Biochimica et Biophysica Acta 1813 (2011) 2026–2035

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Cross talk between smooth muscle cells and monocytes/activated monocytes via CX3CL1/CX3CR1 axis augments expression of pro-atherogenic molecules

Elena Dragomir Butoi [a,*], Ana Maria Gan [a], Ileana Manduteanu [a], Daniela Stan [a], Manuela Calin [a,c], Monica Pirvulescu [a], Rory Ryan Koenen [b], Christian Weber [b], Maya Simionescu [a]

[a] Institute of Cellular Biology and Pathology "Nicolae Simionescu" of the Romanian Academy, Bucharest, Romania
[b] Institute for Cardiovascular Prevention, Ludwig-Maximilians-University Munich, Munich, Germany
[c] Institute of Macromolecular Chemistry "Petru Poni", Iasi, Romania

ARTICLE INFO

Article history:
Received 31 March 2011
Received in revised form 11 August 2011
Accepted 15 August 2011
Available online 22 August 2011

Keywords:
Smooth muscle cell
Monocyte
Fractalkine
CX3CR1
Inflammatory cytokine
Metalloproteinase

ABSTRACT

Objective: In atherosclerotic lesions, fractalkine (CX3CL1) and its receptor (CX3CR1) expressed by smooth muscle cells (SMC) and monocytes/macrophages, mediate the heterotypic anchorage and chemotaxis of these cells. We questioned whether, during the close interaction of monocytes with SMC, the CX3CL1/CX3CR1 pair modulates the expression of pro-atherogenic molecules in these cells.

Methods and results: SMC were co-cultured with monocytes or LPS-activated monocytes (18 h) and then the cells were separated and individually investigated for the gene and protein expression of TNFα, IL-1β, IL-6, CX3CR1 and metalloproteinases (MMP-2, MMP-9). We found that SMC–monocyte interaction induced, in each cell type, an increased mRNA and protein expression of TNFα, IL-1β, IL-6, CX3CR1, MMP-2 and MMP-9. Blocking the binding of fractalkine to CX3CR1 (by pre-incubation of monocytes with anti-CX3CR1 or by CX3CR1 siRNA transfection) before cell co-culture decreased the production of TNFα, CX3CR1 and MMP-9. Monocyte–SMC interaction induced the phosphorylation of p38MAPK and activation of AP-1 transcription factor. Silencing the p65 (NF-kB subunit) inhibited the IL-1β and IL-6 and silencing c-jun inhibited the TNFα, CX3CR1 and MMP-9 induced by SMC–monocyte interaction.

Conclusions: The cross-talk between SMC and monocytes augments the inflammatory response in both cell types as revealed by the increased expression of TNFα, IL-1β, IL-6, CX3CR1 and MMPs. Up-regulation of TNFα, CX3CR1 and MMP-9 is further increased upon interaction of SMC with activated monocytes and is dependent on fractalkine/CXRCR1 pair. These data imply that the fractalkine/CX3RCR1 axis may represent a therapeutic target to impede the inflammatory process associated with atherosclerosis.

1. Introduction

Atherosclerosis, entails in addition to dyslipidemia a complex inflammatory process whose progression is dependent upon an intricate network of cells, cytokine and chemokine signaling [1–3]. Inflammatory cytokines are produced mainly by monocyte/macrophages and lymphocytes, but also by endothelial cells and smooth muscle cells (SMC) after stimulation by inflammatory mediators or toxins [4]. Several cytokines such as TNFα, IL-6 and IL-1β may predict the risk of cardiovascular events [5].

TNFα exerts potent pro-inflammatory effects in atherosclerosis and other metabolic and inflammatory disorders. It is present in human and animal atherosclerotic plaques. In TNFα-deficient apoE−/− mice, the atherosclerotic lesion size in the aortic sinus was significantly smaller than in apoE−/− mice, and this was associated with a decreased expression of ICAM-1, VCAM-1, and MCP-1 [6]. Like TNFα, IL-1β is one of the main pro-inflammatory products of monocytes/macrophages generated in pathologic conditions. Blocking IL-1β in ApoE−/− mice impedes the development of atherosclerosis revealing its role in the plaque formation [7]. IL-6, a significant cytokine, is implicated in the pathology of numerous diseases, including atherosclerosis [8]. IL-6 treatment at supra-physiological concentrations of C57Bl/6 mice and apoE−/− mice (on low-, or high-fat diet) resulted in a fivefold and twofold increase, respectively, in fatty streak size [9].

Vascular cells and monocyte/macrophages secrete matrix metalloproteinases (MMPs). MMP-2 that is constitutively expressed in normal artery SMC, is increased in atherosclerosis concomitantly with the enhanced expression of MMP-9 both in SMC and macrophages [10]. The MMP-9 activity causes the degradation of SMC's basal lamina facilitating

* Corresponding author at: Institute of Cellular Biology and Pathology "Nicolae Simionescu", 8, B.P. Hasdeu Street, P.O. Box: 35-14, Bucharest, Romania. Tel.: +40 21 319 45 18; fax: +40 21 319 45 19.
E-mail addresses: elena.dragomir@icbp.ro (E.D. Butoi), anca.gan@icbp.ro (A.M. Gan), ileana.manduteanu@icbp.ro (I. Manduteanu), daniela.stan@icbp.ro (D. Stan), manuela.calin@icbp.ro (M. Calin), monica.pirvulescu@icbp.ro (M. Pirvulescu), rkoenen@ukaachen.de (R.R. Koenen), cweber@ukaachen.de (C. Weber), maya.simionescu@icbp.ro (M. Simionescu).

0167-4889/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamcr.2011.08.009

their migration to the intima and therefore may be involved in fibrous cap formation; excessive proteolytic activity of MMPs renders the plaque vulnerable and prone to destabilization and rupture [11].

Recent studies indicate that within the plaque, the interaction between SMC and monocytes has a role in the promotion of monocyte retention, foam-cell formation, and in atherogenesis [12]. Moreover, the SMC–monocyte interactions increase the production of atherosclerosis-related factors such as metalloproteinase-1 within both cell types [13] but the pathophysiological consequences of their interaction are scantily characterized. Fractalkine (CX3CL1) is an atypical chemokine that functions as chemoattractant or as adhesion molecule, facilitating monocyte and T cell transmigration in atherosclerotic lesion prone areas [14]. The presence of fractalkine and its cognate receptor was demonstrated in human coronary artery atherosclerotic plaque but not in normal artery [15]. Previous reports have shown that within the atherosclerotic plaque SMC and monocytes interact via the CX3CL1/CX3CR1 axis and that their interaction regulates monocyte survival and differentiation [15–17]. The fractalkine/CX3CR1 axis has been implicated in the pathogenesis of vascular dysfunction and vascular disease. Thus, interrupting the CX3CL1–CX3CR1 binding in vivo has a highly protective effect in animal models of atherosclerosis [18] and that human subjects heterozygous for the CX3CR1-M280 allele, which encodes a defective receptor, have a reduced risk of atherosclerotic cardiovascular disease [19]. Based on these data, we hypothesized that, the interaction between SMC and monocytes via CX3CL1/CX3CR1 axis may have an effect on each cell type and trigger the induction of pro-inflammatory molecules that are important for plaque progression. We report here that direct contact between SMC and monocytes or SMC and lipopolysaccharide (LPS)-activated monocytes increases the expression of TNFα, IL-1β, IL-6, CX3CR1 and MMPs, and that CX3CL1–CX3CR1 binding is involved in the up-regulation of TNFα, CX3CR1 and MMP-9 expression, essential molecules known to affect the progression of atherosclerosis. Activation of monocytes with LPS before co-culture with SMC produces a further increase of TNFα, CX3CR1 and MMP-9.

