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RESEARCH ARTICLE

Differential expression of the fractalkine chemokine receptor (CX_3CR1) in human monocytes during differentiation

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Circulating monocytes (Mos) may continuously repopulate macrophage (MAC) or dendritic cell (DC) populations to maintain homeostasis. MACs and DCs are specialized cells that play different and complementary immunological functions. Accordingly, they present distinct migratory properties. Specifically, whereas MACs largely remain in tissues, DCs are capable of migrating from peripheral tissues to lymphoid organs. The aim of this work was to analyze the expression of the fractalkine receptor (CX_3CR1) during the monocytic differentiation process. Freshly isolated Mos express high levels of both CX_3CR1 mRNA and protein. During the Mo differentiation process, CX_3CR1 is downregulated in both DCs and MACs. However, MACs showed significantly higher CX_3CR1 expression levels than did DC. We also observed an antagonistic CX_3CR1 regulation by interferon (IFN)-γ and interleukin (IL)-4 during MAC activation through the classical and alternative MAC pathways, respectively. IFN-γ inhibited the loss of CX_3CR1, but IL-4 induced it. Additionally, we demonstrated an association between CX_3CR1 expression and apoptosis prevention by soluble fractalkine (sCX_3CL1) in Mos, DCs and MACs. This is the first report demonstrating sequential and differential CX_3CR1 modulation during Mo differentiation. Most importantly, we demonstrated a functional link between CX_3CR1 expression and cell survival in the presence of sCX_3CL1.
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Keywords: dendritic cells; fractalkine receptor; macrophages; monocytes; survival

INTRODUCTION

Monocytes (Mos) represent approximately 5%–10% of peripheral blood leukocytes in humans and mice. They originate from a myeloid precursor in the bone marrow,[1] are released in the circulation and then enter into tissues. The half-life of Mo in blood is believed to be relatively short: approximately 3 days in humans.[2] This short half-life in the blood has fostered the concept that blood Mo may continuously repopulate macrophage (MAC) or dendritic cell (DC) populations to maintain homeostasis and, during inflammation, fulfill critical roles in innate and adaptive immunity.[3,4] Two major fates of Mo differentiation are easily identified and separable: many Mos develop into MACs, and others become DCs.[5] Functionally, MACs robustly degrade material that they engulf[6] and apparently fail to present antigens or initiate a T-cell response,[7,8] release a variety of cytokines upon activation and are thought to be important for the local clearance of dead cells in inflamed and non-inflamed tissues.[9] In contrast, DCs have a poor proteolytic capacity,[6] but are able to phagocytize material from other cells and to process and intracellularly retain peptides/antigens that are subsequently presented by major histocompatibility complex molecules together with appropriate costimulation for T-cell priming.[8,10]

Moreover, MACs and DCs have distinct migratory properties. Whereas MACs largely remain in tissues, DCs are capable of migrating from peripheral tissues to lymphoid organs to interact with T cells and induce an immune response.[11] In this regard, recent research has focused on the mechanisms and

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molecules involved in monocyte recruitment and their differentiation in various tissues.[12,13]

Fractalkine (CX_3CL1), a member of the CX_3C subfamily, is a unique chemokine in which the first two conserved cysteine residues are separated by three non-conserved amino acids.[14] Unlike other chemokines, CX_3CL1 exists in two forms: a membrane-anchored form and a soluble form. Soluble CX_3CL1 (sCX_3CL1) acts as a chemoattractant, whereas the membrane-anchored molecule functions as an adhesion molecule. The membrane anchored form of CX_3CL1 includes a chemokine domain that is tethered to the cell surface *via* a heavily glycosylated mucin-like stalk, followed by a single transmembrane domain and a single cytoplasmic domain.[15,16] The full-length molecule can be cleaved from the cell membrane by tumor necrosis factor-α-converting enzyme, a member of the ADAM (disintegrin and metalloprotease) family, and ADAM10, to produce a soluble form that includes the chemokine domain and most of the stalk region.[15] CX_3CL1 is expressed on the surface of endothelial cells,[17] epithelial cells,[17,18] DCs[19,20] and neurons[21] and is induced by pro-inflammatory cytokines, such as interleukin (IL)-1 and tumor necrosis factor-α.[22,23] The CX_3CL1 receptor, CX_3CR1, is capable of mediating both leukocyte migration and firm adhesion. CX_3CR1 is expressed on a number of leukocytes, including monocytes, T-cell subsets and NK cells,[24,25] and CX_3CR1 expression has been recently described in platelets.[26] However, a comprehensive knowledge about CX_3CR1 expression during monocyte differentiation and the intracellular signals and pathways that underlie this process have not been explored.

The most popular model to study monocyte-derived DCs is to culture blood Mo of either subset[27] in the presence of granulocyte-macrophage colony stimulating factor (GM-CSF) and IL-4[5,28] or other related cytokine cocktails.[29] These DCs have been considered the 'gold standard' DC[30] for assessing maturation and many aspects of human DC biology. Conversely, although a body of evidence suggests that MACs in several organs self-renew without input from blood precursors,[13] the fact that Mos give rise to MACs in virtually any organ in the context of inflammation is undisputed. In this regard, GM-CSF is the growth factor that is traditionally used to differentiate Mos into MACs.[31,32]

In the current study, we investigated CX_3CR1 expression in circulating Mos, Mo-derived DCs or MACs using different *in vitro* cytokine stimulation by monitoring the phenotype of these cells at different stages in culture. Furthermore, CX_3CR1 regulation at the transcriptional level was evaluated by mRNA analysis during Mo differentiation. Finally, the biological relevance of CX_3CR1 expression through cell survival regulation was analyzed and discussed.

MATERIALS AND METHODS

Materials

Endotoxin-free reagents and plastics were used in all experiments. Lipopolysaccharide (LPS, *Escherichia coli* serotype O111:B4), acridine orange, ethidium bromide and propidium iodide (PI) were obtained from Sigma (St Louis, MO, USA). Soluble fractalkine (sCX_3CL1) was obtained from PeproTech (Mexico, DF, Mexico). FITC-conjugated anti human-CX_3CR1 mAb (Clone 2A9-1)—Rat IgG2b, κ—were from BioLegend (San Diego, CA, USA); PC5-conjugated anti human-CD14 mAb (Clone RMO52)—Mouse IgG2a—were from Beckman Coulter (Brea, CA, USA); R-phycoerythrin-conjugated anti human-CD16 mAb (Clone 3G8)—Mouse IgG1, κ—were from CALTAG Laboratories (Burlingame, CA, USA), PE-conjugated anti-human CD23 mAb (Clone M-L233)—Mouse IgG1, κ—, PE-CY5-conjugated anti-human CD86 mAb (Clone IT2.2)—Mouse IgG2b, κ—, PE-conjugated anti human-CD83 mAb (Clone HB15e)—Mouse IgG1, κ—, FITC-conjugated anti-human-CD1a mAb (Clone HI149)—Mouse IgG1, κ—, FITC-conjugated anti-human—human leukocyte antigen (HLA)-DR mAb (Clone L243)—Mouse IgG2a, κ—, FITC-conjugated anti human-CD206 mAb (Clone 19.2)—Mouse IgG1, κ—were from BD Pharmingen (San José, CA, USA); PE-conjugated anti human-CCR2 mAb (Clone 48607)—IgG2B—were from R&D Systems (Minneapolis, MN, USA); Recombinant human IL-4, recombinant human IL-13 and recombinant human interferon (IFN)-γ were from PeproTech. GM-CSF was from Laboratorios Goutier (Laboratorios Goutier S.A., Ciudad de Buenos Aires, Argentina).

