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Cytokines use different intracellular mechanisms to upregulate the membrane expression of CX_3CR1 in human monocytes

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ABSTRACT

Membrane expression of fractalkine (CX_3CL1)-receptor (CX_3CR1) is relevant in monocytes (Mo) because CX_3CR1-CX_3CL1 interactions might participate on both, homeostatic and pathologic conditions. We have previously demonstrated that CX_3CR1 levels are decreased during culture and when Mo are differentiated into dendritic cells, but enhanced when differentiated into macrophages. Regarding soluble factors, lipopolysaccharide (LPS) accelerated the loss of CX_3CR1, while interleukin (IL)-10 and Interferon-gamma (IFN-γ) prevented it. However, the comprehensive knowledge about the intracellular pathways that underlay the level of CX_3CR1 expression in Mo is still incomplete.

In the current work, we studied the effect of anti-inflammatory cytokines (IL-4, IL-13, IL-10), alone or together with IFN- γ on CX_3CR1 expression. We found that only IL-10 and IFN-γ separately were able to prevent CX_3CR1 down-modulation during culture of human Mo. Besides, Mo incubated with IL-10 plus IFN-γ showed the highest CX_3CR1 expression by cell, suggesting cooperation between two different mechanism used by both cytokines. By studying intracellular mechanisms triggered by IL-10 and IFN-γ, we demonstrated that they specifically induced PI3K-dependent serine-phosphorylation of signal transducer and activator of transcription (STAT)3 or STAT1, respectively. Moreover, chemical inhibitors of STAT1 or STAT3 abrogated IFN-γ or IL-10 effects on CX_3CR1 expression. Strikingly, only IL-10 increased *CX_3CR1* mRNA level, as consequence of augmenting mRNA stability. *CX_3CR1* mRNA increase was PI3K-dependent, supporting the causal link between the action of IL-10 at the *CX_3CR1* transcript and CX_3CR1 protein level on Mo. Thus, both cytokines up-regulate CX_3CR1 expression on human Mo by different intracellular mechanisms.

1. Introduction

The unique member of the CX_3C chemokine subfamily is Fractalkine (CX_3CL1), which exists in a soluble or a membrane-anchored form. The latter functions as an adhesion molecule (Bazan et al., 1997), and is expressed on the surface of endothelial and epithelial cells (Lucas et al., 2001), dendritic cells (DC) (Papadopoulos et al., 1999) and neurons (Harrison et al., 1998) and is induced by pro-inflammatory cytokines such as interleukin (IL)-1, tumor necrosis factor (TNF)-α and Interferon-gamma (IFN-γ) (Imaizumi et al., 2004). The full-length molecule can be cleaved from the cell membrane by metalloproteases, resulting in the release of a soluble CX_3CL1 which acts as a chemoattractant. The CX_3CL1 receptor, CX_3CR1, is expressed on several leukocytes, including monocytes (Mo), T cell subsets, and on the major subset of Natural

Abbreviations: CX_3CL1, fractalkine; CX_3CR1, fractalkine receptor; Mo, monocytes; TNF-α, tumor necrosis factor alpha; LPS, lipopolysaccharide; PI3K, phosphatidylinositol-3-kinase; IL-, interleukin-; IFN-γ, Interferon-gamma; STAT, signal transducer and activator of transcription; JAK, Janus kinase; HRP, Horseradish peroxidase; FITC, Fluorescein isthiocyanate; PE, Phycoerythrin; PECy5, Phycoerythrincyanin 5.1; MFI, mean fluorescence intensity; SSC/FSC, side vs. forward scattering; mAb, monoclonal antibody; ActD, actinomycin D; PFA, paraformaldehyde

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Killer cells (Imai et al., 1997). Besides, CX_3CR1 expression has been described in platelets (Postea et al., 2012).The expression of CX_3CR1 is very important for Mo physiology, during homeostasis and disease because CX_3CR1-CX_3CL1 interactions might control the number of circulating Mo by regulating their half-life (Landsman et al., 2009; White et al., 2014; Janssen et al., 2016), but also it promotes the firm adhesion of Mo to endothelial cells. This process is particularly relevant after vascular injury, where CX_3CR1^+ Mo play a central role for endothelium wound healing (Getzin et al., 2018). In addition, it is now clear that specific cell-cell interactions between endothelial cells and invading Mo are key elements that contribute to recruitment of circulating Mo to sites of injury (Apostolakis et al., 2007; Tacke et al., 2007), as well as progression of systemic vasculitis, atherosclerotic diseases and some autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus (Yano et al., 2007; Kasama et al., 2010; Garcia et al., 2013). Moreover, it has been recently reported that in a tumor environment CX_3CR1-CX_3CL1 interactions between circulating Mo and endothelium, are critical to define the transmigration of pro-angiogenic Mo to the tumor (Sidibe et al., 2018). In the central nervous system, CX_3CR1-dependent chemotaxis of microglia to the site of injury has been also described (Prinz and Priller, 2014). Thus, through regulation of CX_3CR1 membrane expression on Mo, it is possible fine-tuning control of Mo survival and recruitment, affecting endothelium healing, inflammatory process, tumor growth, or degenerative diseases. Although the regulation of membrane CX_3CR1 expression on Mo remains poorly understood, we have previously demonstrated that CX_3CR1 levels are decreased in human Mo during culture or during differentiation process to DC (Panek et al., 2015). Besides, while lipopolysaccharide (LPS) accelerated the loss of CX_3CR1, IL-10 and IFN-γ prevented it by a phosphatidylinositol-3-kinase (PI3K)-dependent mechanism (Ramos et al., 2010). However, a comprehensive knowledge about the intracellular signals and pathways that underlay the level of CX_3CR1 expression in Mo is still incomplete.

Immunoregulatory Th2-cytokines such as IL-10, IL-4 and IL-13 have been described to exert anti-inflammatory properties on various cell types, mainly Mo. Binding of IL-10 to the extracellular domain of IL-10 receptor 1 (IL-10R1) activates phosphorylation of the receptor-associated kinases, Janus kinase (JAK)1 and Tyk2 (Makuta et al., 2003; Zhou et al., 2001). These kinases then phosphorylate specific tyrosine (Tyr) residues of IL-10R1, resulting in the recruitment of STAT3 and phosphorylation at residue tyrosine 705 (Y705). Although activation of STAT3 in myeloid cells by IL-10 is required for many anti-inflammatory effects, signal transducer and activator of transcription (STAT)1 can also be activated. Besides, IL-4 and IL-13 are four-helix-bundle short-chain cytokines genetically and structurally related, that use overlapping receptor components (IL-4Rα), share overlapping downstream signaling machinery (e.g., JAK1, JAK2, and STAT6), and drive a lot of common effects in a broad-spectrum cell types, both immune and non-immune (Wills-Karp and Finkelman, 2008).

On the other hand, IFN-γ is the critical cytokine of T helper (Th)1-polarized immune response and one of the most potent Mo activators and inducer of classical macrophage activation. The signaling pathway elicited by IFN-γ begins with receptor dimerization and activation of JAK1 and JAK2 by transphosphorylation. These kinases are responsible for tyrosine phosphorylation of STAT1 (Bancerek et al., 2013). Phosphorylation of tyrosine 701 (Y701) leads to STAT1 homodimerization and translocation into the nucleus, where STAT1–STAT1 dimers bind γ-interferon activated sites located in responsive gene promoters. Although IFN-γ primarily activates STAT1, STAT3 and STAT5 can also be activated (Ramana et al., 2002). In addition, for full transcriptional activation a second phosphorylation event, at serine 727 (S727) in the transactivation domain, is necessary for all STATs, except STAT2 and STAT6 (Bancerek et al., 2013).

When Mo are stimulated with these two types of cytokines (IFN-γ vs. IL-10, IL-4/IL-13) a distinct pattern of gene expression, cell surface molecules, and phenotypic changes occur. As consequence, both types of cytokines often mediate divergent functions and they reciprocally inhibit the action of the other, in spite of some overlapping in the proteins activated by them.