2. Materials and methods

2.1. Materials

Monoclonal antibodies, rabbit anti-human CX3CR1, antiphospho- and total p38MAPK, and anti-human to pc-jun were from Santa Cruz Biotechnology. The FITC labeled anti-human CX3CR1 was from MBL (Biozol, Germany). The monoclonal antibody to fractalkine was from R&D Systems, and anti-actin, the secondary antibodies and all the other reagents were from Sigma Aldrich Chemie GmbH (Germany). Signal West Pico Chemiluminescent Substrate kit was from Pierce (Rockford USA). Rabbit and mouse IgG used for flow cytometry and neutralization studies were from Sigma and BD Biosciences. siRNAs (p65/c-Jun/CX3CR1/scrambled) and siRNA transfection reagent Superfect were obtained from Santa Cruz Biotechnology. siRNA transfection reagent turbofect was from Fermentas.

CD14 MicroBeads were from Miltenyi Biotech. Human TNFα and IL6 enzyme-linked immunosorbent assays were from R&D Systems. RT-PCR reagents and Alexa Fluor594 were from Invitrogen.

2.2. Cell culture

Human aortic SMC were isolated from the media of fetal thoracic aorta and characterized as a pure cell line devoid of any contaminants. The cells exhibited an elongated spindle-shaped morphology, grow as multilayers with the characteristic hills and valley pattern (as assessed by phase-contrast microscopy), and exhibited bundles of cytoplasmic myofilaments and numerous caveolae at the cell periphery (as demonstrated by electron microscopy). In addition immunoblotting and immunohistochemistry experiments revealed that they are positive for smooth muscle alpha-actin, and for vinculin, negative for von Willebrand factor [22], and display functional store-operated channels responsive for capacitative calcium entry [23,24]. SMC were cultured in DMEM as described [20].

Monocyte-like cell line U937 (a kind gift of Professor S.C. Silverstein, Columbia University, New York, USA) were grown in suspension in the RPMI 1640 culture medium containing 5% FCS and were split 1:5, twice a week.

This investigation was carried out according to the principles outlined in the Declaration of Helsinki [21]. The Ethics Committee of the Institute of Cellular Biology and Pathology "Nicolae Simionescu", Bucharest, approved the protocol.

2.3. Experimental design: incubation of monocytes with SMC followed by cell separation

Monocytes or LPS-activated monocytes in suspension (10^6) were added to confluent cultured SMC and incubated (37 °C, 5%CO_2) in RPMI1640 medium for 18 h. The latter time was chosen based on our preliminary data showing that incubation of SMC or monocytes with soluble fractalkine for 18 h was sufficient to induce a statistically significant up-regulation of IL6, VCAM-1 and TNFα expression (Supplementary Fig. 1C, D, E). After 18 h, the non-adherent monocytes were removed and the co-culture represented by the adhered monocytes to SMC was incubated with accutase for 10 min. From the resulting cell suspension, a pure SMC or monocyte population was separated by positive selection of monocytes, using CD14 MicroBeads and a magnetic cell sorting (MACS) separator (Miltenyi Biotech) according to the manufacturer's instructions. The adherent monocytes represented ~20% of the total number of monocytes added to SMC (as quantified from the extracted mRNA). Before starting the cell separation experiments, we checked for the CD14 surface expression of both SMC and monocytes and found that only the latter were CD14 positive (Supplementary Fig. 1A, B). Monocyte purity in each cell preparation was evaluated by flow cytometry using MoFlo MLS flow cytometer (Dako-Cytomation, Fort Collins, CO). Since the RMPI-1640 medium used contains 11 mM glucose that can be considered as a 'hyperglycemia-like' environment, we performed control experiments with monocytes cultured in RPMI without glucose and in the culture medium containing 5.5 and 11 mM glucose concentrations. The results showed that TNFα, MMP-9 and CX3CR1 gene expressions were not affected by these glucose concentrations (Supplementary Fig. 1F).

To increase the expression of fractalkine on SMC surface, in some experiments, before cell interaction, SMC were activated (4 h) with TNFα (10 ng/ml). For blocking studies, monocytes were pre-incubated with human monoclonal anti-CX3CR1 or anti-CCR2 (5 μg/ml) for 30 min (37 °C) and then were interacted with SMC (as above). As negative control, a non-specific immunoglobulin (IgG, BD Bioscience) was used instead of anti-CX3CR1.

2.4. Flow cytometry

Monocytes or accutase-harvested SMC were fixed with ice-cold 3% paraformaldehyde, washed twice with ice-cold phosphate buffered saline and labeled with anti-human CX3CR1 IgG (2 μg/ml) followed by FITC-conjugated anti-rabbit IgG (1/100). The mean CX3CR1-specific fluorescence was corrected for the background as determined with nonspecific rabbit IgG isotypes. For surface expression of CD14, the cells were incubated with a saturated concentration of FITC-conjugated anti-CD14 (30 min, 4 °C). Flow cytometry was performed using a MoFlo MLS flow cytometer.

2.5. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Gene analysis was performed as described [20] (Supplementary material). The mRNA levels of analyzed molecules were normalized relative to GAPDH mRNA levels.

2.6. Western blot

Protein analysis was performed as described [20]. The signals were visualized using SuperSignal West Pico chemiluminescent substrate (Pierce) and quantified by densitometry employing gel analyzer system Luminiscent image analyzer LAS 4000 (Fujifilm) and Image reader LAS 4000 software.

2.7. Transfection of small interfering RNA (siRNA)

The siRNA (p65/c-Jun/scrambled) was transfected into SMC using siRNA transfection reagent Superfect and siRNA (CX3CR1/scrambled) into monocytes using turbofect, according to the manufacturer's protocol. Twenty-four hours after transfection, the cells were harvested and analyzed. Transfection efficiency was evaluated by detection of p65 and c-Jun protein expression using Western blot assay. After transfection, the interaction between SMC and monocytes, followed by cell separation, was performed as described.

2.8. Gelatin zymography assay

Conditioned medium collected from cultured monocytes, SMC or from interacted monocyte–SMC was electrophoresed under non-reducing conditions on 10% polyacrylamide gels containing 1 mg/ml gelatin as substrate. After electrophoresis, the gels were renatured in 2.5% Triton X-100 (2×30 min) and then incubated (18 h, 37 °C) in 50 mM Tris–HCl, pH 7.4, containing 10 mM $CaCl_2$ and 0.2 mM PMSF; subsequently the gels were stained with 0.2% Coomassie brilliant blue R-250 and de-stained with 10% acetic acid and 25% methanol. The white bands against the blue background indicated the presence of gelatinolytic activity. Image acquisition was done with Image Master VDS and LisCap software (Amersham Pharmacia Biotech). Computerized densitometry was employed to evaluate the relative enzymatic activity (TotalLab software — Amersham Pharmacia Biotech, Wien).

2.9. TNFα and IL6 quantification

TNFα and IL-6 antigen were quantified in the cell-conditioned media using an ELISA assay (R&D systems, UK) according to the manufacturer's instructions. The detection limit was 5 pg/ml.

2.10. Real time PCR

Quantification of TNF-α, MMP-9 and CX3CR1 mRNA expression was done by amplification of cDNA using SYBR Green I chemistry (Supplementary material). The relative quantification was performed by comparative CT method and expressed as arbitrary units [25].

