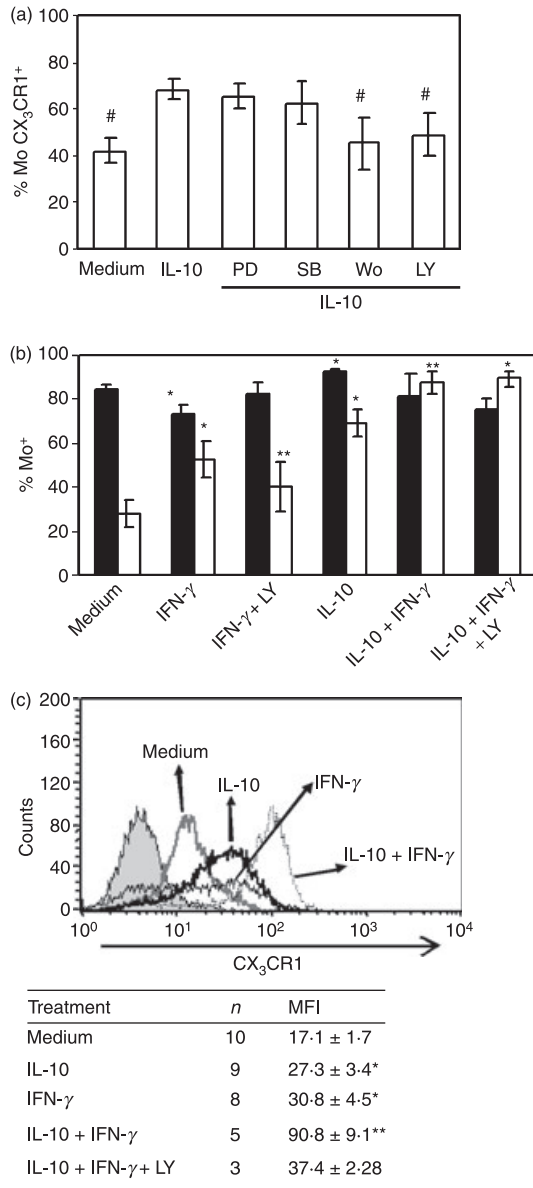
**Figure 3.** Influence of mitogen-activated protein kinase (MAPK) or phosphatidylinositol 3-kinase (PI3K) chemical inhibitors on cytokine-mediated CX₃CR1 regulation. (a) Purified Mo ($1 \times 10^6$/ml) were pre-incubated for 1 hr with the corresponding kinase-inhibitors (PD98059, 20 μM; SB203580, 30 μM; Wortmannin, 100 nM; LY294002, 25 μM) before interleukin-10 (IL-10; 10 ng/ml) addition, and cultured for 20 hr. The results are expressed as the percentage of CX₃CR1-positive cells. Each bar represents the mean ± SEM of five to seven healthy donors. #$P < 0.05$ statistically different compared with IL-10 alone. (b) Purified Mo ($1 \times 10^6$/ml) were cultured for 20 hr with medium or interferon-γ (IFN-γ; 240 U/ml) alone or with LY294002 (25 μM); and/or IL-10 (10 ng/ml). Then, CD14 and CX₃CR1 expression was analysed by flow cytometry. The results are expressed as the percentage of CX₃CR1-positive (white bars) or CD14-positive (black bars) cells into the gate of Mo. Each bar represents the mean ± SEM of five to seven healthy donors, except for medium and IFN-γ bars which each correspond to 11 blood samples. *$P < 0.05$; statistically different compared with the same parameter of Mo in medium; **$P < 0.05$ statistically different compared with IFN-γ alone. (c) Representative histograms of CX₃CR1 expression after culture with medium, IL-10, IFN-γ or both cytokines for 20 hr. Filled histogram corresponds to isotype-control. Data in the table are the mean ± SEM of CX₃CR1 mean fluorescence intensity (MFI) of $n$ experiments. *$P < 0.05$ statistically different compared with medium; **$P < 0.05$ statistically different compared with IFN-γ alone.

| Treatment | $n$ | MFI |
|---|---|---|
| Medium | 10 | 17·1 ± 1·7 |
| IL-10 | 9 | 27·3 ± 3·4* |
| IFN-γ | 8 | 30·8 ± 4·5* |
| IL-10 + IFN-γ | 5 | 90·8 ± 9·1** |
| IL-10 + IFN-γ+ LY | 3 | 37·4 ± 2·28 |