Thus, the aim of our study was to determine if IL-10, IL-4 and IL-13 alone or together with IFN-γ were able to modulate CX_3CR1 expression; and to elucidate signaling pathways and intracellular mechanisms triggered by those cytokines that lead to prevent CX_3CR1 down-modulation during culture.

2. Materials and methods

2.1. Ethics statements

This study was approved by the Ethics Committee from Academia Nacional de Medicina de Buenos Aires. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

2.2. Reagents and antibodies

Endotoxin-free reagents and plastics were used in all experiments. Lipopolysaccharide (LPS, *E. coli* serotype O111:B4), actinomycin D (ActD), Phorbol 12-myristate 13-acetate (PMA), sodium orthovanadate ($NaVO_4$), sodium fluoride (NaF) and bovine serum albumin (BSA) were obtained from Sigma (St Louis, MO). Phenylmethylsulphonyl fluoride (PMSF), protease inhibitors aprotinin, leupeptin, pepstatin A, MAPK inhibitors SB203580 (p28) and PD98059 (ERK1/2), and the PI3K inhibitor LY294002, were purchased from Calbiochem-Novabiochem (La Jolla, CA). STAT1 inhibitor adenosine was purchased from Sigma-Aldrich (St Louis, MO). STAT3 inhibitor JSI-124 (cucurbitacin I) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and dissolved in ethanol initial stock concentration at 10 mM. Recombinant human IL-4, IL-10, IL-13, and IFN-γ were from PeproTech (PeproTech Mexico, DF, México). All tissue culture flasks, dishes and multiwell plates were Falcon (Orange Scientific, Graignette Business Park, Belgium).

FITC-conjugated anti human-CX_3CR1 mAb (Clone 2 A9-1) -Rat IgG2b, κ – and the respective isotype control were purchased from BioLegend (San Diego, CA, USA); PECy5-conjugated anti human-CD14 mAb (Clone RMO52) – Mouse IgG2a- was from Beckman Coulter (Brea, CA, USA), and the PECy5 Mouse IgG2a, κ isotype control mAb from Biolegend.

For western blotting assays, the primary antibodies anti-phospho-STAT1 (pY701) and anti-β-Actin were obtained from Cell Signaling (Danvers, MA, USA) and anti-STAT1 was from Santa Cruz Biotechnology. Secondary antibodies were horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Caltag, Burlingame, CA) and HRP sheep anti-mouse IgG (Amersham, Aylesbury, UK). For detection of phosphoproteins by flow cytometry, we used the following antibodies: polyclonal primary antibody anti-STAT1 (pS727) from Cell Signaling Technology, secondary antibody FITC-conjugated goat anti-rabbit IgG from Sigma-Aldrich and PE-Mouse anti-STAT3 (pS727) Clone 49/p-STAT3 from BD Phosphoflow (San José, CA, USA).

2.3. Isolation and culture of human peripheral blood monocytes (Mo)

Mo were isolated from freshly prepared buffy coats that were between 2–4 h old, kindly provided by the Garrahan Hospital's Hemotherapy Service (Ciudad de Buenos Aires, Argentina). Blood samples were obtained from healthy donors after written informed consent. Blood was seeded on successive Ficoll-Hypaque (Ficoll Pharmacia, Uppsala, Sweden; Hypaque, Wintthrop Products, Buenos Aires, Argentina) and Percoll (GE Healthcare, Uppsala, Sweden) gradients, as previously described (Panek et al., 2015; Ramos et al., 2010). Viability of Mo was > 96% as determined by Trypan blue exclusion test and CD14 staining of Mo revealed that their purity was > 85%. Finally, Mo were suspended at 1×10^6 cells/ml in RPMI-1640 (Hyclone

Laboratories Inc., Logan, Utah) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Natocor, Córdoba, Argentina), and 1% Antibiotic–Antimycotic solution (Gibco, Invitrogen, San Diego, CA). When indicated, Mo were pre-incubated with specific inhibitors (LY294002, 25 uM; SB20358, 30 uM; PD98059, 20 uM during 1 h or with Adenosine 200 uM, JSI-124, 2 uM) during 30 min and then in medium alone or with IFN-γ (500 U/ml); or IL-10 (10 ng/ml) or IL-4 (50 ng/ml); or IL-13 (50 ng/ml), each one alone or in combination with IFN-γ.

2.4. Flow cytometric analysis

2.4.1. Membrane antigens (CX_3CR1 and CD14)

After treatments, Mo were incubated with the specific conjugated mAb for 30 min at 4 °C. In all cases, isotype-matched antibodies were assayed in parallel and the threshold level for the fluorescence of positive cells was set for each sample from a difference between curves obtained from the specific mAb and isotype control mAb staining. Mo were identified and gated according to their side vs. forward scattering (SSC/FSC) dot-plot profiles and positive staining for CD14. The fluorescence was measured on 10,000 events using the Cell Quest program on a Becton Dickinson FACScan and the results were expressed as percentage of positive cells for each antigen or the mean fluorescence intensity (MFI) of each antigen per cell.

2.4.2. Phospho-specific flow cytometric analysis of STAT1 and STAT3 at S727

Purified Mo (1.0×10^6 cells/ml) were cultured in RPMI medium without FBS for 30 min at 37 °C to improve specific signal of phosphoproteins. Later on, Mo were stimulated or not for 30 min at 37 °C with IFN-γ (500 U/ml) and/or IL-10 (10 ng/ml). When indicated, before treatments Mo were pre-incubated for 60 min with PI3K inhibitor (LY294002, 25 μM). LPS (1 μg/ml) and PMA (50 ng/ml) were used as positive controls of S727-STAT1 or S727-STAT3 phosphorylation, respectively. After stimulation, cells were fixed with FIX Buffer Phosphoflow I (BD Phosphoflow -San José, CA, USA) for 10 min at 37 °C, then pelleted and permeabilized with Perm Buffer III (BD Phosphoflow -San José, CA, USA) for 30 min on ice. After washing them twice in washing buffer (PBS- 1 × 0.5% BSA), they were suspended in 200 μl of blocking buffer (PBS- 1 × 1.5% BSA) and incubated for 10 min at RT for blockade. For STAT1 detection, they were stained with primary antibody anti-phospho-STAT1 (pS727) (final dilution 1:100) at RT for 1 h, washed twice as before and blocked with goat serum (final dilution 1:100) at RT for 10 min. Afterwards, cells were stained with secondary antibody FITC-conjugated goat anti-rabbit IgG (final dilution 1:500) at RT for 30 min, in the dark. Alternatively, they were directly stained with primary antibody PE-conjugated anti-phospho-STAT3 (pS727) (final dilution 1:200) at RT for 1 h. Finally, cells under both treatments were washed twice and suspended in PBS 1 × 0.5% paraformaldehyde (PFA) for FACS analysis. 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).

2.5. RNA isolation and quantitative real time reverse transcriptase-polymerase chain reaction (RT-qPCR)

Total RNA was obtained from 5×10^6 Mo after purification, and from Mo after incubation in medium alone or with different cytokines, during different times. In some experiments Mo were preincubated for 60 min with kinase inhibitor (LY294002, 25 μM) before cytokine addition. Total RNA was extracted with NucleoSpin RNA II Kit (Macherey-Nagel, Bethlehem, PA, USA). For each sample, cDNA was synthesized from 1 μg of total RNA (pretreated with DNaseI during extraction) with Accuscript high Fidelity 1st Strand cDNA synthesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. Relative quantification of *CX_3CR1* mRNA expression and the housekeeping glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was measured by RT-qPCR, using a LightCycler 2.0 instrument with the Fast-Start DNA Master SYBR Green I real-time PCR kit according to the manufacturer's instructions (Roche Applied Science, Indianapolis, IN, USA) (Ramos et al., 2010).