Human peripheral blood Mo isolation and culture

Mos were isolated from freshly prepared buffy coats that were 2–4 h old and were 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 was obtained. Briefly, peripheral blood mononuclear cells were obtained by standard density gradient centrifugation on Ficoll-Hypaque (Ficoll Pharmacia, Uppsala, Sweden; Hypaque, Wintthrop Products, Buenos Aires, Argentina). Subsequently, Mos were further isolated using a Percoll (GE Healthcare, Uppsala, Sweden) gradient centrifugation, as previously described.[33] Mo viability was >96%, as determined by the trypan blue exclusion test, and CD14 staining of Mo revealed that their purity was >85%. Finally, Mos were suspended at 10^6/ml in RPMI-1640 (Hyclone Laboratories Inc., Logan, UT, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Natocor, Córdoba, Argentina), and antibiotic–antimycotic liquid (Gibco, Invitrogen, San Diego, CA, USA).

Human DCs and MAC preparation

Mos were purified as described above (purity>85%) and cultured up to 7 days at a concentration of 1×10^6/ml in complete RPMI media that was supplemented with 30 ng/ml GM-CSF to differentiate the cells into MACs or with 50 ng/ml GM-CSF and 20 ng/ml IL-4 or IL-13 to differentiate the cells into immature DCs (iDCs) in six-well culture plates (Corning Inc., New York, NY, USA) with or without 1000 U/ml IFN-γ. On day 3, fresh media containing the growth factors were added. On day 7, the cells were harvested, washed twice with PBS, and either used for the experiments or aliquoted for further differentiation. Where

indicated, iDCs were further cultured in the presence of 500 ng/ml LPS+1000 U/ml IFN-γ for 48 h to induce maturation.

Unless indicated otherwise, MACs were polarized with 400 U/ml IFN-γ+100 ng/ml LPS for M1 differentiation or 25 ng/ml IL-4 alone for M2 differentiation in complete RPMI media for 3 additional days.[34]

Cellular apoptosis quantification by Annexin-V binding and flow cytometry

Mos were purified as described above and cultured overnight at a concentration of 1.5×10^6/ml in complete RPMI medium that was supplemented with 1% FCS. An aliquot of purified Mo was cultured for 7 days in RPMI medium that was supplemented with 30 ng/ml GM-CSF to differentiate the cells into MACs or with 50 ng/ml GM-CSF and 20 ng/ml IL-4 to differentiate the cells into iDCs in six-well culture plates (Corning Inc.). On the last day, medium containing growth factors was removed and replaced with RPMI medium that was supplemented with 1% FCS with or without 1 μg/mL sCX_3CL1. After 24 h, the cells were harvested, washed twice with PBS, and used for cellular apoptosis quantification. Annexin-V binding to cells was performed using an apoptosis detection kit (APOAF 50TST; Sigma). In brief, the cells were labeled with annexin-V-FITC for 15 min at 4 °C and with PI immediately before the evaluation of fluorescence by flow cytometry (FACScan flow cytometer; Becton Dickinson, San Jose, CA, USA). The collected data were analyzed using the CellQuest analysis software (Becton Dickinson), as previously described. The results are reported as a percentage of Annexin-V-positive cells.

Quantification of cellular apoptosis and viability by fluorescent microscopy

Quantification of Mo apoptosis after overnight incubation with or without sCX_3CL1 was performed as previously described using the fluorescent DNA-binding dyes acridine orange (100 mg/ml) to determine the percentage of Mo that had undergone apoptosis and using ethidium bromide (100 mg/ml) to differentiate between viable and nonviable cells.[35] To assess the percentage of Mo showing morphological features of apoptosis, at least 200 cells were scored in each experiment.

Flow cytometric analysis

Mos were identified and gated according to their side *vs.* forward scattering (SSC/FSC) dot-plot profiles and positive staining for CD14. Each cell population was incubated with a 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 based on the difference between the curves obtained from the specific and isotype control mAb stainings. 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.

RNA isolation and quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR)

Total RNA was obtained from fresh Mos after purification and from iDCs at 72 h of culture during differentiation. Total RNA from MACs was obtained after differentiation at day 7, and total RNA from M1 or M2 polarized MACs was extracted at day 10, which included 7 days of differentiation and 3 days of polarization.

Total RNA was extracted with the NucleoSpin RNA II Kit (Macherey-Nagel, Bethlehem, PA, USA) from 5×10^6 Mos or 3×10^6 Mo-derived DCs or MACs from healthy blood donors. For each sample, cDNA was synthesized from 1 μg of total RNA (pre-treated with DNaseI during extraction) with the Accuscript High Fidelity 1st Strand cDNA synthesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. Relative quantification of CX_3CR1 and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression was measured by qRT-PCR 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).

PCR amplifications were performed in a final volume of 20 μl, which contained 4 mM MgCl and 0.75 μM of each of the required primers. PCR was performed with an initial denaturation step of 10 min at 95 °C, followed by 45 cycles of a qRT-PCR protocol (10 s at 95 °C, 10 s annealing at 58 °C and 15 s extension at 72 °C). The SYBR Green fluorescent DNA binding dye was monitored after each cycle at 81 °C. The 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. The results were expressed as a concentration ratio between the target gene and the GAPDH mRNA levels. Products were not generated in control reactions in which reverse transcriptase was omitted during cDNA synthesis.

The primers were V28 CX_3CR1: forward 5′-TGACTGGCAGATCCAGAGGTT-3′, reverse 5′-GTAGAATATGGACAGGAACAC-3′ (product size 163 bp);[36] GAPDH: forward 5′-CCCTTCATTGACCTCAACTAC-3′, reverse 5′-TGAGTCCTTCCACGATACC-3′ (product size: 416 bp).[37]

To check the primer specificity, the PCR products were electrophoresed on 2% agarose gels and visualized with ethidium bromide staining.

Statistical analysis

Statistical significance between more than two groups was tested using the non-parametric, one-way Kruskal–Wallis test. Comparative analyses between two groups were performed using the non-parametric, unpaired, two-tailed Mann–Whitney *U* test.

RESULTS

Freshly isolated Mos downregulate CX_3CR1 during culture

As previously described,[38] the loss of CX_3CR1 expression during ON culture was observed in Mos that were purified from buffy coats or peripheral blood both in the presence of FCS or autologous serum, which allowed us to rule out artificial activation that was derived from the purification procedure.