2.11. Statistical analysis

The data obtained from the experiments were expressed as the means ± standard deviation (SE). Statistical evaluation was carried out by one-way ANOVA test. The p value for multiple comparisons was calculated using one-way ANOVA and Bonferroni test from OriginPro7.5 software. $p<0.05$ was considered statistically significant.

3. Results

3.1. The interaction between SMC and monocytes increases the TNFα gene expression and TNFα protein release in the conditioned media

The expression of TNFα in SMC and monocytes was assessed before and after cell–cell interaction followed by individual cell separation (as described in the experimental design). We found that prior to their contact, monocytes and especially SMC express a low level of TNFα mRNA. Conversely, upon monocyte–SMC interaction, the TNFα gene expression increased significantly in both cell types (Fig. 1A).

To track the role of CX3CL1–CX3CR1 axis in the increased TNFα production upon monocyte–SMC interaction, before co-culture, monocytes were pre-incubated for 30 min with anti-CX3CR1 to block the cell-to-cell interaction via CX3CL1–CX3CR1 binding. These experiments showed that the TNFα expression was significantly reduced in both, monocytes and SMC (Fig. 1A) demonstrating the dependence of TNFα expression on CX3CL1–CX3CR1 interaction. The control experiments revealed that: i) pre-incubation of monocytes with a nonspecific immunoglobulin (IgG) or anti-CCR2 (MCP-1 receptor) followed by interaction with SMC, did not change the TNFα expression (Supplementary Fig. 2A); ii) the TNFα expression was not changed by incubation of monocytes (without co-culture) with anti-CX3CR1 (Supplementary Fig. 2D); iii) after 18 h in co-culture no change in the proliferation of SMC or monocytes was detected by PCNA Western blot experiments, (Supplementary Fig. 2E). These experiments indicated that the increase

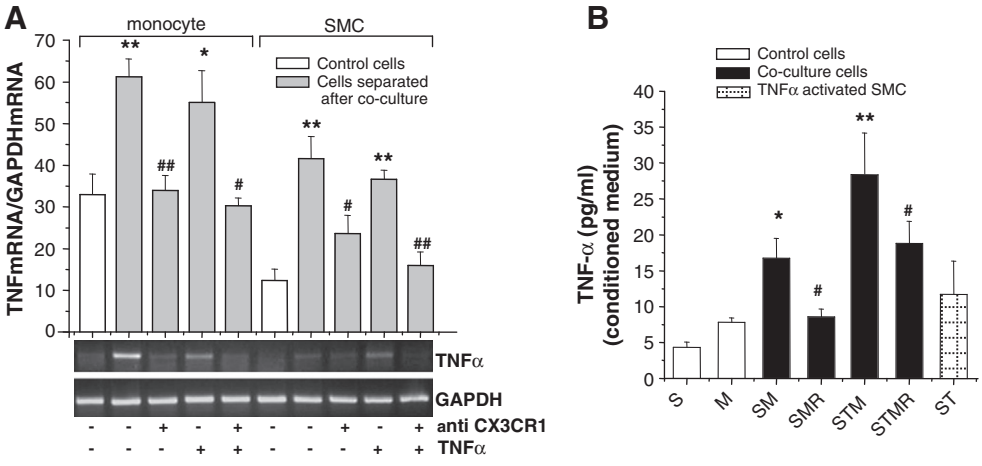

Fig. 1. Monocyte–SMC interaction increases TNFα gene expression in both cells. A. Monocytes were co-cultured with SMC, then separated and TNFα mRNA was quantified in isolated monocytes and SMC. White columns: control monocytes and SMC; gray columns: isolated monocytes or SMC after their co-culture. Interactions of monocytes with SMC or with TNFα-activated SMC increase the TNFα gene expression in both cell types. Pre-incubation of monocytes with anti-CX3CR1 before cell interaction decreases the TNFα expression. n = 4. B. TNFα protein released in the culture media of SMC (S), monocytes (M), SMC co-cultured with monocytes (SM), SMC interacted with monocytes previously exposed to anti-CX3CR1 (SMR), TNFα-stimulated SMC co-cultured with monocytes (STM), TNFα-stimulated SMC interacted with anti-CX3CR1-exposed monocytes (STMR) and TNFα-activated SMC (ST). Note that the level of TNFα protein is increased in the conditioned media from co-cultured SMC–monocytes and decreased when SMC were co-cultured with anti-CX3CR1-exposed monocytes. n = 3, $^{*}p<0.05$, $^{**}p<0.01$ co-cultured vs. control (separately cultured) cells, $^{\#}p<0.05$; $^{\#\#}p<0.01$ co-cultured cells vs. co-cultures in which monocyte CX3CR1 was blocked.

in the expression of TNFα in SMC and monocytes was dependent on cell–cell interaction, and was not due to a raise in cell proliferation.

To find out if the SMC pre-stimulation produces a greater effect, the cells were exposed to TNFα (that increases the CX3CL1 expression) prior to the interaction with monocytes. The experiments showed that the interaction of TNFα-stimulated SMC with monocytes increased the TNFα expression in monocytes and SMC to a level comparable to that obtained for non-stimulated SMC (Fig. 1A).

Blocking of CX3CR1 on monocytes by incubation with anti-CX3CR1 before interaction with TNFα-activated SMC reduced the TNFα mRNA expression to the control level, in both cell types (Fig. 1A).

To assess if monocyte–SMC interaction induces the release of TNFα protein, we determined the presence of TNFα in the culture medium using an ELISA assay. As shown in Fig. 1B, TNFα released in the conditioned media upon SMC–monocyte interaction (lane SM) increased significantly compared to TNFα values in the culture media of control SMC (S) or monocytes (M). Pre-incubation of monocytes with anti-CX3CR1 before the interaction with SMC, reduced the released TNFα to control level (Fig. 1B, SMR) demonstrating that this effect was dependent on CX3CL1–CX3CR1 binding.

Pre-stimulation of SMC with TNFα followed by the cell interaction induced a significant release of TNFα in the culture medium as compared to unstimulated SMC (Fig. 1B, lane STM). Blocking the CX3CR1 before cell interaction significantly reduced the TNFα concentration in the conditioned medium (Fig. 1B, lane STMR). Interestingly, when SMC were stimulated with TNFα (without further interaction with monocytes) the release of TNFα in the culture medium was lower (50%) compared to the values obtained for interacted cells. This revealed that the SMC–monocyte interaction is a prerequisite for increased release of the cytokine. Together, these data indicate that TNFα produced during SMC–monocyte interaction is dependent on CX3CL1–CX3CR1 axis.

3.2. SMC–monocyte interaction modulates IL-1β and IL-6 mRNA and protein expression independent of CX3CL1–CX3CR1 axis

In culture, monocytes and aortic SMC displayed a baseline expression of IL-1β mRNA; however, upon their interaction, IL-1β mRNA and protein expression increased significantly in both cell types (Fig. 2A and B).

In contrast to IL-1β, cultured monocytes did not express detectable level of IL-6 mRNA. Moreover, monocyte–SMC interaction did not affect IL-6 expression in monocytes (data not shown) but increased significantly the IL-6 mRNA in SMC (Fig. 2C) and IL-6 protein released in the culture medium (Fig. 2D, lane SM). Blocking of CX3CL1–CX3CR1 binding by pre-incubation of monocytes with anti-CX3CR1 did not affect IL-6 or IL-1β expression induced by cell–cell interaction. In TNFα-activated SMC, the release of IL-6 was comparable to the control SMC (Fig. 2D, ST and S). These results demonstrated that although SMC–monocyte interaction increased IL6 and IL-1β expression, the CX3CL1–CX3CR1 axis is not involved in their induction.