PCR amplifications were performed in a final volume of 20 μl containing 4 mM $MgCl_2$ and 0.75 μM each of the required primers. PCR was performed with an initial denaturation step of 10 min at 95 °C, followed by 45 cycles of the following protocol: 10 s at 95 °C, 10 s annealing at 58 °C, 15 s extension at 72 °C. The fluorescent DNA binding dye SYBR Green was monitored after each cycle at 81 °C. LightCycler 2.0 was used to determine the crossing point for individual samples. Serial dilutions of a positive control sample of cDNA were prepared in duplicate to generate standard curves. Relative standard curves describing the PCR efficiency of target gene and *GAPDH* were created and used to perform efficiency-corrected quantification with the LightCycler Relative Quantification Software version 4.0. Results were expressed as a concentration ratio between the *CX_3CR1* mRNA and *GAPDH* mRNA levels. Products were not generated in control reaction in which reverse transcriptase was omitted during cDNA synthesis (Panek et al., 2015).

The primer sequences used are listed in Table 1.

After amplification was complete, a melting curve was generated by heating slowly at 0.1 °C per second from 50 °C to 98 °C, with continuous collection acquisition of fluorescence.

2.6. Real time RT-qPCR analysis of mRNA stability: half-life calculation

To evaluate the half-life of *$CX_3CR1(V28)$* mRNA, freshly isolated Mo (1×10^6 cells/ml) were incubated for 2 h in medium with or without IL-10 (10 ng/ml). Then, ActD (1 μg/ml) was added to ensure transcriptional shutoff and total RNA was extracted at different times. Relative *$CX_3CR1(V28)$* mRNA levels were measured by RT-qPCR analysis and normalized to *GAPDH* mRNA levels. The initial *$CX_3CR1(V28)$* mRNA level (at time 0 of ActD addition), was set at 100%. Decay curves were plotted using GraphPad Prism 5.0 software. Data were fitted with one-phase exponential decay model. The mRNA half-life ($t_{1/2}$) in every

Table 1
Primer sequences.

Gene	Primer sequences	PCR product size	GeneBank accession number
CX_3CR1 (V28)	Fw 5′-TGACTGGCAGATCCAGAGGTT- 3′ Rev 5′-GTAGAATATGGACAGGAACAC-3′ (Pachot et al., 2008)	164 bp	NM_001337.3
Total CX_3CR1	Fw 5'-CCGCCAACTCCATGAACAACC-3'	212 bp	NM_001171174.1 NM_001171171.1
	Rev 5'-CAGGGGGAGTAGGAAGCCAAGAA-3'		NM_001171172.1 NM_001337.3
GAPDH	Fw 5′-CCCTTCATTGACCTCAACTAC-3′	418 bp	NM_001256799.2 NM_001289745.2
	Rev 5′-TGAGTCCTTCCACGATACC-3′ (Koziolek et al., 2007)		NM_001289746.1 NM_002046.6

condition was calculated by the equation $t_{1/2} = \ln(2)/k$ (where k is the mRNA decay constant) and represents the time at which 50% of the mRNA is degraded.

2.7. Statistics

Unless otherwise indicated, statistical significance between more than two groups was tested using non-parametric, one-way Kruskal-Wallis test followed by Dunn's test. Comparative analyses between two groups were performed using a nonparametric, unpaired, 2-tailed Mann-Whitney *U* test. For RT-qPCR studies, statistical significance analysis were performed by ANOVA test followed by Bonferroni´s Multiple Comparison test and for time course assay, by 2-way ANOVA followed by Bonferroni Post test.

3. Results

3.1. CX_3CR1 modulation by cytokines

As previously described, Mo purified from buffy coats or peripheral blood lose CX_3CR1 surface expression during ON culture (Panek et al., 2015; Ramos et al., 2010). Not only we confirmed that IFN-γ and IL-10 prevented CX_3CR1 down-modulation by flow cytometric studies, but also we showed that CX_3CR1 membrane expression after ON incubation with both cytokines was significantly higher than in fresh Mo (Fig. 1A–D). On the other hand, the other anti-inflammatory cytokines, IL-4 and IL-13 were unable to modify CX_3CR1 expression during ON culture (Fig. 1A and B). By analyzing the MFI of CX_3CR1 expression per cell, we demonstrated that IFN-γ and IL-10 when added simultaneously, showed a cooperative effect on CX_3CR1 expression, because the two-way ANOVA test evidenced a significant interaction between both cytokines (IFN-γ and IL-10) ($p < 0.05$) (Fig. 1C and D). In contrast, IL-4 and IL-13 did not affect the IFN-γ–induced CX_3CR1 expression, either when incubated simultaneously (Fig. 1A and B) or preincubated 60 min before IFN-γ (data not shown). It is important to highlight that CD14 expression was not significantly affected by these treatments, except by IFN-γ alone that reduced CD14 expression, as previously reported (Payne et al., 1992) (Supplementary Fig. 1).

Because several cytokines can also activate the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol-3-kinase (PI3K), in addition to the JAK/STAT pathway (Makuta et al., 2003; Zhou et al., 2001; Ramana et al., 2002; Kaur et al., 2008; Stark and Darnell, 2012), Mo were incubated ON with IFN-γ in the presence of MAPK or PI3K inhibitors. We found that neither ERKs nor p38 MAPK inhibitors, PD98059 or SB203580 respectively, impaired IFN-γ or IL-10 regulatory effects (Fig. 2A and B). Although PD98059 partially and not statistically significant blocked the CX_3CR1 upregulation induced by IFN-γ when was incubated in combination with IL-10 or IL-4 (Fig. 2A and B). However, specific PI3K inhibitor LY294002, blocked the effect of IFN-γ and IL-10 on CX_3CR1 expression. Besides, LY294002 also blocked IFN-γ effect when Mo were incubated in the presence of IFN-γ simultaneously with IL-10, IL-4 or IL-13 (Fig. 2A and B). Pre-treatment of cells with LY294002 alone or together with IFN-γ did not significantly affect FSC/SSC characteristics or regulation of CD14 expression (Data not shown). These results lead us to conclude that among anti-inflammatory cytokines only IL-10 up-regulated CX_3CR1 membrane expression alone or in cooperation with IFN-γ through a PI3K-dependent mechanism.

3.2. CX_3CR1 modulation by cytokines involves STAT activation at serine

Then, we investigated the intracellular signaling pathways triggered by IFN-γ and IL-10 driving to regulation of CX_3CR1. Looking for a common pathway underlying the prevention of CX_3CR1 loss, which could be blocked by LY, and taking into account that Akt is a serine/threonine-kinase enzyme, we next evaluated the capacity of IL-10 and IFN-γ, separately or together to induce phosphorylation at S727 of STAT1 and/or STAT3 by flow cytometric studies. Mo incubated with LPS or PMA were assayed in parallel, as positive controls of phospho-serine-activation of STAT1 or STAT3, respectively (Kovarik et al., 1999). Interestingly, only IFN-γ induced serine phosphorylation of STAT1, and only IL-10 induced serine phosphorylation of STAT3 in Mo (Fig. 3A and B). It should be noted that the results of S727-STATs phosphorylation in Mo upon incubation with both cytokines did not raise a significant interaction by the two-way ANOVA test, meaning that phosphorylation of S727-STAT1 by IFN-γ is not affected by IL-10; and similarly phosphorylation of S727-STAT3 by IL-10 is not affected by IFN-γ. (Fig. 3A and B).

Most relevant, inhibition of PI3K pathway with LY294002 prevented IFN-γ -induced phosphorylation of STAT1 at S727 (Fig. 3C), and IL-10-induced phosphorylation of STAT3 at S727 (Fig. 3D). As it can be observed, LY294002 did not affect basal phosphorylation at S727-STAT1 by IL-10 or basal phosphorylation at S727-STAT3 by IFN-γ (Fig. 3C and D). In order to investigate the cause-effect relationship between STAT activation and CX_3CR1 regulation by each cytokine, we used chemical STAT inhibitors. In fact, adenosine was demonstrated to inhibit S727-phosphorylation of STAT-1 induced by IFN-γ in macrophages (Barnholt et al., 2009) and JSI-124 is a known inhibitor of STAT3 in different cell types (Ishdorj et al., 2010; Yang et al., 2017). As shown in Fig. 3E and F, adenosine (200 uM) significantly inhibited up-regulation of CX_3CR1 induced by IFN-γ, and JSI-124 (2 uM) significantly inhibited the increase in CX_3CR1^+-Mo induced by IL-10, respectively. All together these results suggest that this alternative activation is Akt-dependent, cytokine specific and that a fully activation of STAT1/STAT3 pathways play a central role during CX_3CR1 regulation in this cellular type.