During incubation, the Mos underwent changes in both morphology and surface antigen expression, depending on the culture conditions. Freshly isolated Mos initially appeared small and round, but after 7 days in GM-CSF-conditioned medium, they became adherent and increased in both size and granularity. In contrast, Mos cultured in GM-CSF+IL-4 conditioned medium for 7 days became large cells and grew in multiple semi-adherent colonies and exhibited cytoplasmic projections. These morphological changes were also observed in the different dot plot graphic of FSC *vs.* SSC profiles by flow cytometry (Figure 1a). Next, we analyzed the CD14, CD16 and CD1a cell marker expression patterns (Figure 1a and b). We observed that Mos that were cultured with GM-CSF (30 ng/ml) for 7 days upregulated CD16 expression and downregulated CD14 expression, which was consistent with a change in the phenotype from Mo to MAC. In contrast, Mos cultured with GM-CSF (50 ng/ml)+IL-4 (20 ng/ml) for 7 days, downregulated CD14 and upregulated CD1a, consistent with a change in phenotype from Mos to iDCs (Figure 1a and b). Next, we analyzed whether CX_3CR1 expression was altered during Mo differentiation into either MACs or iDCs. Freshly isolated Mos had high CX_3CR1 expression, which was completely lost after 7 days of incubation in DC-differentiating conditions (Figure 1c and d). It is important to note that CX_3CR1 expression was significantly downregulated at 24 h and that by 72 h, no CX_3CR1 was found on the iDC surfaces (Figure 1e). At this time point, the CD1a differentiation marker began to rise, and the expression of CD14 was partially downregulated.

In contrast, MAC CX_3CR1 expression after 7 days of culture was higher than that observed at day 1 of culture, although at this time point, CD16 began to rise and CD14 was partially downregulated (data not shown). However, despite the partial restoration of CX_3CR1 expression in MACs after 7 days of culture in the presence of GM-CSF, the percentage of MACs that were positive for CX_3CR1 was lower than in the fresh Mo (Figure 1e).

These data indicate that CX_3CR1 is differentially expressed on human Mos, MACs and DCs and that one consequence of monocyte differentiation towards DCs is the selective loss of CX_3CR1 protein from the cell surface.

DC maturation

Based on the above results, we decided to further examine the regulation of CX_3CR1 expression during DC maturation. Mature DCs (mDCs) were induced by treatment of iDCs with 500 ng/ml LPS+1000 U IFN-γ for 48 h. The results showed that the mDCs were characterized by increased HLA-DR, CD86 and CD83 expression compared with iDCs (Figure 2a and b). CD86 molecule expression represents the main co-stimulatory axis for immune reactions. The induction of high CD83 and HLA-DR levels in mature DCs simply shows that they are functional.[39] Even after this maturation process, CX_3CR1 expression remained negligible in the DC population (Figure 2c).

Alternative protocol for Mo differentiation towards iDCs

Although the most common differentiation protocol of human Mos towards DCs uses GM-CSF plus IL-4, some groups have reported that IL-13 can also differentiate Mos into DCs equally or even more efficiently than IL-4.[40] Both cytokines have structural and functional similarities.[41] The results depicted in supplemental Figure 1a confirm that IL13 plus GM-CSF was efficient in differentiating Mos into iDCs and that the induced phenotype was similar to IL-4-driven iDCs.

Under this alternative differentiation protocol, the iDCs showed no CX_3CR1 membrane expression from day 3 of culture (Supplementary Figure 1b). In conclusion, during the differentiation process to DCs, Mos lose CX_3CR1 from their cellular membranes and expression cannot be restored by maturation stimuli.

Classical and alternative activation of MACs differentially affects CX_3CR1 expression

The above data indicate that CX_3CR1 is differentially expressed on MACs and DCs. The differential cytokine composition between both culture conditions was IL-4 (or IL-13), which was present in the DC differentiation medium. The effects of IL-4 (or IL-13) on MACs have been a major focus of immunologists. When MACs are stimulated with IL-4 (or IL-13) in the absence of IFN-γ (and/or TLR ligands), a distinct pattern of gene expression, cell surface markers, secreted cytokines and chemokines occurs relative to those induced by IFN-γ ($\pm$TLR ligands) stimulation. Gordon and Taylor[13] described the former as 'alternative activation' of MACs to distinguish it from the latter, ''classical activation''.[42] Therefore, we determined CX_3CR1 expression following MAC activation by culturing these cells for 72 h in the presence of 25 ng/ml of IL-4 to drive 'alternative activation' (M2) or 100 ng/ml LPS+400 U/ml IFN-γ to drive classical activation (M1).[13] Flow cytometry analysis revealed that MACs treated with IFN-γ+LPS upregulated the MAC maturation marker HLA-DR and downregulated CD206, confirming the M1 phenotype (Figure 3a and b). Conversely, IL-4 treatment induced enhanced CD206 and HLA-DR expression.[42] Additionally, these cells expressed CD23 antigen, which is specifically induced by IL-4. Finally, we analyzed CX_3CR1 expression in both types of activated MACs by flow cytometry. The results in Figure 3c and d show that IL-4 induced the downregulation of CX_3CR1 membrane expression on MACs. In contrast, MACs that were classically differentiated in the presence of LPS+IFN-γ for 72 h upregulated CX_3CR1 expression. Altogether, these results strongly suggest that classical and alternative MAC activation antagonistically modulates CX_3CR1 expression.