## CX₃CR1 modulation by IL-10 did not involve MAPK, but rather PI3K activation

Since MAPK and PI3K pathways play a major role in IL-10-mediated induction of several cell surface receptors,[17–19] Mo were treated with IL-10 in the presence of MAPK or PI3K inhibitors for 20 hr. We found that none of the MAPK inhibitors impaired IL-10 regulatory effect (Fig. 3a). However, PI3K inhibitors Wortmanin (Wo, 100 nM), or the more specific LY294002 (25 μM), impaired the effect of IL-10 on CX₃CR1 expression (Fig. 3b). It may be noted that treatment of cells with LY294002 alone did not affect cell viability and did not induce any variation in basal CX₃CR1 and CD14 expression when incubated alone (data not shown).
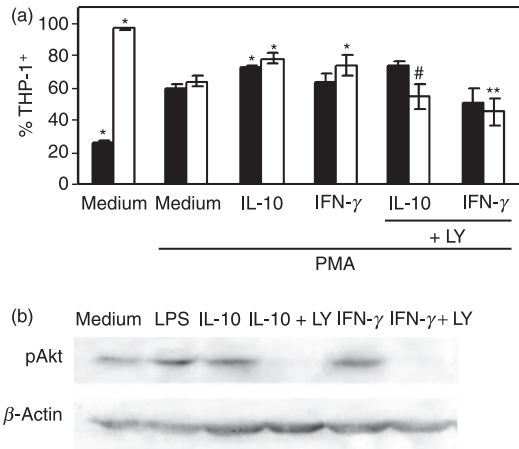
## IFN-γ prevented CX₃CR1 down-modulation during Mo culture

Interferon-γ and IL-10 usually have antagonistic effects on Mo so we investigated IFN-γ action on CX₃CR1 modulation in Mo. Surprisingly, overnight incubation of Mo in the presence of 240 U/ml IFN-γ blocked the down-modulation of CX₃CR1, through a PI3K-dependent mechanism similar to that of IL-10 modulation (Fig. 3b). In contrast, and in line with previous reports, IFN-γ significantly down-modulated CD14 expression (Fig. 3b).When Mo were incubated with both IFN-γ and IL-10, we observed a synergistic effect, which was revealed not only by a higher percentage of CX₃CR1⁺ cells but also by a significant increase in the mean fluorescence intensity (MFI) (Fig. 3c). When LY294002 was assayed in Mo treated with IFN-γ and IL-10, a partial blocking effect was observed in the increase in CX₃CR1 MFI (Fig. 3b,c).

## IL-10 blocked CX₃CR1 down-modulation in mature THP-1 cells

To study intracellular signals in a pure cell population we examined the regulation of CX₃CR1 expression in THP-1 cells. The treatment of these cells with 5 ng/ml PKC-activating phorbol ester (PMA) for 48 hr is a widely accepted procedure for maturation of Mo.[20] Hence, PMA-treated

Figure 4. Phorbol 12-myristate 13-acetate (PMA) -maturated THP-1 cells down-modulated $CX_3CR1$ expression. (a) THP-1 cells ($1 \times 10^6$/ ml) were differentiated with PMA (5 ng/ml) for 48 hr, then they were incubated with medium, interleukin-10 (IL-10; 10 ng/ml) or interferon-$\gamma$ (IFN-$\gamma$; 240 U/ml) during 20 hr. Some cell samples were pre-incubated for 1 hr with LY294002 (25 μM). All samples were analysed for $CX_3CR1$ and CD14 expression by flow cytometry. The results are expressed as the percentage of $CX_3CR1$-positive (white bars) or CD14-positive (black bars) THP-1 cells. Each bar represents the mean ± SEM of five independent experiments. *$P < 0.05$ statistically different compared with the same parameter of THP-1 cells in medium + PMA; **$P < 0.05$ statistically different compared with IFN-$\gamma$ alone; #$P < 0.02$ statistically different compared with IL-10 alone. (b) LY294002 impaired IL-10- or IFN-$\gamma$-dependent Akt activation. PMA-maturated THP-1 cells ($1 \times 10^6$/ml) were pre-incubated during 60 min with medium or LY294002 (25 μM), and 60 min with IL-10 (10 ng/ml) or IFN-$\gamma$ (240 U/ml). Then protein cell extracts were assayed for Akt phosphorylation by immunoblotting as described in the Materials and methods. Cells incubated with lipopolysaccharide (1 μg/ml) during 60 min were assayed as a positive control.