3.3. IL-10 but not IFN-γ increases CX_3CR1 mRNA expression in Mo

Since cytokine regulatory effects are often mediated through the activation of gene transcription and subsequent protein expression, we evaluated *CX_3CR1* mRNA expression in Mo incubated during different times in medium, IFN-γ, IL-10 or the combination of both cytokines.

Four transcript variants encoding two different isoforms have been found for this gene (Barlic et al., 2004), and functionally independent promoters have been described for driving differential transcription of *CX_3CR1* in different cells (Garin et al., 2002). The most represented variant in Mo is V28 (Garin et al., 2003). Thus, we performed quantitative analysis of V28 variant of *CX_3CR1 ($CX_3CR1(V28)$)* mRNA.

We studied the effect of adding IFN-γ and/or IL-10 in the culture on *$CX_3CR1(V28)$* expression in Mo at different time points. Because cultured Mo diminished *$CX_3CR1(V28)$* mRNA at short times, consistently with the significant reduction of membrane expression of the protein observed after ON culture (Panek et al., 2015; Ramos et al., 2010), all values of *$CX_3CR1(V28)$* mRNA in experimental conditions were normalized to the expression of the reference *GAPDH* and relating to the mRNA value in medium at the same time point.

Mo exhibited an almost 2-fold increase in *$CX_3CR1(V28)$* mRNA at 2 h after IL-10 treatment (Fig. 4A). The significant increase in the level of *$CX_3CR1(V28)$* mRNA was observed up to 8 h after IL-10 treatment (Fig. 4B).

In sharp contrast, IFN-γ did not enhance *$CX_3CR1(V28)$* transcription. Moreover, IFN-γ- treated Mo showed the lowest level of *$CX_3CR1(V28)$* mRNA compared to all other experimental groups. The amount of *$CX_3CR1(V28)$* mRNA in Mo treated simultaneously with IL-10 and IFN-γ was intermediate between the mRNA levels obtained by both treatments separately, resulting significantly different from each one. These results demonstrated that although both cytokines increased CX_3CR1 protein expression on the Mo membrane, only IL-10 enhanced *$CX_3CR1(V28)$* transcript level in a time-dependent manner. It is important to point out that none of the cytokines, alone or in combination, altered *GAPDH* values in Mo (Supplementary Fig. 2).

As IFN-γ could be affecting the mRNA level of another *CX_3CR1*

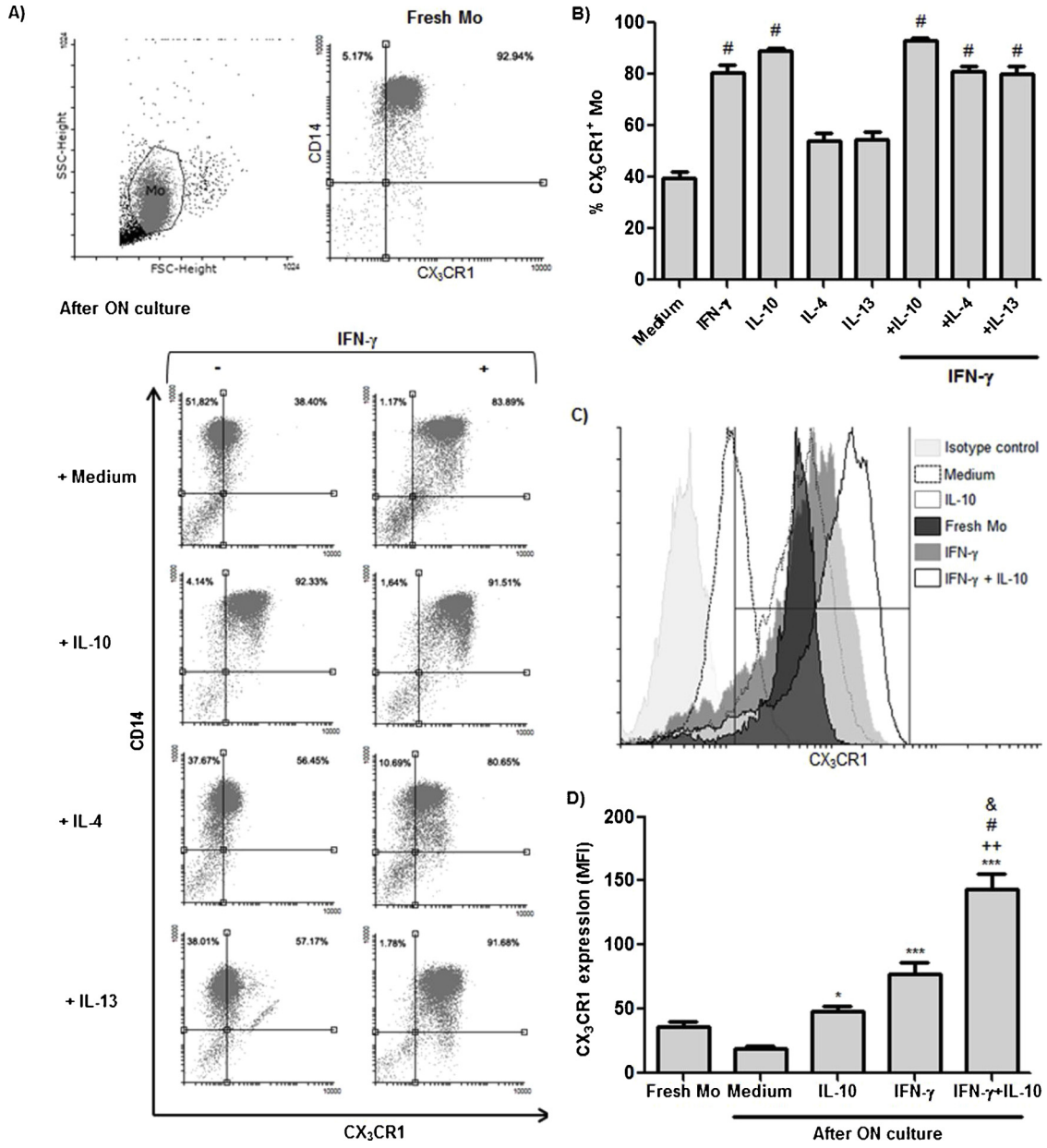

Fig. 1. IL-10 and IFN-γ prevent the down-modulation of CX_3CR1 expression on peripheral blood Monocytes. Purified Mo (1×10^6 cells/ml) from different donors were incubated in medium alone or with IL-10 (10 ng/ml), or IL-4 (50 ng/ml) or IL-13 (50 ng/ml) or IFN-γ (500 U/ml) alone or in combination with each IL for 18 h at 37 °C. Then, CX_3CR1 and CD14 expression were analyzed by flow cytometry before ON culture (Fresh Mo) or after incubation for 18 h at 37 °C (ON culture). Mo were distinguished on the basis of their forward-/side-scatter properties and CD14 expression. **A)** Representative dot-plot graphs of Mo under each condition are shown. **B)** The results are expressed as the percentage of CX_3CR1-positive Mo. Each column represents the mean ± SEM from 7 to 10 different donors. # $p < 0.001$ vs. medium, by Kruskal Wallis ($p < 0.0001$) followed by Dunn test. **C)** Representative histograms from fresh Mo, and Medium, IL-10, IFN-γ, and IFN-γ + IL-10 after ON culture are shown. **D)** Results are expressed as the MFI of CX_3CR1expression on Mo. Each column represents the mean ± SEM from 7 to 10 different donors. *$p < 0.05$ and ***$p < 0.001$ vs. Medium, #$p < 0.05$ vs. IL-10, ++$p < 0.01$ vs fresh Mo, by Kruskal-Wallis ($p < 0.0001$) followed by Dunn's test. & Interaction between IL-10 and IFN-γ was analyzed by the two-way ANOVA test ($p < 0.05$).

mRNA variant (and subsequently the CX_3CR1 membrane expression), we quantified all protein-encoding transcripts by using primers within the coding region of exon 5, which is common for all *CX_3CR1* transcripts (Garin et al., 2002). Results depicted in Fig. 4C and D show similar mRNA regulation and kinetic curve by cytokines than when *$CX_3CR1(V28)$* was measured.