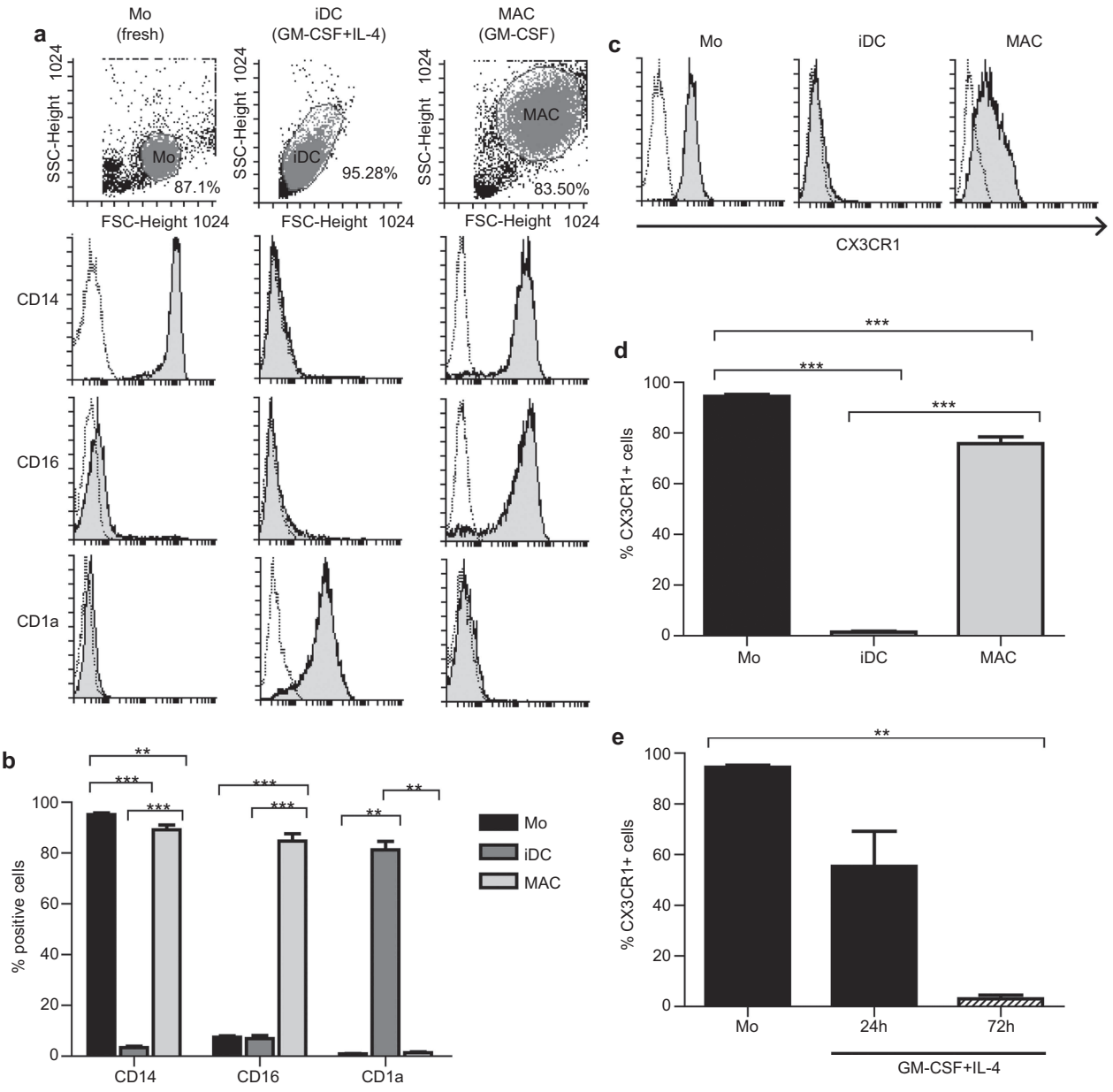

Figure 1 CX_3CR1 expression changes in different Mo-derived populations. Freshly isolated Mos were either immediately stained or after culturing for 7 days with GM-CSF (30 ng/ml) or GM-CSF (50 ng/ml)+IL-4 (20 ng/ml) to differentiate Mos into MACs or iDCs, respectively. Cells were stained with fluorescein isothiocyanate PC5-conjugated anti-CD14 mAb, PE-conjugated anti-CD16 mAb and FITC-conjugated anti-CD1a mAb. (**a**) Morphology changes between freshly isolated Mos, iDCs and MACs were determined by flow cytometric analysis of scatter (SSC) *vs.* forward (FSC). A representative dot plot of each cellular population according to the culture condition is shown in the left panel. Flow cytometric analysis of the cell surface expression of CD14, CD16 and CD1a was performed by gating the monocytic, iDC or MAC populations according to size and granularity in the SSC *vs.* FSC dot plot. Representative histograms of membrane expression of each antigen marker in the Mo, iDC and MAC populations are shown in the right panel. Dotted histograms represent the isotype controls. (**b**) The percentage of CD14-, CD16- and CD1a-positive cells in the freshly isolated Mos, iDCs and MACs are shown. Each bar represents the mean±s.e.m. from 5–10 different donors. $^{**}P<0.01$ and $^{***}P<0.001$, by the Kruskal–Wallis test ($P<0.001$) followed by the Mann–Whitney *U* test were calculated for each marker individually. Mos, iDCs or MACs were also stained with a FITC-conjugated anti-CX_3CR1 mAb. Cells were analyzed for CX_3CR1 expression changes by flow cytometry. (**c**) Representative histograms of membrane CX_3CR1 expression in Mos, iDCs and MACs are shown. Dotted histograms represent the isotype controls. (**d**) The percentages of CX_3CR1 positive cells in Mo, iDC and MAC populations are shown. Each bar represents the mean±s.e.m. from 7–10 different donors. $^{***}P<0.001$, according to the Kruskal–Wallis test followed by the Mann–Whitney *U* test. (**e**) Changes in CX_3CR1 expression at different culture times. Each bar represents the mean±s.e.m. from 4–10 different donors. $^{**}P<0.01$, by the Kruskal–Wallis test ($P<0.001$) followed by the Mann–Whitney *U* test. iDC, immature dendritic cell; MAC, macrophage; Mo, monocyte; PC5, phycoerythrin–cyanin 5.1; PE, phycoerythrin.