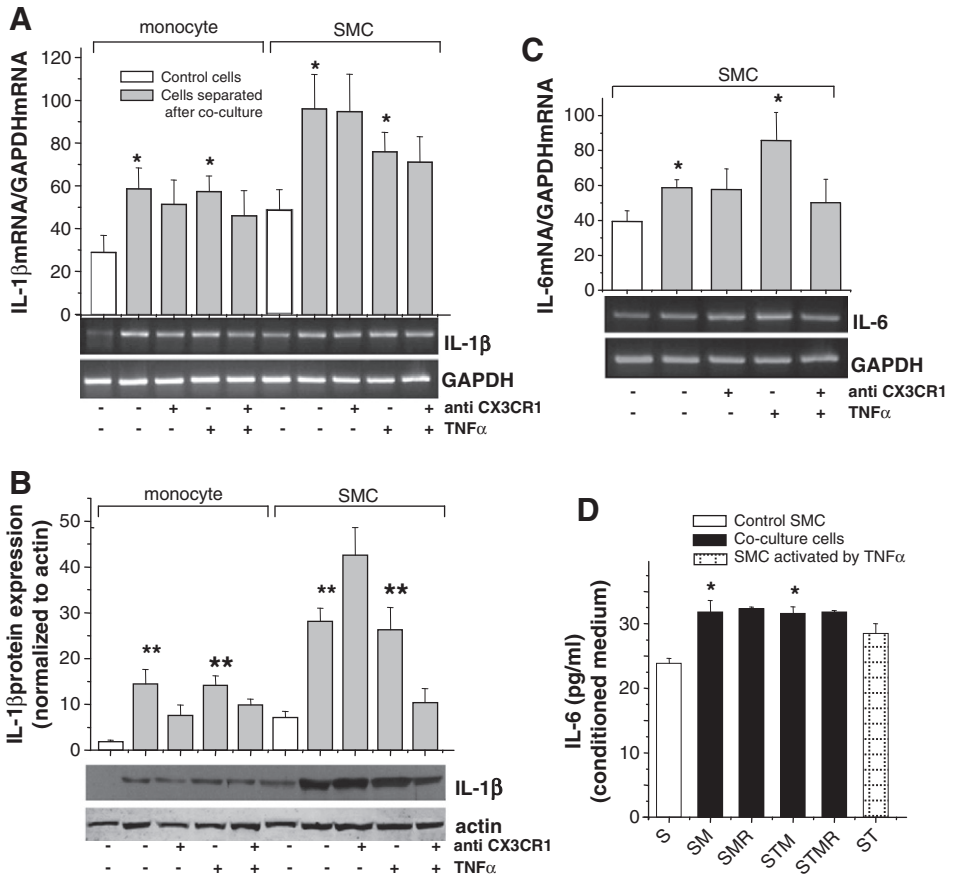

Fig. 2. Effect of monocyte–SMC interaction on IL-1β and IL-6 expression. A, B. IL-1β mRNA and protein expression in control cells (white columns) or separated cells after their co-culture (gray columns). Interactions of monocytes with SMC or with TNFα-activated SMC increase the IL-1β gene and protein expression in both cell types. Pre-incubation of monocytes with anti-CX3CR1 before cell co-culture does not affect the IL-1β gene or protein expression. n = 4, *p < 0.05 co-cultured vs. control (separately cultured) cells, **p < 0.01 co-cultured vs. control (separately cultured) cells. C. IL-6 mRNA expression in SMC. The cells isolated after interaction with monocytes exhibit an increased expression of IL-6 mRNA compared with control SMC (white columns). Pre-incubation of monocytes with anti-CX3CR1 does not affect the IL-6 mRNA induced by cell interaction. n = 4, *p < 0.05 interacted SMC vs. control SMC. D. IL-6 protein released in the conditioned medium (CM) of resting SMC (S) or interacted with monocytes in various conditions. Same abbreviations as in Fig. 1B. Note that the IL6 protein in the CM of resting monocyte is undetectable whereas in the CM of interacted cells (SM) is increased compared with the protein detected in CM of control SMC (S) n = 3, *p < 0.05 separately cultured (S) vs. interacted cells (SM, STM).

3.3. SMC–monocyte interaction increases the MMP-2 and MMP-9 expression; MMP-9 induction is a function of CX3CL–CX3CR1 pair

Cultured SMC expressed low level of MMP-2 mRNA and no MMP-9 mRNA, whereas monocytes expressed low levels of both MMPs (Fig. 3A and B). Upon SMC–monocyte interaction, both MMPs increased significantly in each cell type. Likewise, MMP-9 protein was not detectable in the conditioned media of monocytes or SMC (Fig. 3C, lanes S, M) but increased significantly in the conditioned media collected from co-cultured cells (Fig. 3C, lane SM). Interestingly, in TNFα-activated SMC (prior to interaction with monocytes) the MMP-9 gene and protein expression were considerably increased in both cell types (Fig. 3A) and in co-culture media (Fig. 3C, lane STM), respectively. Pre-stimulation with TNFα did not affect the MMP-2 gene expression (Fig. 3B). Blocking the CX3CR1 on monocytes reduced the MMP-9 gene expression in monocytes and SMC, and the protein released in the conditioned co-culture media, whereas no effect was detected in MMP-2 mRNA or protein expression. Incubation of monocytes with a nonspecific IgG or anti-CCR2, before cell co-culture, did not affect the MMP-9 expression induced by cell interaction (Supplementary Fig. 2B). Moreover, adding the anti-CX3CR1 on monocytes (without cell interaction) did not affect the MMP-9 expression (Supplementary Fig. 2D). These results highlight that the CX3CL1–CX3CR1 axis has a role in the production of MMP-9.

3.4. CX3CR1 mRNA and protein expression is increased upon SMC–monocyte interaction and is dependent on CX3CL1–CX3CR1 pair

Since CX3CL1 induces its own expression and increases monocyte–SMC adhesion [26] and CX3CR1 is expressed by both monocytes and SMC [15,27], we questioned whether CX3CL1–CX3CR1 binding also modulates the expression of CX3CR1. We observed that the interaction of SMC with monocytes induced a significant up-regulation of CX3CR1 gene and protein expression in each cell type (Fig. 4A and B) compared to their expression in controls, non-interacted cells. Stimulation of SMC with TNFα before cell interaction, led to a further increase in the gene and protein expression of CX3CR1 in SMC (Fig. 4A and B). Pre-treatment of monocytes with anti-CX3CR1, before cell co-culture reduced the CX3CR1 expression in both cell types, suggesting that the CX3CR1 modulation is dependent on CX3CL1–CX3CR1 binding. Pre-incubation of monocytes with a nonspecific immunoglobulin (IgG) or anti-CCR2, followed by interaction with SMC, did not affect the CX3CR1 expression (Supplementary Fig. 2C).