The causal link between the action of IL-10 at the *CX_3CR1* transcript level and their action on CX_3CR1 expression on Mo membrane was analyzed by quantification of *CX_3CR1* mRNA in Mo preincubated with LY294002 before IL-10 addition, alone or together IFN-γ. We observed that preincubation of Mo with PI3K inhibitor counteracted the IL-10-induced increase of the specific mRNA level (Fig. 5). Similar results were obtained measuring *CX_3CR1 (V28)* variant (Fig. 5A) or total *CX_3CR1* mRNA (Fig. 5B). These results suggest that the modulation of *CX_3CR1* mRNA expression level by IL-10 is PI3K-Akt-dependent.

3.4. IL-10 enhances CX_3CR1 (V28) mRNA expression increasing the stability of the transcripts

The increase of *$CX_3CR1(V28)$* steady-state transcripts in Mo treated with IL-10 could be mediated by different mechanisms. Since it has been reported that the expression of several chemokine receptors are

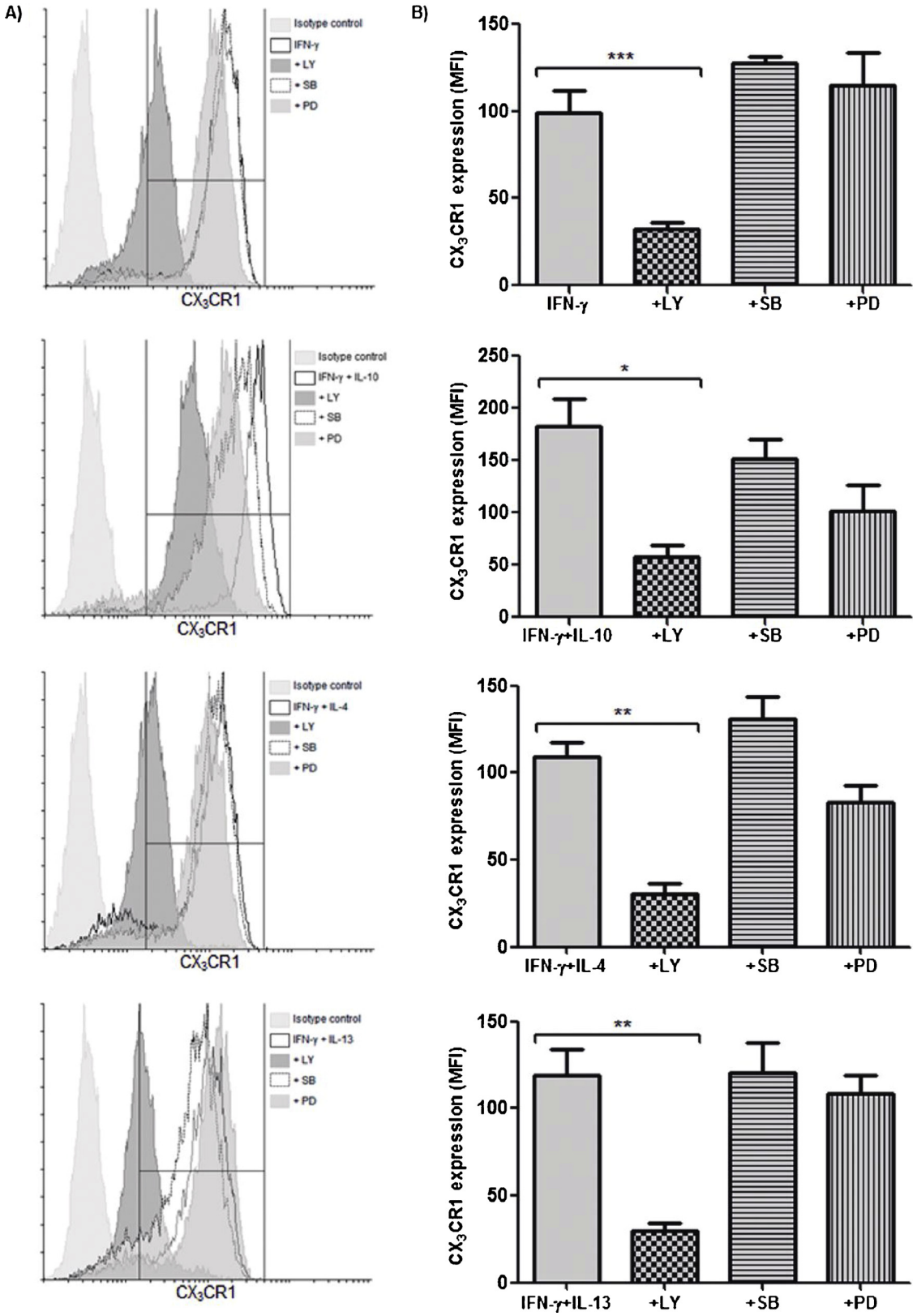

Fig. 2. Influence of MAPK or PI3K chemical inhibitors on cytokine-mediated CX_3CR1 regulation. Purified Mo (1×10^6/ml) were pre-incubated or not for 1 h with the corresponding kinase-inhibitors (LY294002, 25 μM; SB203580, 30 μM; PD98059, 20 μM) before incubation with medium or IFN-γ (500 U/ml) alone or in combination with IL-10 (10 ng/ml), or IL-4 (50 ng/ml) or IL-13 (50 ng/ml) for 18 h. CX_3CR1 expression was analyzed by flow cytometry. **A)** Representative histograms of Mo under each cytokine treatment with and without inhibitors are shown. **B)** Results are expressed as the MFI of CX_3CR1 expression on Mo. Each column represents the mean ± SEM from 5 to 8 different donors. *$p < 0.05$, **$p < 0.01$ and *** $p < 0.001$ vs. control with no inhibitors, by Kruskal Wallis ($p < 0.05$) followed by Dunn test.