THP-1 cells significantly enhanced CD14 (approximately 20–60%) and decreased $CX_3CR1$ cell surface expression (approximately 90–60%) (Fig. 4a). These results reproduce the phenotype alterations observed in fresh Mo cultured overnight. Subsequently, we demonstrated that IL-10 and IFN-$\gamma$ increased $CX_3CR1$ expression in maturated THP-1 cells, in a way similar to that observed in human Mo. Moreover, the specific PI3K inhibitor LY294002 counteracted IL-10 and IFN-$\gamma$ modulatory effects on $CX_3CR1$ expression, whereas MAPK inhibitors had no effect (Fig. 4a).

Then, we evaluated Akt activation upon IL-10 and IFN-$\gamma$ treatment by western blot (WB) to corroborate that PI3K was involved in cytokine up-regulation of $CX_3CR1$.[20] The results shown in Fig. 4(b) indicate that both IL-10 and IFN-$\gamma$ induced a strong phosphorylation of Akt in THP-1 cells at 30 min post-stimulation. In addition, this effect was blocked by LY294002.
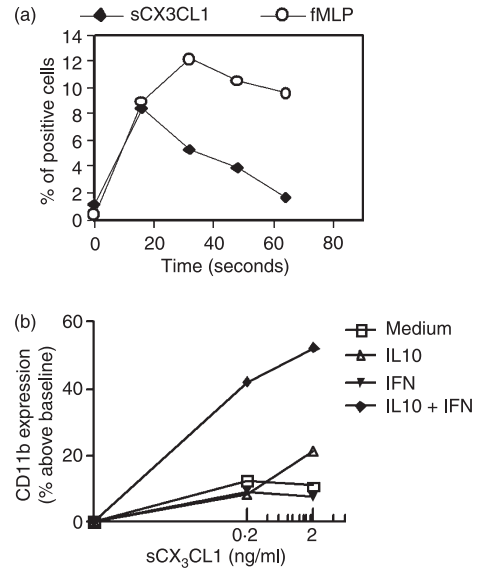


Figure 5. $CX_3CL1$-dependent functionality. (a) Peripheral blood mononuclear cells (PBMC) were loaded with Fluo3-AM, as indicated in the Materials and methods. Then cells were treated with $sCX_3CL1$ (100 ng/ml) or fMLP ($10^{-7}$ M). Changes in $[Ca^{2+}]_i$ were followed by monitoring the change in the percentage of positive cells inside the monocyte (Mo) gate. The figure shows one representative experiment out of three. (b) Purified Mo ($1 \times 10^6$ cells/ml) cultured with medium, interleukin-10 (IL-10; 10 ng/ml), interferon-$\gamma$ (IFN-$\gamma$; 240 U/ml) or IL-10 + IFN-$\gamma$ for 20 hr, were incubated with $CX_3CL1$ (0·2; 2 ng/ml) for 15 min at 37°. Mo were gated out based on forward and side scatter parameters. CD11b expression, measured as mean fluorescence intensity (MFI), was expressed as percentage above baseline (i.e. incubation with each cytokine without $sCX_3CL1$). The figure shows one representative experiment out of three.

## $CX_3CR1$ membrane expression correlated with $CX_3CL1$-dependent functionality

A common feature of chemokine receptors is their capacity to mobilize calcium upon interaction with their specific ligand. As a consequence, incubating Fluo3-AM PBMC with 100 ng/ml $sCX_3CL1$ resulted in a rapid increase in $[Ca^{2+}]_i$, reaching peak values within 10– 20 seconds and declining thereafter to baseline levels (Fig. 5a). The increase was concentration dependent in the 10–100 ng/ml range, and reached a maximum at 100 ng/ml. However, $[Ca^{2+}]$ mobilization could not be tested in purified Mo treated with cytokines to correlate the $CX_3CR1$-dependent function with changes in its membrane expression, probably as the result of a previous $[Ca^{2+}]$ mobilization during the purification and cytokine incubation periods.