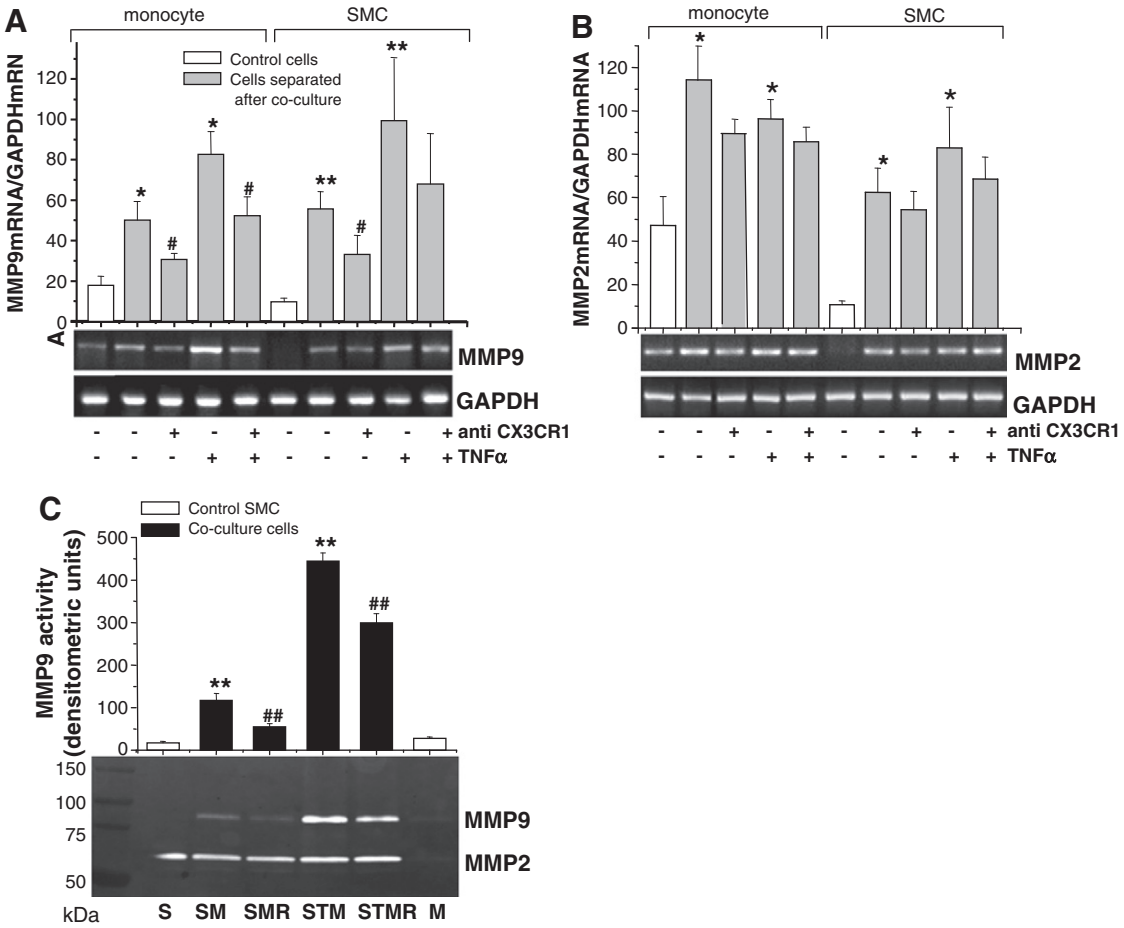

Fig. 3. Effect of monocyte–SMC interaction on MMP-9 and MMP-2 mRNA and enzymatic activity. A. Gene expression of MMP-9 in monocytes and SMC. Co-culture of SMC with monocytes increases the mRNA expression in both cell types (gray columns) compared with control cells (white columns). Activation of SMC with TNFα before co-culture with monocytes produced an additional increase in MMP-9 mRNA. Pre-incubation of monocytes with anti-CX3CR1 before interaction with SMC or TNFα-activated SMC decreases the MMP-9 mRNA induced by cell co-culture. n = 4. B. MMP-2 mRNA expression increases upon interaction of monocytes with SMC or TNFα-activated SMC. The CX3CR1 blocking on monocytes has no significant effect on MMP-2 mRNA. n = 4. C. Enzymatic activity of MMP-2 and MMP-9 (SDS-PAGE zymography) assessed in the conditioned media of cultured SMC (S), monocytes (M), SMC co-cultured with monocytes (SM) or with monocytes pre-incubated with anti-CX3CR1 (SMR); TNFα-stimulated SMC co-cultured with monocytes (STM) or with monocytes pre-incubated with anti-CX3CR1 (STMR). Note that MMP-9 (but not MMP-2) protein is significantly increased in the conditioned media collected from co-cultured cells (SM) and from co-culture of TNFα-activated SMC with monocytes (STM). Blocking the CX3CR1 on monocytes reduces the MMP-9 protein released in the co-culture conditioned medium, whereas no effect was detected in MMP-2 protein. n = 3, $^{*}p<0.05$; $^{**}p<0.01$ interacted cells vs. separately cultured cells; $^{\#}p<0.05$; $^{\#\#}p<0.01$ interacted cells in receptor blocking experiments vs. interacted cells.

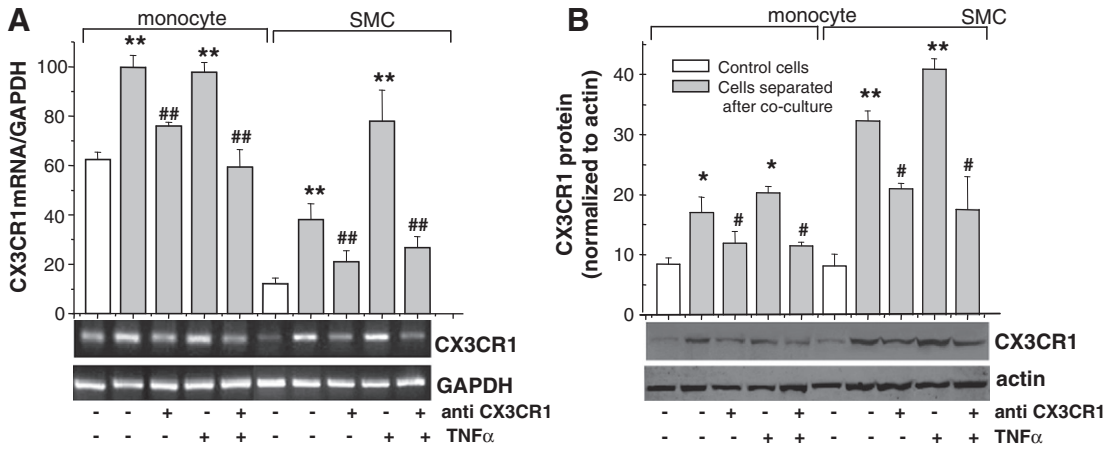

Fig. 4. The interaction between monocytes and SMC increases CX3CR1 expression in both cells. CX3CR1 mRNA (A) and protein (B) expression in isolated monocytes and SMC after their co-culture in various conditions (gray lanes) and in control monocytes and SMC (white lanes). Note that the co-culture of cells increases the CX3CR1 mRNA and protein expression in both cell types. Activation of SMC with TNFα before cell interaction produces a further increase of CX3CR1 expression in SMC. Pre-incubation of monocytes with anti-CX3CR1 decreases the CX3CR1 expression. n = 3, $^{*}p<0.05$; $^{**}p<0.01$ interacted cells vs. separately cultured cells; $^{\#}p<0.05$; $^{\#\#}p<0.01$ interacted cells in blocking experiments vs. interacted cells.