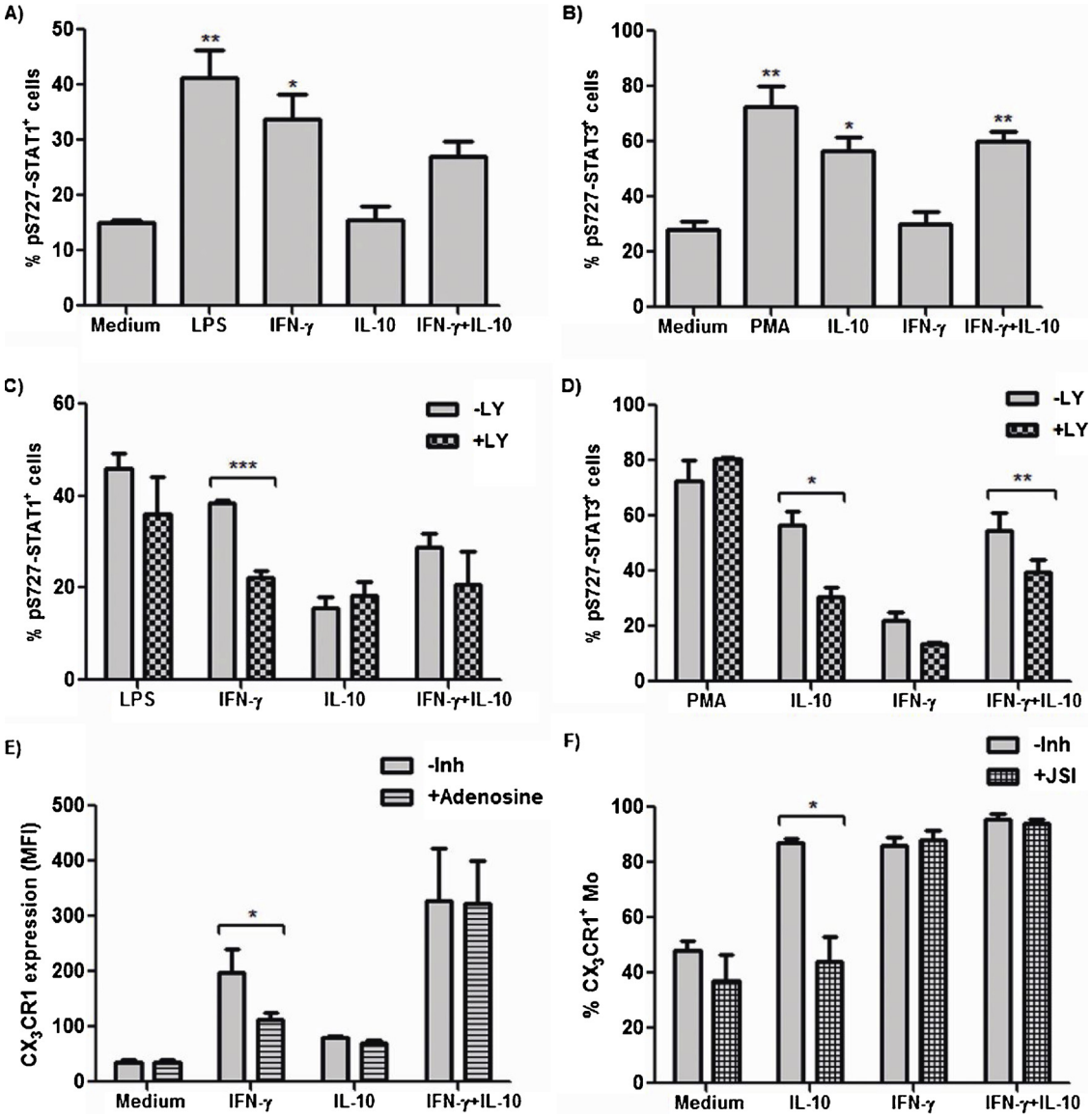

Fig. 3. Serine phosphorylation of STATs by IFN-γ and IL-10. Influence of STATs chemical inhibitors on cytokine-mediated CX_3CR1 regulation. Purified Mo (1×10^6 cells/ml) were incubated with medium alone or with IL-10 (10 ng/ml) and/or IFN-γ (500 U/ml) for 30 min. When indicated, Mo were pre-incubated with the inhibitors LY294002 (25 μM, 1 h) (**C and D**), adenosine (200 μM, 30 min) (**E**) or JSI-124 (2 μM, 30 min) (**F**). **A and C)** Results are expressed as percentage of pS727-STAT1-positive cells. Each column represents the mean ± SEM from 3 to 4 different experiments. Mo treated with LPS (1 μg/ml) were assayed in parallel as a positive control. **A)** *p < 0.05 and **p < 0.01 vs. medium, by Kruskal Wallis (p < 0.01) followed by Dunn test. Interaction between IL-10 and IFN-γ was not significant by two-way ANOVA test. **C)** ***p < 0.001 vs. control -LY, by Mann Whitney *U* test. **B and D)** Results are expressed as percentage of pS727-STAT3-positive cells. Each column represents the mean ± SEM from 3 to 6 different experiments. Mo treated with PMA (50 ng/ml) were assayed in parallel as a positive control. **B)** *p < 0.05 and **p < 0.01 vs. medium, by Kruskal Wallis (p < 0.0001) followed by Dunn test. Interaction between IL-10 and IFN-γ was not significant by two-way ANOVA test. **D)** *p < 0.05 and **p < 0.01 vs. control -LY, by Mann Whitney *U* test. **E and F)** CX_3CR1 and CD14 expression were analyzed by flow cytometry. Mo were distinguished on the basis of their forward-/side-scatter properties and CD14 expression. **E)** Results are expressed as the MFI of CX_3CR1 expression on Mo. Each column represents the mean ± SEM from 3 to 4 different donors. *p < 0.05, by Mann Whitney *U* test. **F)** Results are expressed as the percentage of CX_3CR1-positive Mo. Each column represents the mean ± SEM from 3 to 4 different donors. *p < 0.05, by Mann Whitney *U* test.

selectively regulated by pro- or anti-inflammatory signals affecting the stability of their mRNA (Penton-Rol et al., 1998), we evaluated the effects of IL-10 on *$CX_3CR1(V28)$* mRNA stability. Mo were cultured in the presence or absence of IL-10 for 2 h when transcription was subsequently stopped by adding ActD. RNA was extracted at different times and *$CX_3CR1(V28)$* mRNA quantified by RT-qPCR. Our results show that in the absence of IL-10, the estimated half-life of the transcript was approximately 34 min while treatment with IL-10 increased the *$CX_3CR1(V28)$* mRNA half-life to 63 min (Fig. 5C).

4. Discussion

The immune system utilizes at least fifty cytokines to trigger signals in the immune microenvironment. However, cells only possess four JAK and seven STAT proteins to deliver these signals. Downstream of the same STAT protein, extremely diverse physiologic events can occur (Delgoffe et al., 2011). But the contrary is also true: through different transduction pathways, similar biological events can occur. In this context, we found that IFN-γ and IL-10 through the activation of different intracellular mechanisms lead to a remarkable up-regulation of CX_3CR1 expression on Mo, compared not only to the expression at the