Then, to assess whether variations in $CX_3CR1$ expression might impact Mo functionality, we investigated the up-regulation of CD11b, which has been previously demonstrated to be a sensitive marker of $sCX_3CL1$-mediated Mo activation.[8] We observed a slight increase in CD11b-

expression with 2 ng/ml sCX$_3$CL1 in Mo incubated for 20 hr with IL-10, as compared with Mo cultured in medium (Fig. 5b). However, when Mo were incubated for 20 hr in the presence of both cytokines (IL-10 + IFN-$\gamma$) simultaneously, there was a clear increase in CD11b compared with all the other treatments. These data suggest that up-regulation of CX$_3$CR1 was accompanied by a significant increase in the sCX$_3$CL1-dependent response.

## Discussion

Emerging evidence shows that the regulation of chemokine receptor expression during cell activation or deactivation is as important as the regulation of chemokine production for tuning the chemokine system.[21]

In this study, we addressed the regulation of Mo CX$_3$CR1 membrane expression during maturation and the influence of bacterial factors and cytokines on this process. We observed the selective down-modulation of CX$_3$CR1 during overnight culture of Mo, which was not associated with apoptosis or cellular death and which resulted from an active process dependent on protein synthesis. In addition, PMA-matured THP-1 cells mimic the down-modulation of CX$_3$CR1 observed in cultured human Mo. Similarly, it has been reported that the maturation process itself is the main factor in the selective loss of CCR2 gene expression during Mo maturation using the PMA-differentiated THP-1 cell line model.[22–24].

Previous studies on mononuclear phagocytes have shown that exposure to microbial agents or inflammatory mediators (e.g. LPS, IL-1 and TNF-$\alpha$) induces specifically the down-modulation of certain CC chemokine receptors (CCR1, CCR2, CCR5),[22,25,26] and that anti-inflammatory agents, such as IL-10, have an opposite effect.[21] In line with these reports, we found that LPS accelerated the down-regulation of CX$_3$CR1 during Mo culture, and that IL-10 strongly prevented it. Moreover, IL-10 increased CX$_3$CR1 expression in PMA-matured THP-1 cells. The IL-10 effect was time- and dose-dependent, and specific for IL-10 because other Mo deactivating factors such as IL-4 and TGF-$\beta$ did not affect CX$_3$CR1 expression. In addition, IFN-$\gamma$, which promotes Mo maturation,[24,27] also prevented CX$_3$CR1 down-modulation in Mo and increased CX$_3$CR1 expression in PMA-matured THP-1 cells. Moreover, IFN-$\gamma$ and IL-10 together showed a synergistic effect, leading Mo to reach a maximum CX$_3$CR1 expression that was even higher than the level in fresh Mo. These results suggest that, besides sharing some intracellular signalling pathways, both cytokines may be acting through alternative and additional signalling or by sensitizing Mo to the other cytokine-mediated signalling.

Two important mechanisms of chemokine-receptor regulation have been described. One of them involves intracellular storage and its rapid mobilization and internalization.[28] Although almost no information is available about CX$_3$CR1 regulation in Mo, a recent study has shown that MCP-1 induces a transient increase in CX$_3$CR1 expression on Mo surface at 15 min, which suggests mobilization from intracellular pools to the plasma membrane rather than *de novo* synthesis of receptor protein.[29] Following this enhancement a marked and rapid down-regulation is observed at 60 min, which is an indirect evidence of the existence of an active degradation mechanism. Under our experimental conditions, we did not observe such early increase with IL-10 or IFN-$\gamma$ treatment (data not shown). However, through the evaluation of surface and intracellular CX$_3$CR1 expression, we concluded that during culture of non-stimulated Mo the rate of CX$_3$CR1 membrane appearance is lower than the rate of degradation or loss. In contrast, in IL-10-stimulated Mo the rate of formation is higher than the rate of degradation, regardless of the mechanism involved.