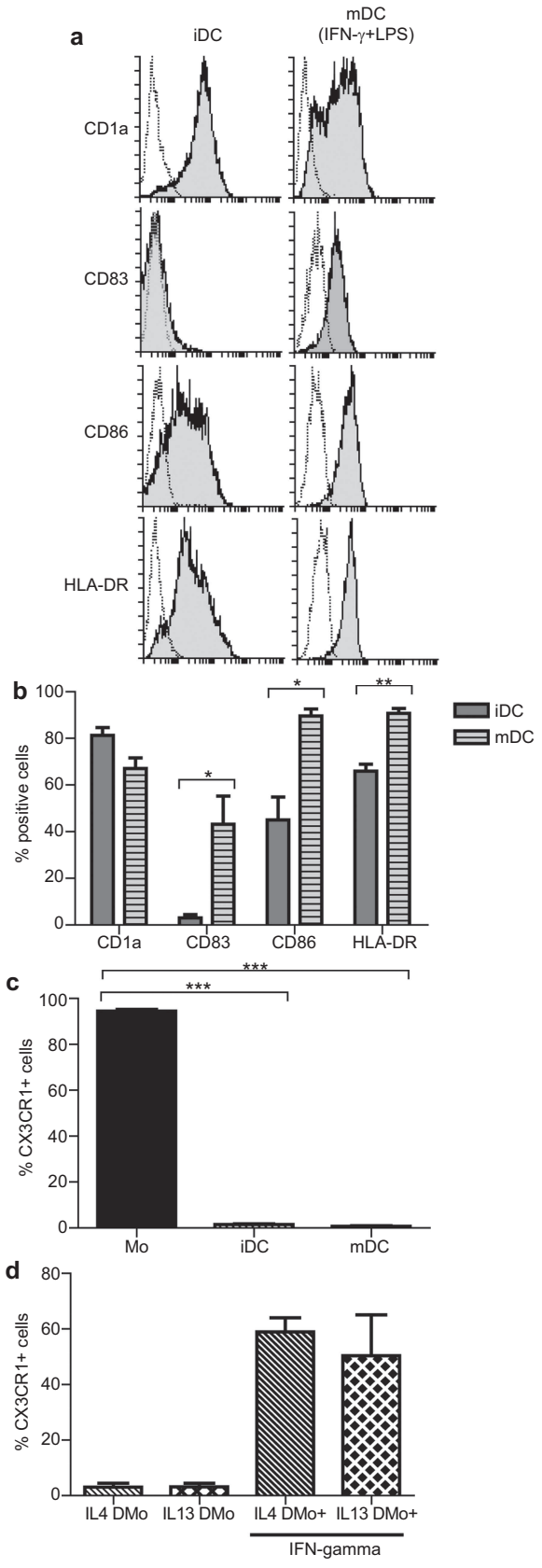

Figure 2 CX_3CR1 expression in DC after maturation. Mo-derived iDC were further cultured in presence of LPS (500 ng/ml)+1000 U/ml de IFN-γ for 48 h to obtain mDC. Subsequently, the cells were stained with FITC-conjugated anti-CD1a mAb, PE-conjugated anti-CD83 mAb, PE-Cy5-conjugated anti-CD86 mAb and FITC-conjugated anti-HLA-DR mAb and then analyzed by flow cytometry. (**a**) Representative histograms for CD1a, CD83, CD86 and HLA-DR membrane expression in iDC or mDC are shown. Dotted histograms represent the isotype controls. (**b**) The percentages of positive cells for CD1a, CD83, CD86 and HLA-DR in iDC or mDC are shown. Each bar represents the mean±‴ s.e.m. from 4–6 different donors. *$P<0.05$ and **$P<0.01$, by Mann–Whitney *U* test calculated for each marker individually. (**c**) CX_3CR1 expression in Mo, iDC or mDC. The results are expressed as percentage of positive cells. Each bar represents the mean±s.e.m. from 7–10 different donors. ***$P<0.001$, according to Kruskal–Wallis test ($P<0.001$) followed by the Mann–Whitney *U* test. (**d**) Mo were cultured up to 3 days in the presence of GM-CSF (50 ng/ml)+IL-4 or IL-13 (20 ng/ml) with or without IFN-γ (1000 U/ml). Subsequently, the cells were stained with FITC-conjugated anti-CX_3CR1 mAb and then analyzed by flow cytometry. The percentages of CX_3CR1 positive cells in every cell population are shown. Each bar represents the mean±s.e.m. from 2–4 different donors. DC, dendritic cell; GM-CSF, granulocyte-macrophage colony stimulating factor; HLA, human leukocyte antigen; IFN, interferon; LPS, lipopolysaccharide; mDC, mature dendritic cell; PE, phycoerythrin.

Presence of IFN-γ during DC differentiation abrogated CX_3CR1 downregulation

Because MACs activated in the presence of IFN-γ upregulated CX_3CR1 expression, we further investigated whether IFN-γ was able to abrogate the loss of CX_3CR1 during DC differentiation. Mos were incubated with IFN-γ (1000 U/ml) from the first until the third day of the DC-differentiating protocol. Figure 2d shows that DCs differentiated in the presence of IFN-γ inhibited the downregulation of CX_3CR1. In contrast, we previously showed that iDCs that maturated for 48 h in the presence of IFN-γ+LPS did not reverse the CX_3CR1 downregulation that occurred during DC differentiation. These results suggest that IFN-γ was able to attenuate CX_3CR1 downregulation during the first stages of DC differentiation and induced its expression in MACs.

CX_3CR1 gene expression in Mos, MACs and DCs

Because we established that CX_3CR1 protein was differentially regulated during Mo differentiation into DCs or MACs, we next determined whether this regulation occurs at the transcription level. For this purpose, CX_3CR1 mRNA expression was analyzed by reverse transcription coupled with qRT-PCR. Using primers specific for the CX_3CR1 V28 variant, we obtained a 163 bp PCR product from freshly isolated Mos (Figure 4a). Thus, we performed a quantitative analysis of CX_3CR1 mRNA on freshly isolated Mos, Mos cultured for 3 days in GM-CSF+IL-4/IL-13, Mos cultured for 7 days in GM-CSF and MACs polarized for 3 additional days with IFN-γ+LPS or IL-4. Under these conditions, the freshly isolated Mos showed high CX_3CR1 mRNA levels that were drastically decreased during DC or MAC differentiation. Consistent with the CX_3CR1 membrane expression, CX_3CR1 mRNA in MACs was higher than in DCs. Interestingly, we still detected CX_3CR1 mRNA in DCs, whereas CX_3CR1 protein was undetectable. Conversely, the further IL-4-dependent alternative activation