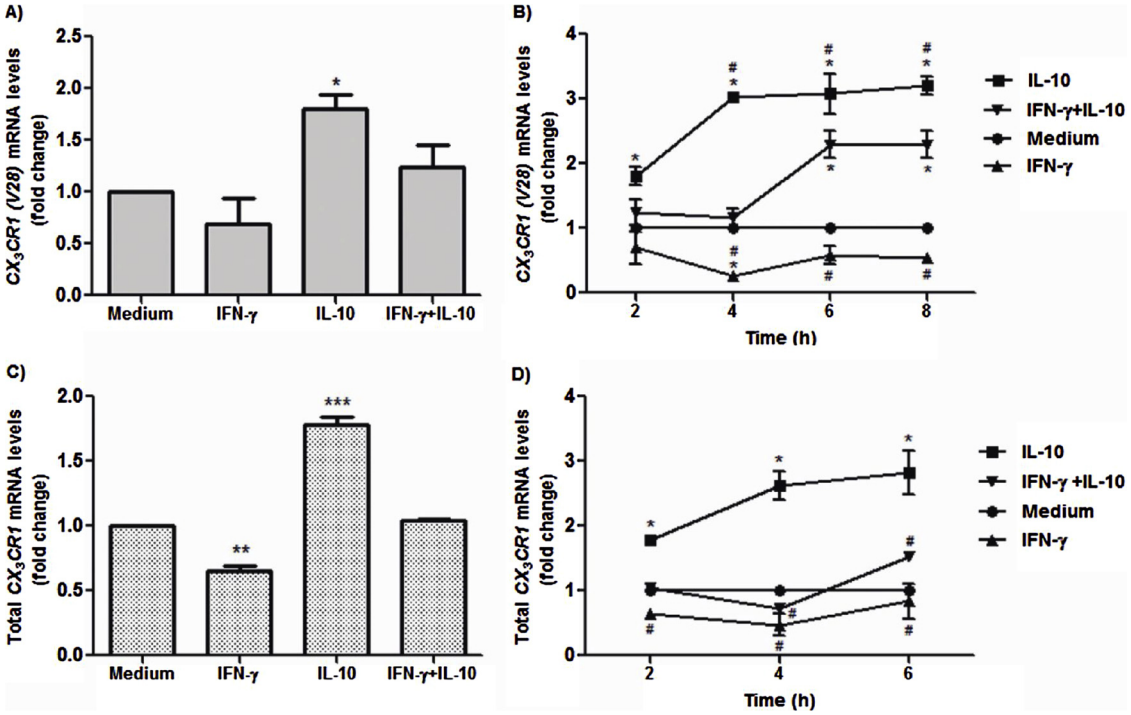

Fig. 4. Effect of IFN-γ and IL-10 on *CX_3CR1* mRNA expression level in Mo Purified Mo (1×10^6 cells/ml) were incubated in medium alone or with IL-10 (10 ng/ml) and/or IFN-γ (500 U/ml). After different times, total RNA was extracted and cDNA was prepared. Then, **A** and **B)** *$CX_3CR1(V28)$*, or **C** and **D)** Total *CX_3CR1* expression were measured by real time RT-qPCR and levels were normalized to those of the *GAPDH*. The results are presented as fold change in the specific mRNA level relative to medium control samples obtained at the same time point. Results are expressed as the mean ± SEM of 3 different healthy donors. **A** and **C)** Two hours of incubation time, A) *$p < 0.05$ vs. medium, by ANOVA ($p < 0.02$) followed by Bonferroni´s Multiple Comparison test. C)** $p < 0.01$ and ***$p < 0.001$ vs. medium, by ANOVA ($p < 0.001$) followed by Bonferroni´s Multiple Comparison test. **B** and **D)** Time course of *$CX_3CR1(V28)$* **(B)** or total *CX_3CR1* **(D)** mRNA expression upon treatments. **B)***$p < 0.05$ vs. medium, #$p < 0.01$ vs. IFN-γ + IL-10, by two-way ANOVA followed by Bonferroni Post test. **D)** *$p < 0.05$ vs. medium and #$p < 0.01$ vs. IL-10, by two-way ANOVA followed by Bonferroni Post test.

same time point after culture, but also to the expression on fresh Mo. In contrast, IL-4 and IL-13 were not able to modulate CX_3CR1 expression separately or together with IFN-γ, at least at short incubation times.

Cooperation between IFN-γ and IL-10 is intriguing because several authors have shown antagonistic actions between them and even the reprogramming IL-10 activity by IFN-γ, or vice versa. However, some exceptions have also been described. In fact, both cytokines lead to the over-expression of Fc and CX_3CR1 receptors on membrane of myeloid cells (Ramos et al., 2010; Liu et al., 2005). We can speculate that both types of receptors mediate key functions that must be guaranteed in contexts as dissimilar as those immersed in a Th-1 and Th-2 type response. We showed that IFN-γ- and IL-10-mediated CX_3CR1 up-regulation was not dependent on MAPK, but PI3K-dependent. This is not entirely surprising since, PI3K activates the enzyme Akt in Mo after IL-10 and/or IFN-γ incubation (Ramos et al., 2010), and PI3K is required for efficient induction of CXC chemokine receptor 3 (CXCR3) on T cells upon IFN-γ activation (Barbi et al., 2008).

JAK/STAT signaling pathway is pivotal for biologic responses stimulated by IL-10 or IFN upon interaction with their specific receptors on membrane cells (Stark and Darnell, 2012). But also all of the STAT proteins, except STAT2 and STAT6, carry one or more conserved serine phosphorylation sites that is required for optimal transcriptional activity (Bancerek et al., 2013; Decker and Kovarik, 2000), and mutation of these serine residues results in decreased binding of STATs to target genes. The critical importance of S727 phosphorylation for the biological impact of IFN-γ is further supported by studies with mediators of inflammation (LPS, TNF-α), which cooperate with IFN-γ in the activation of macrophages through JAK-independent ser-phosphorylation (Decker and Kovarik, 2000; Ito et al., 1999).Thus, we investigated ser-phosphorylation of STAT1 and STAT3 by IFN-γ and IL-10. We found that this phosphorylation was specific for each cytokine: while IFN-γ only induced ser-phosphorylation of STAT1, only IL-10 induced ser-phosphorylation of STAT3. Although IL-10 partially counteracted STAT1 ser-phosphorylation triggered by IFN-γ when incubated together, this effect was not statistically significant and two-way ANOVA analysis showed no interaction between both cytokines.

The mechanisms of S727- STATs phosphorylation are not yet well understood. S727 lies within the MAPK consensus motif $PX_n(S/T)P$; in agreement with this, it has been reported that S727 of STAT3 is phosphorylated by the H7-sensitive or PD98059-sensitive pathway upon IL-10 signaling; similarly, S727 of STAT1 was reported to be phosphorylated by MAPK upon IFN-γ signaling (Bancerek et al., 2013). In this regard, we found that PD98059, inhibitor of ERKs MAPK, slightly and not significantly diminished the effect of IL-10 + IFN-γ on CX_3CR1 up-regulation on Mo. Most significantly, ser-phosphorylation of STATs by each cytokine was impaired by the incubation of Mo in presence of LY, suggesting that PI3K is directly or indirectly involved. Thus, although we concluded that S727 of STAT1 and STAT3 are phosphorylated by IFN-γ or IL-10, respectively, through a PI3K/Akt dependent pathway, we could not discard that ERKs activation is also involved in S727 STAT phosphorylation when both cytokines are present. Although the signaling cascade is not completely defined, our results are in agreement with recent evidence about JAK proteins can also activate other signaling molecules, especially from the PI3K family, and PI3K acts in a novel pathway that plays an important role in signaling in response to IFN-γ (Chow and Gale, 2015).

Furthermore, chemical inhibitors of S727-STAT1 (adenosine) or STAT3 (JSI-124) activation were able to inhibit the regulation of CX_3CR1 expression by IFN-γ or IL-10, respectively, supporting the cause-effect relationship between ser-phosphorylation of STATs and cytokine biological effect on CX_3CR1 expression. This result strongly supports that over-expression of CX_3CR1 was secondary to the