3.5. The effect of CX3CR1 silencing on TNFα, MMP-9 and CX3CR1 expression induced by interaction of SMC with LPS-activated monocytes

It is known that after the endothelial transmigration, the monocytes become activated, up-regulate a broad spectrum of inflammatory molecules and MMPs [28] and may interact with the intimal cells. In this context, we analyzed the interaction of activated monocytes with SMC. Monocytes were activated (6 h) by exposure to 100 ng/ml LPS (a concentration reported to activate human monocytes and induce TNFα release [29]) and then the cells were co-cultured with SMC for 18 h. To specifically block the CX3CR1 expression, in some experiments activated monocytes and SMC were transfected with CX3CR1 siRNA, or negative control siRNA and next co-cultured. The real time-PCR results showed that the interaction of SMC with LPS-activated monocytes increased the expression of TNFα, MMP-9 and CX3CR1 in both cell types (Fig. 5A, B, C), as compared to control cells (without co-culture). Interestingly co-culture of SMC with activated monocytes induced a higher expression of TNFα and MMP-9 versus co-culture of SMC with non-activated monocytes (Supplementary Fig. 3A, B), while the CX3CR1 expression was not modified (not shown).

CX3CR1 silencing significantly reduce the expression of TNFα, MMP-9 and CX3CR1 expression in each cell type (Fig. 5A, B, C), confirming the results obtained by pre-treatment of monocytes with anti-CX3CR1. Cell transfection with negative control siRNA had no effect on the expression of inflammatory molecules induced by co-culturing LPS-activated monocyte with SMC.

3.6. Role of NF-kB and AP-1 in the induction of inflammatory molecules upon SMC–monocyte interaction

We further explored the regulating factor(s) that may be activated in SMC upon their interaction with monocytes. First, we analyzed if SMC–monocyte interaction induces the activation of p38MAPK or ERK. Investigations of p38MAPK phosphorylation (using Western blot assay) showed that the SMC–monocyte interaction activated the p38MAPK in each cell type (Fig. 6A). No significant activation of ERK1/2 was detected (data not shown). Blocking of CX3CR1 on monocytes before interaction with SMC impaired activation of p38MAPK in SMC, suggesting that fractalkine–CX3CR1 axis activates the p38MAPK signaling pathway (Fig. 6A).

Next, we investigated the activation of AP-1 transcription factor induced by cell–cell interaction and found that the phospho c-jun was activated in both, SMC and monocytes (Fig. 6B). The c-jun activation in SMC was dependent on CX3CL1–CX3CR1 pair, since blocking of CX3CR1 on monocytes impaired its activation.

To test directly whether AP-1 or NF-kB is involved in the up-regulation of TNFα, IL-6, IL-1β, CX3CR1 and MMP expression in aortic SMC, the endogenous expression of NF-kB (p65) and AP-1 (c-jun) was blocked with target-specific siRNA prior to SMC–monocyte interaction. Transfection of SMC with either oligomer followed by interaction with monocytes, suppressed accumulation of target mRNA, p65 or c-jun. As a result, the protein expression of p65 was reduced by 68% and c-jun subunit was decreased by 45% in cells transfected with p65 siRNA and c-jun siRNA, respectively (Fig. 6C1 and C2). The expression of p65 and c-jun in SMC transfected with the negative control oligomers and interacted with monocytes was not changed. After verifying that the transfection experiments blocked the endogenous expression of NF-kB and AP-1, we tested the TNFα, IL-6 and IL-1β, CX3CR1 and MMP-9 expression in transfected cells. SMC transfection with negative control siRNA had no effect on inflammatory molecules, induced by monocyte–SMC interaction (Fig. 6D, E). In contrast, c-jun knockdown strongly decreased the TNFα mRNA (Fig. 6D1), MMP-9 mRNA expression (Fig. 6E1) and CX3CR1 mRNA (Fig. 6E2) indicating that AP-1 activation in SMC is involved in the induction of these molecules upon cell–cell interaction. As shown in Fig. 6D2, D3, silencing of p65 reduced the gene expression of IL-6 and IL-1β in SMC interacted with monocytes.

4. Discussion and conclusion

Recent data indicate that vascular SMC and monocytes–macrophages are not merely innocent coexisting neighbors in the plaque, but their interaction enhances monocyte procoagulant activity and production of atherosclerosis-related factors such as MMP-1, MCP-1 and IL-6 that aggravate the inflammatory process [13,30]. The adhesive interaction between monocytes and SMC is enhanced by growth factors through a process independent of VCAM-1 and ICAM-1 expressed by SMC [26]. Consequently, other adhesion molecules and chemokines may be instrumental in the adhesion process. Immunocytochemistry data demonstrated that within the human plaque, CX3CL1 on SMC co-localizes with macrophage CX3CR1 [15]. We predicted that the close interaction between monocytes and the vessels' resident SMC reflects a cross talk between these cells, which may have repercussion on the plaque evolution, i.e. increased production of cytokines or MMPs. Thus, we evaluated the induction of pro-atherogenic molecules in SMC and in monocytes, consequent to their direct contact, and the role of the CX3CL1–CX3CR1 axis in the process. Our results showed that the interaction between