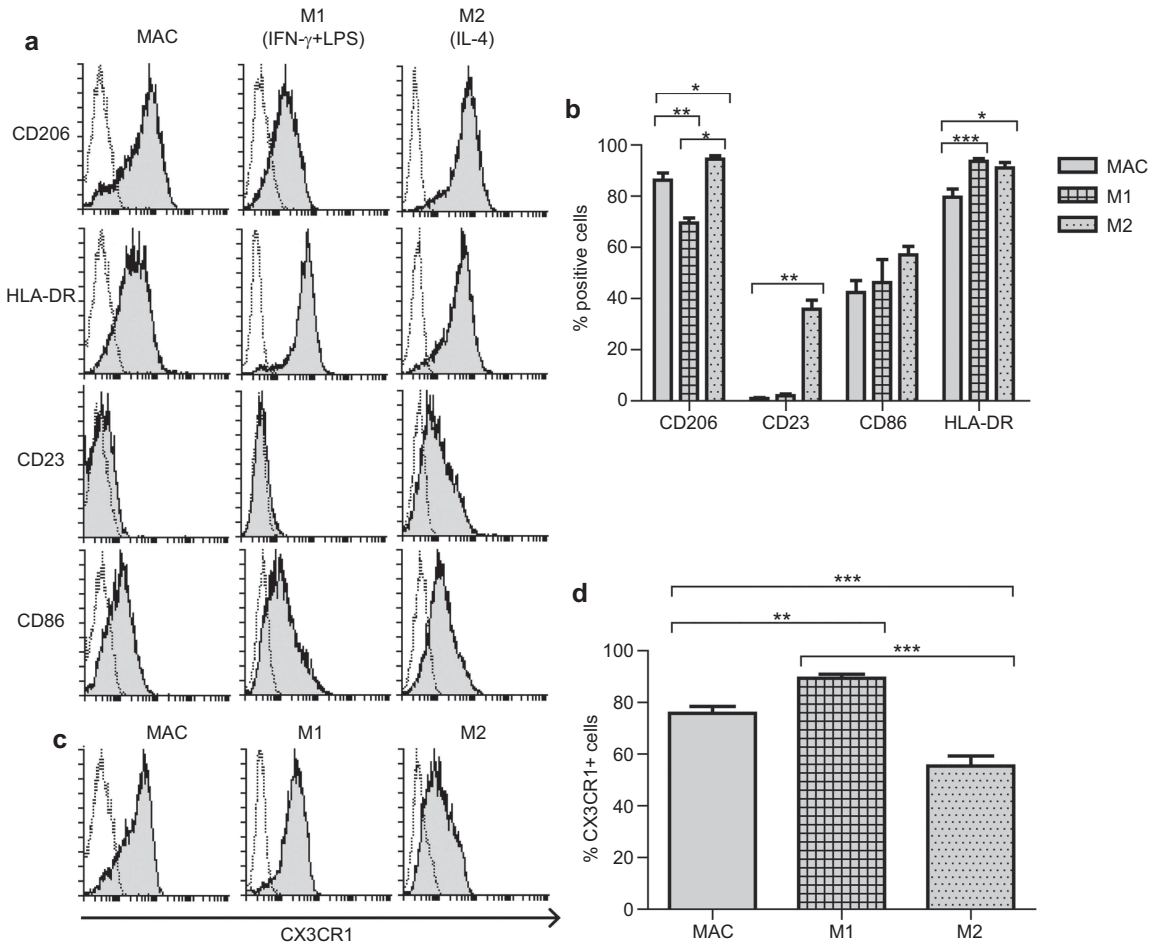

Figure 3 CX_3CR1 membrane expression in MACs before and after activation. MACs were further cultured for 72 h either in presence of LPS (100 ng/ml)+IFN-γ (400 U/ml) to obtain classically activated MACs (M1) or IL-4 (25 ng/ml) to obtain alternative-differentiated MACs (M2). Subsequently, the cells were stained with FITC-conjugated anti-CD206 mAb, PE-conjugated anti-CD23 mAb, PCy5-conjugated anti-CD86 mAb and FITC-conjugated anti-HLA-DR mAb and then analyzed by flow cytometry. (**a**) Representative histograms of membrane surface marker expression in MAC, M1 and M2 cell populations are shown. Dotted histograms represent the isotype controls. (**b**) The percentage of positive cells for CD206, CD23, CD86 and HLA-DR in MAC, M1 and M2 cell populations are shown. Each bar represents the mean±s.e.m. from 4–9 different donors. $*P<0.05$, $**P<0.01$ and $***P<0.001$, by the Kruskal–Wallis test ($P<0.05$) followed by the Mann–Whitney *U* test. In another set of experiments, MAC, M1 and M2 populations were stained with FITC-conjugated anti-CX_3CR1 mAb. (**c**) Representative histograms for CX_3CR1 membrane expression in the MAC, M1 and M2 cell populations are shown. Dotted histograms represent the isotype controls. (**d**) The percentage of CX_3CR1-positive cells in the MAC, M1 and M2 cell populations are shown. Each bar represents the mean±s.e.m. from 9–12 different donors. $**P<0.01$ and $***P<0.001$, by the Kruskal–Wallis test ($P<0.0001$) followed by the Mann–Whitney *U* test. HLA, human leukocyte antigen; IFN, interferon; LPS, lipopolysaccharide; MAC, macrophage; PE, phycoerythrin.

of MACs downregulated CX_3CR1 mRNA (Figure 4b). Altogether, these results suggest that the decrease in CX_3CR1 protein expression was concomitant with the downregulation of CX_3CR1 mRNA.

Biological function of the CX_3CR1–CX_3CL1 interaction

Finally, to assess the biological impact of the differences in CX_3CR1 expression on the Mo-derived populations, we investigated sCX_3CL1-dependent survival in fresh Mos, iDCs and MACs because the CX_3CR1–CX_3CL1 interaction has been previously proposed to be a signal for Mo survival.[43] Morphological assessment of Mo apoptosis showed a significant decrease in the apoptotic rate of Mo cultured overnight with sCX_3CL1 compared with control Mos that were incubated in medium (Figure 5a). Because 1 μg/ml of CX_3CL1 was sufficient to significantly delay Mo apoptosis, this concentration of ligand was used in the following experiments.

A similar result was observed by flow cytometry through DNA staining with PI and binding of phosphatidylserine on the cell surface by annexin-V (Figure 5b). Thus, the percentage of annexin V-positive Mos significantly decreased, and PI/annexin V double-negative cells significantly increased in the presence of sCX_3CL1. However, there was no difference in the apoptotic rate of iDCs when they were incubated overnight with or without sCX_3CL1 (Figure 5b). When MACs were similarly treated with sCX_3CL1, a slight but not significant decrease

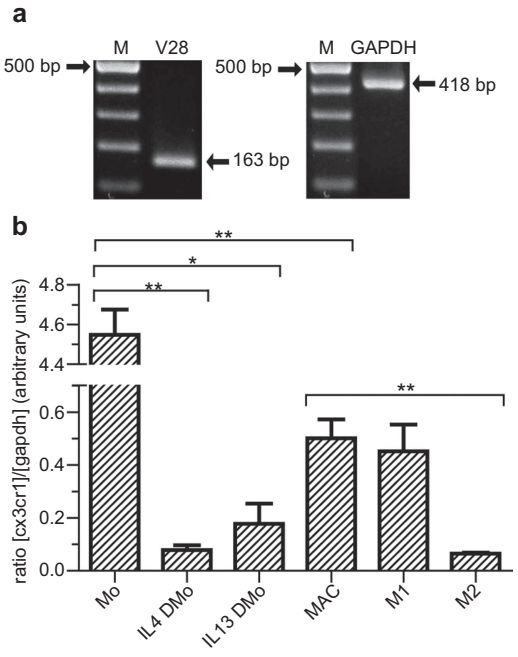

Figure 4 CX_3CR1 gene expression in Mos and Mo-derived DCs and MACs. Total RNA was prepared from freshly isolated Mo and cells that had been cultured for three days with GM-CSF (50 ng/ml) + IL-4 or IL-13 (20 ng/ml) to differentiate Mo into iDCs (IL-4 DMo or IL-13 DMo), or for 7 days with GM-CSF (30 ng/ml) to differentiate Mo into MACs. MACs were further cultured for 72 h either in presence of LPS (100 ng/ml) and IFN-γ (400 U/ml) to obtain classically activated MACs (M1), or IL-4 (25 ng/ml) to obtain alternative-differentiated MACs (M2). Subsequently, cDNA was prepared and real-time PCR was performed using specific primers for V28 variant of CX_3CR1 and GAPDH. (**a**) The specificity of the primers is shown. PCR products obtained in the Mo were electrophoresed on 2% agarose gel and visualized with ethidium bromide staining. M is a 100 bp DNA ladder. (**b**) CX_3CR1 mRNA expression in the different cell populations was analyzed by real-time RT-PCR. Data were normalized against GAPDH gene expression. The results are expressed in arbitrary units. Each bar represents mean±s.e.m. of 3–5 different donors, each one assessed in quadruplicate. $*P<0.05$, $**P<0.01$, by the Kruskal–Wallis test ($P<0.001$) followed by the Mann–Whitney *U* test. DC, dendritic cell; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; iDC, immature dendritic cell; GM-CSF, granulocyte-macrophage colony stimulating factor; IFN, interferon; MAC, macrophage; Mo, monocyte; RT-PCR, reverse transcriptase-polymerase chain reaction.

in the apoptotic rate was observed (Figure 5b). These results indicate that the interaction of sCX_3CL1 with its receptor is in fact an efficient survival signal and suggest that the differential expression of this receptor in the cell membrane offers the possibility to differentially regulate the half-life of Mo-derived cells.