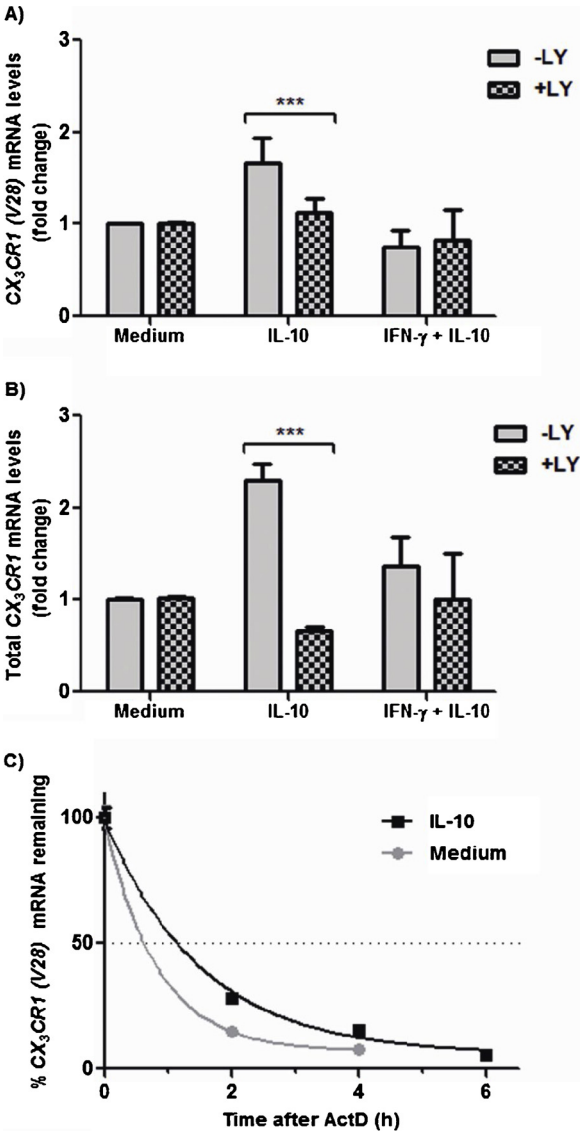

Fig. 5. Effect of PI3K chemical inhibitor on IL-10 mediated *CX_3CR1* mRNA induction. Effect of IL-10 on *CX_3CR1* mRNA stability. **A and B)** Purified Mo (1×10^6 cells/ml) were incubated in medium, IL-10 (10 ng/ml) alone or with IFN-γ (500 U/ml). After two hours, total RNA was extracted and cDNA was prepared. *$CX_3CR1(V28)$* **(A) and** total *CX_3CR1* **(B)** expression were measured by real time RT-qPCR and levels were normalized to those of the *GAPDH*. The results are presented as fold change in the specific mRNA level relative to medium control samples obtained at the same time point. Results are expressed as the mean ± SEM of 3 different healthy donors. ***$p < 0.001$ vs. IL-10 by Mann Whitney *U* test. **C)** Purified Mo (1×10^6 cells/ml) were incubated for 2 h in medium alone or with IL-10 (10 ng/ml). ActD (1 μg/ml) was then added to cells to inhibit transcription, and RNA was isolated at the indicated times (2, 4 or 6 h) and cDNA was prepared. Relative quantification of *$CX_3CR1(V28)$* and *GAPDH* mRNA were measured by RT-qPCR. A decay curve for remaining *$CX_3CR1(V28)$* mRNA levels is shown. Values were normalized to *GAPDH* levels and represented as a percentage of *$CX_3CR1(V28)$* mRNA levels at time 0 of ActD addition. Data represent the mean ± SEM from 2 independent experiments. The half-life for a given mRNA represents the required time to decay at 50% of its initial abundance: Medium $t_{1/2=}$ 34 min; IL-10 $t_{1/2=}$ 63 min. The decay rate constants were significantly different: $p < 0.01$ vs. medium, by Extra sum-of-squares F test.

activation of different signaling pathways in which the only common step was the dependence on Akt phosphorylation.

Regarding how STAT activation leads to regulation of CX_3CR1 expression, two important mechanisms of chemokine receptor regulation have been described. One of them acts at the level of receptor intracellular storage, mobilization to the membrane and/or its proteolytic degradation. The second mechanism is at mRNA level, and involves both transcription induction and/or stability enhancement. Both mechanisms have been described for CX_3CR1 regulation. MCP-1 induces a transient increase of this receptor in Mo as consequence of the mobilization of intracellular pool to the plasma membrane (Green et al., 2006). But also, *CX_3CR1* mRNA has been reported to be induced in human fibroblasts by H_2O_2 (Koziolek et al., 2007), and in microglia after ischemia (Tarozzo et al., 2003). Furthermore, IL-10 *in vivo*-treated psoriatic patients show high levels of *CX_3CR1* mRNA in Mo (Jung et al., 2004). On the other hand, other authors have reported that LPS *in vitro* reduces *CX_3CR1* mRNA in peripheral mononuclear cells, while IL-10 has no effect (Pachot et al., 2008).

CX_3CR1 is one of the largest and complex chemokine receptor gene, composed by five exons and four introns spanning over 18 kb (Barlic et al., 2004; Garin et al., 2002). The alternative splicing of these exons render different *CX_3CR1* variants, among which V28 is the most abundant in Mo (Garin et al., 2002, 2003). Several potential transcription factor-binding sites are found in the *CX_3CR1* and particularly upstream from exon 4, such as GATA-binding site (GATA), nuclear factor of activated T-cells (NFAT), CCAAT/enhancer-binding protein (C/EBP), octamer-binding factor 1 (OCT1) and STATs (Garin et al., 2002). We found that Mo cultured in medium showed a significant decrease of CX_3CR1 protein from the surface, associated to a time-dependent decrease in *$CX_3CR1(V28)$* levels. Thus, when we assayed *CX_3CR1* mRNA in presence of IFN-γ and/or IL-10, we expressed the results as fold change respect to the expression in medium alone at the same time point. Interestingly, we found that IL-10 significantly increased *$CX_3CR(V28)$* levels since two hours, by enlarging the half-life time of *$CX_3CR1(V28)$* mRNA almost two-fold. Similarly, it was reported that IL-10 upregulates expression of functional CCR1, 2, and 5 receptors in human Mo, by the prolongation of their mRNA half-life, indicating that mRNA stability is a crucial set point for chemokine receptor regulation (Sozzani et al., 1998). Finally, we showed that the increase of *CX_3CR1* mRNA level by IL-10 was dependent on the PI3K signaling pathway, supporting the causal link between the action of IL-10 at the *CX_3CR1* transcript level and their action at CX_3CR1 protein expression level on Mo.

In sharp contrast, we found that IFN-γ increased CX_3CR1 protein expression through a PI3K and pS727-STAT1 dependent pathway, but reduced *$CX_3CR1(V28)$* mRNA levels. Considering that IFN-γ action is mediated mainly through the modulation of gene expression, we tested the total *CX_3CR1* mRNA using specific primers that hybridize in the common region to all variants (Garin et al., 2002, 2003). The results showed the same cytokine-mediated effects on both RT-qPCR at all evaluated time-points, supporting that IFN-γ enhances CX_3CR1 protein in the membrane of Mo by an alternative mechanism not associated with the transcription of *CX_3CR1*. In this regard, another possible explanation for CX_3CR1 membrane up-regulation is that IFN-γ also down regulates the expression of a yet unknown protease which target is CX_3CR1 protein. The sustained expression of CX_3CR1 after incubation in the presence of protein synthesis inhibitors is an indirect evidence that a protein synthesis-dependent degradation mechanism is involved in the down-modulation of CX_3CR1 (Ramos et al., 2010). Other study also proposed the existence of a similar degradation mechanism triggered by MCP-1 since 60 min (Green et al., 2006). Thus, we suggest that during IFN-γ culture the rate of CX_3CR1 membrane expression is higher than the rate of receptor degradation or loss.

In conclusion, we demonstrated that IL-10 induces increased levels of CX_3CR1 protein and mRNA in Mo, as consequence of augmenting *CX_3CR1* mRNA levels. Both effects were inhibited by a PI3K inhibitor, LY. In addition, IL-10 ser-phosphorylation of STAT3 was inhibited by LY, and inhibitors of STAT3 activation abrogated IL-10 regulatory effect on CX_3CR1 protein expression. These results strongly suggest that PI3K and STAT3 regulate *CX_3CR1* gene expression, and second, that PI3K-

mediated phosphorylation of serine is required for full stimulation of STAT3 for *CX_3CR1* mRNA stabilization.

The incubation of Mo simultaneously with IFN-γ and IL-10 resulted in the highest CX_3CR1 protein expression by cell, even when *CX_3CR1* mRNA expression was significantly lower than Mo incubated with IL-10 alone, supporting the concept that IFN-γ contributes to up-regulation of CX_3CR1 by inhibiting some degradation pathway and thus, two different mechanisms cooperate to over-express CX_3CR1 in membrane of human Mo.

5. Conclusions

This study demonstrates for the first time that CX_3CR1 protein expression in the membrane of Mo results at least from two different regulatory mechanisms, one driven by IL-10 secondary to stabilization of *CX_3CR1* mRNA and the other driven by IFN-γ probably by reducing CX_3CR1 protein degradation. Considering the central involvement of CX_3CL1/CX_3CR1 signaling in the pathogenesis of vasculitis and atherosclerosis, the knowledge on CX_3CR1-regulation would make possible the development of therapeutic strategies aimed at interfering with this pathway.

Authorship

CP, CB, MR, RF-B, MP conceived and designed the experiments and wrote the paper.

CP, GP, AB, MM performed the experiments and all authors analyzed the data.

All authors contributed to manuscript revision, read and approved the submitted version.

Disclosures

The authors report no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.molimm.2019.01.003.

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