The second mechanism involved in chemokine receptor regulation is at messenger RNA (mRNA) level, through both transcription induction and stability enhancement of the corresponding mRNA.[30] In particular, mRNA for CX$_3$CR1 has been reported to be regulated in different cells and under different situations. In this regard, Koziolek *et al.*[31] have shown a strong mRNA induction in human fibroblasts by H$_2$O$_2$, a mediator of oxidative stress. Similar results have been reported in microglia after ischaemia.[32] Furthermore, recent reverse transcription–polymerase chain reaction assays have demonstrated that IL-10 treatment induces high levels of CX$_3$CR1 mRNA in Mo.[33] On the other hand, other authors have reported that LPS reduces CX$_3$CR1 mRNA in PBMC, while IL-10 has no effect.[8] In this context, when protein synthesis inhibitors were used simultaneously with IL-10 no additive effects were observed, leading us to hypothesize that CHX could be abrogating the *de novo* synthesis necessary for IL-10 modulation of CX$_3$CR1. Interleukin-10 could directly induce the *de novo* synthesis of either CX$_3$CR1, or some protein involved in its degradation step, and so interfere with the circulating circuit of this receptor from the intracellular pool to the plasma membrane. However, further studies with IL-10 and IFN-$\gamma$ treatment should be carried out on CX$_3$CR1 mRNA in Mo to achieve a final conclusion.

Interleukin-10 is secreted by T helper type 2 (Th2) cells and plays a major role in the inhibition of Mo/macrophage function.[34] In contrast, IFN-$\gamma$ is produced by natural killer and T cells, and plays an important role in orienting responses toward a Th1 pattern through macrophage activation.[35,36] Consequently, IL-10 and IFN-$\gamma$ generally have divergent effects on monocytic function. In fact, in the presence of IFN-$\gamma$, IL-10 is less effective at suppressing cytokine and chemokine production, and down-regulating major histocompatibility complex class II expression.[37] The reciprocal is equally and often reported, i.e. IL-10 inhibits IFN-$\gamma$-mediated induction of early response

genes.[38] Therefore, the results reported in this manuscript showing that IL-10 and IFN-$\gamma$ have similar and even synergistic effects on Mo-CX$_3$CR1 regulation have scarce antecedents.[39,40] Most importantly, the synergistic effect between IL-10 and IFN-$\gamma$ observed in CX$_3$CR1 membrane expression correlated with the enhancement in CX$_3$CR1 functionality.

Interferon-$\gamma$ and IL-10 bind to their cognate receptors and initiate a signal that results in the activation of janus kinase (JAK) and signal transducer and activator of transcription (STAT) proteins, leading to transcription of early response genes.[41,42] In addition, it has been shown that JAK proteins can also activate other signalling molecules, specially from the PI3K family.[18,43] In this regard, we demonstrated the activation of the survival enzyme Akt-1, the signalling molecule downstream of PI3K by IL-10 and IFN-$\gamma$ treatment. Moreover, chemical inhibition of PI3K abrogated IL-10 and IFN-$\gamma$-mediated up-regulation of CX$_3$CR1 in both Mo and PMA-maturated THP-1 cells, supporting the conclusion that Akt is involved in the signalling pathway that regulates CX$_3$CR1 expression by IL-10 and IFN-$\gamma$. A number of phosphorylation targets for Akt are now emerging,[43] providing alternative routes by which IL-10 and IFN-$\gamma$ can potentially act. These include the activation of nuclear factor-$\kappa$B,[44] and the Akt capability to translocate to the nucleus where it may influence protein transcription and the cell cycle.[45] Moreover, several reports agree that Akt may be responsible for serine-phosphorylation of STAT1 in response to IFN-$\gamma$ or IL-10.[46] The simultaneous serine-phosphorylation of these transcription factors by Akt seems to be necessary for the complete or enhanced transcriptional activity of STATs on the CX$_3$CR1 promoter. Further studies are necessary to completely elucidate which are the signalling molecules and the transcription factors involved in the up-regulation of CX$_3$CR1 by these cytokines.

Although the biological role of the variations in CX$_3$CR1 levels in the Mo membrane is still poorly understood, it has been recently reported that a high expression of CX$_3$CR1 correlates with an increased functional capacity, assayed as adherent or chemotactic responses.[29] Here, we have similarly shown that CX$_3$CR1 expression correlates with CD11b up-regulation secondary to sCX$_3$CL1 incubation. This effect may have biological consequences because the recruitment of Mo subsets into tissues requires firm arrest and attachment onto vascular endothelia under shear stress conditions. This recruitment is mediated by specific combinations of adhesion molecules and chemokine receptors.[47]

In conclusion, this study further reinforces the concept that locally produced cytokines or bacterial products may regulate the kinetics, composition and functional status of the leucocyte infiltrate by affecting both chemokine production and receptor expression.[48]

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## Disclosures

The authors have no conflicts of interest to disclose.

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