DISCUSSION

Considerable evidence suggests a major role for the CX_3CR1–CX_3CL1 axis in Mo–endothelium interactions under physiological or inflammatory conditions. However, little is known about the regulation of CX_3CR1 at different stages during monocytic differentiation and maturation. CX_3CL1 is prominently expressed by multiple non-hematopoietic cell types, including muscle, neurons, renal cells, epithelial and endothelial cells, under inflammatory and pathological conditions.[15,23,44–50] Under those conditions, CX_3CL1 may facilitate the adhesion and transmigration of monocytes.[51] Additionally, it has been recently proposed that a direct and evolutionary conserved role for CX_3CR1–CX_3CL1 interactions is involved in Mo survival.[43] Although chemokines were originally defined as chemoattractants, a growing body of evidence indicates their additional involvement in the control of cell survival.[52] Landsman *et al.*[43] suggested that CX_3CR1-survival signals can provide a mechanistic explanation for one of the few known phenotypes of CX_3CR1- and CX_3CL1-deficient mice (i.e., their relative protection from diet-induced atherosclerosis). Supporting this hypothesis, we demonstrated that sCX_3CL1 is in fact an efficient survival signal to monocytes. Thus, CX_3CR1 expression could participate not only by promoting cell-to-cell interactions with an inflamed endothelium, but also by increasing Mo survival.

Accordingly, the importance of the CX_3CR1–CX_3CL1 axis in pathogenesis has been highlighted in several studies. It is involved in atherosclerosis,[26,41–42,45,46] vascular and renal inflammation[37,48,53] and cancer,[50] it stimulates actin reorganization in microglia[49,54] and it promotes leukocyte–renal endothelial cell interactions during hemolytic uremic syndrome, thereby contributing to the renal microvascular dysfunction and thrombotic microangiopathy.[55,56]

To improve our understanding of the biological role of the interaction between fractalkine and CX_3CR1 in monocytic populations, we analyzed CX_3CR1 surface protein expression as well as mRNA expression in freshly Mo, MAC and DC populations.

Heterogeneity among human monocytes is well established.[4] There are two main subsets, which include the 'classical' $CD14^{++}/CD16^{-}$ and 'non-classical' $CD14^{+}/CD16^{+}$ monocytes, both of which express CX_3CR1. However, the $CD14^{+}/CD16^{+}$ subset characteristically expresses higher levels.[57] Although the $CD14^{+}/CD16^{+}$ subset expresses higher CX_3CR1 levels, it is a very small population in healthy individuals, and it shows considerable heterogeneity.[4] We analyzed CX_3CR1 expression during differentiation in the whole population of peripheral human monocytes because the separate analysis of the different subsets is technically impracticable due to the complexity of these populations. Therefore, we observed that CX_3CR1 was rapidly downregulated during monocyte differentiation. However, significant differences in CX_3CR1 expression exist between DCs and MACs. Lower CX_3CR1 cell surface expression levels were observed from 24 h after differentiation into DCs, and negligible CX_3CR1 protein expression was observed after 72 h of culture or during DC maturation. Consistently, CX_3CR1 mRNA was profoundly downregulated, although it was not totally absent in DCs, suggesting differentiation-associated downregulation both at the transcriptional and translational level. In agreement with these results, there was no protection against apoptosis mediated by sCX_3CL1 in DCs,

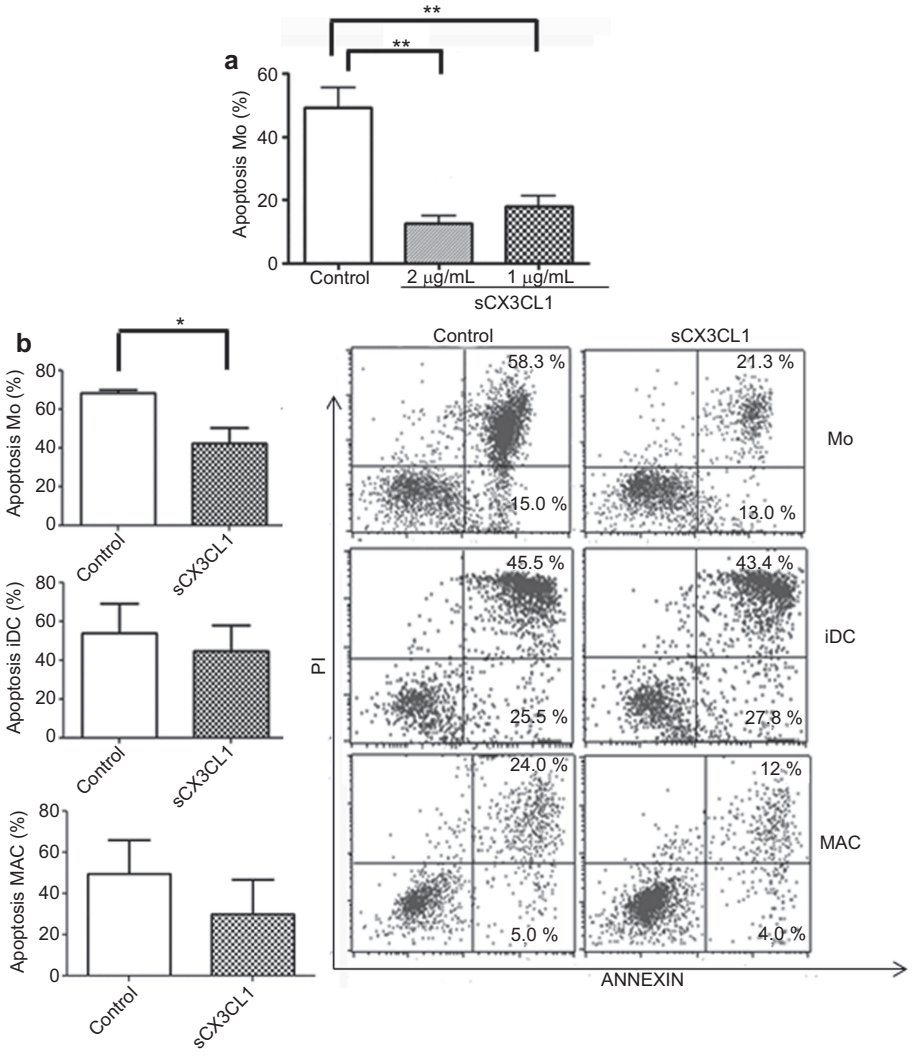

Figure 5 Effect of soluble CX_3CL1 (sCX_3CL1) on the Mo, DC and MAC apoptotic rate. (**a**) Mos were cultured in medium with or without (control) sCX_3CL1, at the indicated concentration for 24 h at 37 °C. Apoptosis was measured by fluorescence microscopy, as indicated in the section on 'Materials and methods'. Each bar represents the mean±s.e.m. from 3–5 different donors. $^{**}P<0.005$, according to the Kruskal–Wallis test ($P<0.005$) followed by the Mann–Whitney *U* test. (**b**) Apoptosis from Mos, iDCs or MACs was measured in parallel by flow cytometry as detailed in the Materials and Methods. Representative PI *vs.* annexin V double dot plots of Mos, iDCs and MACs incubated in medium with or without (control) sCX_3CL1 (1 μg/ml), 24 h at 37 °C. Each bar represents the mean±s.e.m. of annexin V-positive cells for each population from 3–5 different donors. $^{*}P<0.05$, according to the Mann–Whitney *U* test. DC, dendritic cell; iDC, immature dendritic cell; MAC, macrophage; Mo, monocyte; PI, propidium iodide.