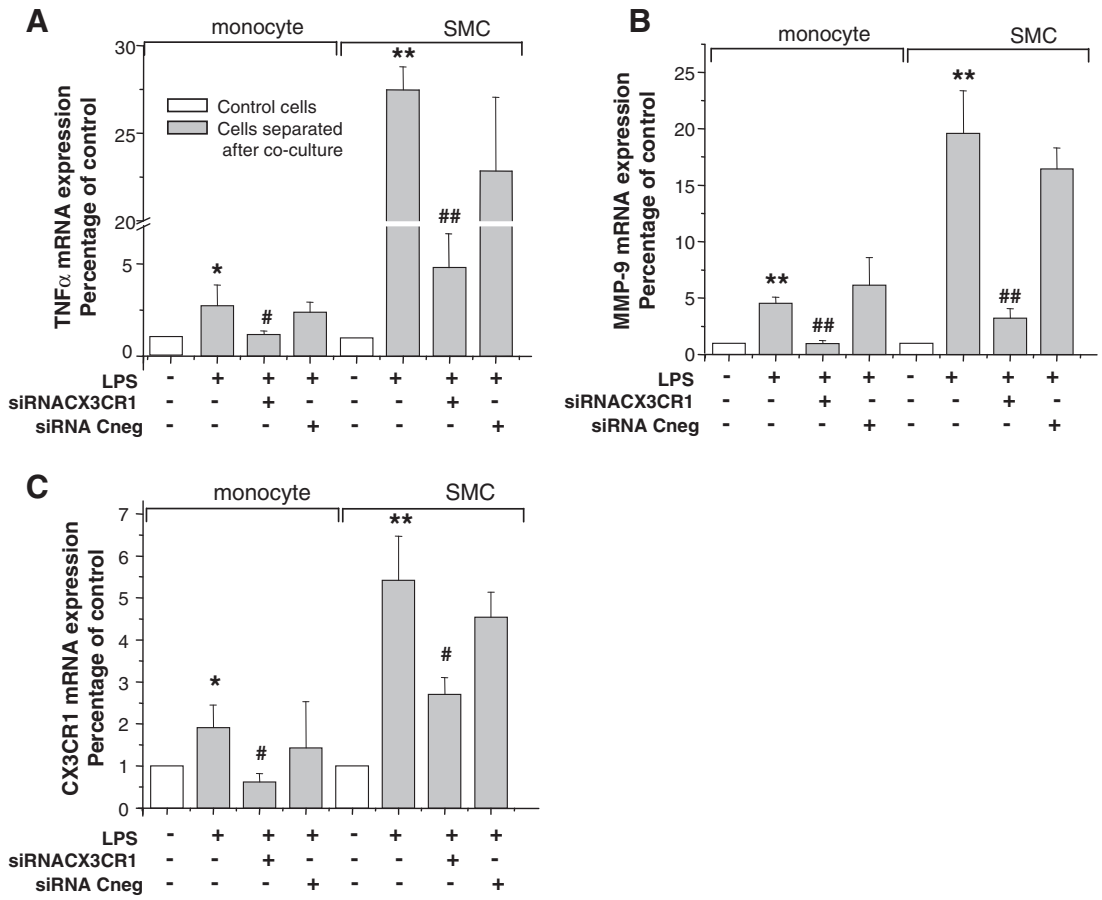

Fig. 5. Co-culture of LPS-activated monocyte with SMC increases the TNFα, MMP-9 and CX3CR1 expression in both cell types; effect of CX3CR1 silencing. Monocytes were activated with LPS (100 ng/ml) and co-cultured with SMC (18 h), then separated and the TNFα (A), MMP-9 (B) and CX3CR1 (C) mRNA were quantified by real time PCR. White columns: control monocytes and SMC; gray columns: monocytes or SMC isolated after their interaction. Note that the interaction of LPS-activated monocytes with SMC increases the TNFα, MMP-9 and CX3CR1 expression in both cells. Knockdown of CX3CR1 by siRNA transfection before cell interaction significantly decreases the TNFα, MMP-9 and CX3CR1 expression. Transfection of negative control siRNA does not affect the mRNA expression. n = 3; $^{*}p<0.05$; $^{**}p<0.01$ interacted cells vs. separately cultured cells; $^{\#}p<0.05$; $^{\#\#}p<0.01$ interacted cells in receptor blocking experiments vs. interacted cells.

monocytes and SMC generated in each cell type the up-regulation of TNFα, IL-6, IL-1β, CX3CR1, MMP-2 and MMP-9 gene and protein expression; CX3CL1–CX3CR1 axis triggers the expression of TNFα, MMP-9 and CX3CR1 whereas IL-6, IL-1β, and MMP-2 expression is independent of this ligand–receptor pair.

Cytokines, like TNFα and interleukins, are key molecules implicated in the chronic vascular inflammation associated to atherosclerosis [31]. Our data show a significant up-regulation of TNFα, IL-1β and IL-6 gene and protein expression in both monocytes and SMC, after their interaction. Incubation of monocytes with anti-CX3CR1 prior to the interaction with SMC impeded significantly the TNFα expression in both cell types, but not the IL-1β and IL-6 mRNA. Interestingly, the TNFα expression is further increased when SMC were interacted with LPS-activated monocytes. This additional increase can be due to the augmented release of soluble factors and/or a raise in CX3CR1 expression in activated monocytes. These assumptions are in line with previous reports that demonstrated that oxidized linoleic acid components (at 24 h) induce human monocytes to mature and undergo a chemokine receptor switch: CCR2 off, CX3CR1 on [27]. Silencing of CX3CR1 significantly reduced the TNFα expression in both cell types, indicating that the CX3CL1–CX3CR1 axis is involved in TNFα induction upon interaction between SMC and monocytes/activated monocytes.

Since blocking of CX3CL1–CX3CR1 binding did not affect the IL-6 and IL-1β expression, we presume that other adhesion molecule–receptor interaction (such as ICAM-1/LFA-1, VCAM-1/VLA-4) which is involved in monocyte adhesion to SMC [16,32], or soluble factors (such as TNFα) produced during co-culture may be implicated in their up-regulation upon cell interaction. Moreover, the non-adherent monocytes present in the conditioned media of co-culture may produce soluble factors that can influence the cross talk between monocytes and SMC. This assumption is supported by a report showing that the increased IL-6 and MCP-1 production in conditioned media in SMC–monocyte co-culture is mediated by soluble factors and IL-6 trans-signaling [30].

Basal level of MMP-9 expression in SMC is low, but TNFα induces its up-regulation via activation of NF-kB and AP-1 [33,34]. Our experiments revealed that the monocyte–SMC interaction by CX3CL1–CX3CR1 binding promotes the gene and protein expression of MMP-2 and MMP-9 in each cell type. Moreover, co-culture of SMC with activated monocytes, induced an additional increase in MMP-9 in both cell types. Blocking the monocyte CX3CR1 (by anti-CX3CR1 or CX3CR1 knockdown) before cell interaction, significantly reduces the MMP-9 mRNA in both, monocytes and SMC. In contrast, the MMP-2 mRNA and protein expression was not affected by CX3CR1 blocking on monocytes, suggesting that other factors produced during monocyte–SMC interaction are involved, as is the case of MMP-1 whose increase, was demonstrated to be dependent on soluble factors besides the direct cell–cell contact [13].

CX3CR1 and CX3CL1 are present in the atherosclerotic lesions [35] mediating the heterotypic anchorage between monocytes/macrophages and SMC [17]. Moreover, the increased expression of CX3CR1 on monocytes leads to their differentiation toward macrophages [27,36]. In this context, our data showing that SMC–monocyte interaction up-regulates the expression of CX3CR1, together with the