which correlates with the absence of CX_3CR1 expression. Although some reports have addressed mRNA CX_3CR1 expression in DCs, and a CX_3CL1-dependent function, the corresponding protein has not been shown.[39,58] In contrast, it has been reported that DCs express the corresponding ligand, chemokine CX_3CL1, which was increased during DC maturation (particularly by CD40 ligation).[20] Stable CX_3CR1 expression during the DC maturation process would contradict the maturation state that is dependent on the expression of other CC-chemokine receptors, which may ensure the directional traffic of immature DCs from blood to target sites.[59] The controversial conclusion about CX_3CR1 protein expression in DCs could be derived from the presence of CX_3CR1 mRNA, which was also observed under our experimental conditions, or the use of DCs from different origins or obtained through different purification protocols. Conversely, and in line with the CX_3CR1–CX_3CL1 survival signal hypothesis, DCs are generally short-lived and are replaced by blood-bone precursors.[60,61] Additionally, CX_3CR1 downregulation may be involved in restricting the 'reverse migration' of differentiated monocytes back into the blood stream, thus facilitating capture within the tissues. In contrast, MACs express intermediate CX_3CR1 protein level and show a partial survival signal by sCX_3CL1. Moreover, CX_3CR1 can be increased in MACs by IFN-γ during the classical activation pathway or when monocytes are exposed to IFN-γ early during the DC-differentiation protocol. It has

been shown that under these conditions, the stimulatory capacity of DCs are negatively regulated, leading to a tolerogenic phenotype as assayed by cell surface molecule expression, an altered transcriptional profile and cytokine production.[62] In this regard, intermediate levels of CX_3CR1 expression between iDCs and tolerogenic DCs could contribute to phenotypically distinguishing between these types of DC. Interestingly, the mechanism of IFN-γ-mediated effects on monocyte-derived DCs has been shown to involve the inhibition of STAT-6 phosphorylation and the downregulation of membrane IL-4α chain subunit expression.[63] These results are in line with previous data demonstrating that the overnight incubation of Mo in the presence of IFN-γ blocks the downmodulation of CX_3CR1 in Mo.[38] The only difference between MACs and DCs that are differentiated from Mos was IL-4 signaling. Moreover, the alternative differentiation of MACs that are mediated by this cytokine was also accompanied by the downregulation of CX_3CR1 surface expression and mRNA levels. Therefore, these data suggest that the differential CX_3CR1 expression on MACs and DCs might be IL-4-dependent. IL-4 binds to the high-affinity IL-4Rα and then heterodimerizes with either the common γ chain (γC) or the IL-13Rα1 chain, forming type I and type II receptor complexes, respectively.[64] IL-13, however, binds with relatively low affinity to the IL-13Rα1 chain and then heterodimerizes with the IL-4Rα chain to form a type II receptor complex. Ligand engagement of receptor complexes activates Janus kinases (JAKs) associated with the cytoplasmic domains of the different chains. JAK3 is constitutively associated with γC through interactions with canonical box 1 and box 2 sequence motifs (JAK-binding motifs). The activated kinases initiate downstream signaling cascades that recruit substrate proteins, such as IRS-1 (in non-hematopoietic cells) or IRS-2 (in hematopoietic cells), STAT6 and others, for tyrosine phosphorylation by the JAKs.[65]

IL-4 produced by Th2 lymphocytes is known to exert anti-inflammatory effects on Mos or MACs. IL-4 acts through activation of the Jak/STAT signaling pathway and inhibits release of pro-inflammatory cytokines by Mo.[66] Our data demonstrate that IL-4 also rapidly downregulates the expression of CX_3CR1 mRNA, accompanied by a decrease in the cell surface expression of this receptor. The latter may somewhat precede that of the mRNA, suggesting that regulation at the translation level may also be involved. The negative regulation of CX_3CR1 by IL-4 points to opportunities for the therapeutic modulation of CX_3CR1 expression.

Under the rationale that IFN-γ and IL-4/IL-13-dependent signals oppositely regulate CX_3CR1 mRNA and protein expression, it is important to consider that both cytokines are the major exponents of Th1 and Th2 responses, respectively. Recent evidence indicates that receptor expression determines the spectrum of action of chemokines in Th1 and Th2 cells and that CX_3CR1 is preferentially expressed on the former, which is consistent with the observation that Th1 cells, but not Th2 cells, respond to CX_3CL1.[67]

Interestingly, a Th1 cytokine pattern predominates in inflammatory situations that drive expansion of Th1 cells, and by acting as both an adhesion molecule and a chemoattractant, CX_3CL1 recruits monocytes, NK cells and T lymphocytes to the endothelium. The present results further support the hypothesis that fractalkine (CX_3CL1) is involved in an amplification circuit of polarized Th1 responses[68] by prolonging the half-life of CX_3CR1 positive cells.

Conversely, Th2 cytokines, such as IL-4 and IL-13, induce an 'alternative' activation program (M2) in MACs. M2 MACs dampen inflammatory and adaptive Th1 responses by producing anti-inflammatory factors (IL-10, transforming growth factor-β and IL-1 receptor antagonist) by scavenging debris and by promoting angiogenesis, tissue remodeling and repair. Recently, the knowledge about MAC plasticity has been enlarged through transcriptome-based network analysis, demonstrating differences inside those so-called 'alternatively activated' MACs (M2a, M2b and M2c) and the capacity to switch from an activated M1 state back to one of the M2 states, and *vice versa*, upon specific signals.[69,70] In line with this study, the present work would contribute to define MAC activation profiles and to the development of new therapeutic strategies in human disease.

Though the actual physiological role of the differential expression of CX_3CR1 in the cell membrane of Mo-derived cells is incompletely understood, the results presented herein suggest that Mo differentiation and the antagonistic regulatory capacity of IFN-γ and IL-4 offer an additional way to control cell survival and function. Thus, by improving our understanding of the regulatory mechanisms that govern CX_3CR1 expression on monocyte lineage cells, we can better appreciate how monocyte recruitment, survival and activation are controlled during the immune response.

CONFLICTS OF INTEREST

None of the authors have any potential financial conflict of interest related to this manuscript.

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