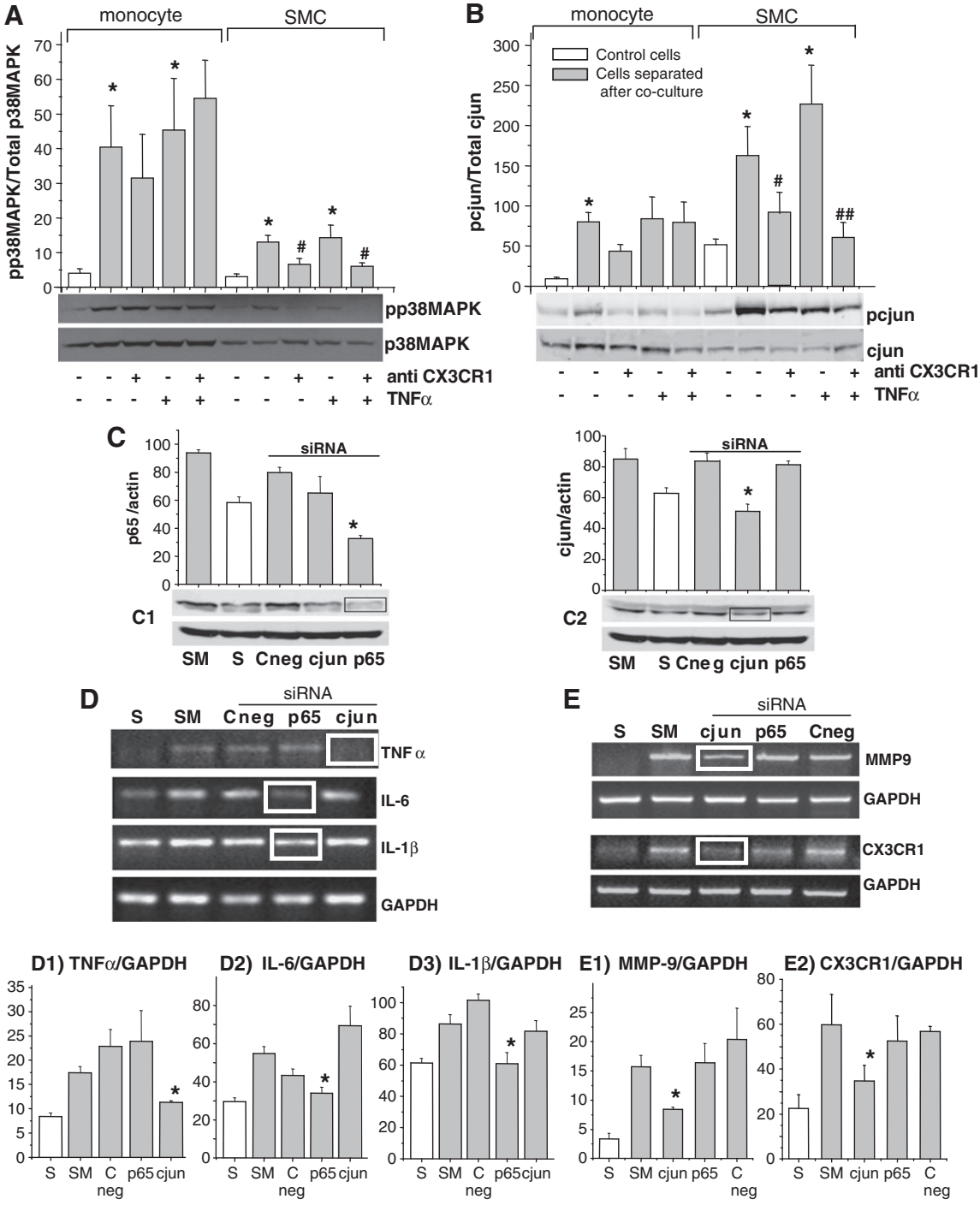

Fig. 6. Factors activated by monocyte–SMC interaction involved in the regulation of inflammatory molecules and MMP-9. A. Evaluation (Western blot) of phospho p38MAPK in monocytes and SMC before (white columns) and after monocyte–SMC co-culture (gray columns). The cell interaction activates p38MAPK in both, monocytes and SMC. Blocking of CX3CR1 on monocytes prior to the interaction with SMC does not affect the p38MAPK activation in monocytes but is significantly impaired in SMC; (n = 3). B. Phospho c-jun protein in monocytes and SMC before and after cell interaction. Compared to controls (white columns), monocyte–SMC interaction increases pc-jun in monocytes and SMC (gray columns). SMC-pre-stimulation with TNFα before cell interaction does not increase pc-jun in monocytes or SMC. Blocking CX3CR1 on monocytes before cell interaction decreases pc-jun expression in SMC, not in monocytes. n = 3, *p < 0.05; interacted cells vs. control cells. #p < 0.05; ##p < 0.01 interacted cells in blocking experiments vs. interacted cells. C. Evaluation of NF-kB and AP-1 knockdown in SMC transfected with scrambled, c-jun and p65 siRNA, then interacted with monocytes and isolated. Transfection of SMC with either oligomer followed by interaction with monocytes, suppresses accumulation of target mRNA, p65 (C1) or c-jun (C2). *p < 0.05; interacted cells vs. transfected and next interacted cells. D. E. Role of NF-kB and AP-1 in the regulation of TNFα, IL-6, IL-1β, MMP-9 and CX3CR1 gene expression. SMC were transfected, co-cultured with monocytes and then separated. The gene expression is shown in control SMC (S), SMC co-cultured with monocytes (SM) and transfected SMC co-cultured with monocytes (Cneg, p65, c-jun). Note that C-jun knockdown decreases the TNFα mRNA (D1), MMP-9 mRNA (E1) and CX3CR1 mRNA (E2). Silencing of p65 reduces the gene expression of IL-6 and IL-1β in SMC interacted with monocytes (D2, D3). n = 3, *p < 0.05.

results indicating a significant increase (~20 times) in CD36 expression in isolated monocytes after interaction with SMC (data not shown), suggest that the contact between these cells participates to the differentiation of monocytes toward the macrophage-phenotype.

TNFα increases the fractalkine expression in SMC [26]. Therefore, in some experiments, to favor the cell–cell contact via CX3CL1–CX3CR1 axis, before co-culture, SMC were stimulated with TNFα. We found that the interaction between TNFα-activated SMC and

monocytes generated an increased induction only for MMP-9, CX3CR1 and TNFα (at protein level) and not for IL-1β or IL-6. The results underline that TNFα, MMP-9 and CX3CR1 regulation is dependent on CX3CL1–CX3CR1 pair.

Since it was reported that soluble fractalkine is released from activated SMC [37], we questioned whether CX3CL1 shedding may take place during the interaction between monocytes and SMC. To uncover if soluble factors interfere in the induction of the molecules which demonstrated to be dependent on CX3CL1–CX3CR1 binding we analyzed the effect of conditioned medium (CM) isolated from SMC on monocyte and of CM from monocytes to SMC. The experiments showed that the CM from monocytes does not affect the TNFα, MMP-9 and CX3CR1 expression in SMC, but the CM from SMC increased the expression of TNFα and MMP-9 in monocytes (Supplementary Fig. 3C, D, F). These results indicate that soluble factors released by SMC, affect the TNFα and MMP-9 expression in monocytes.

AP-1 and NF-kB are inducible transcription factors critical for the expression of many genes involved in the inflammatory response [38]. Our experiments revealed that these transcription factors are implicated in the induction of cytokines and MMPs upon monocyte–SMC interaction. Silencing of p65 (NF-kB subunit) led to the down-regulation of IL-6 and IL-1β whereas c-jun (AP-1 subunit) silencing led to down-regulation of TNFα, CX3CR1, and MMP9. Interestingly, AP-1 is involved in the induction of molecules that are CX3CL1–CX3CR1 dependent, suggesting that this pair trigger the signaling pathway that leads to activation of AP-1 in SMC. This is also supported by the Western blot results showing that blocking CX3CR1 on monocytes prior to interaction with SMC reduced phospho c-jun activation.

It was shown that MMP-9 and CX3CR1 are regulated by activation of AP-1 and NF-kB transcription factors by a process that is dependent on phosphorylation of different MAPK [33,39,40]. We found that p38MAPK activation increased significantly in both, SMC and monocytes. Moreover, in SMC, the p38MAPK activation was dependent on CX3CL1–CX3CR1 binding, suggesting its role in the activation of p38MAPK pathway.

The MAPK cascade is a common signaling pathway by which G protein-coupled receptors initiate functional cellular responses [41], with a role in the regulation of TNFα, IL-6, and IL-1β [42]. The p38MAPK regulate the c-Jun transcription in response to stress, cytokine, and mitogenic stimuli [43] and contribute to NF-kB activation [44]. Hence, we presume that the activation of p38MAPK by monocyte–SMC interaction activates the NF-kB and AP-1 transcription factors that stimulate the subsequent production of cytokines and MMPs.

Evaluation of the oxidative stress in cells after their interactions showed that the intracellular reactive oxygen species was significantly increased in SMC and decreased in monocytes (Supplementary Fig. 4A). Moreover, we found that the monocyte–SMC interaction did not induce apoptosis of monocytes (Supplementary Fig. 4B). Blocking the CX3CR1 on monocytes before the interaction with SMC had no effect on monocyte apoptosis, indicating that the CX3CL1–CX3CR1 interaction is not involved in the apoptotic process. These results corroborate well with previous reports showing that upon interaction between SMC and monocytes, the former protect monocytes from apoptosis by a process dependent on VCAM-1 signaling [36].

Study limitation: In this study we have used a human aortic SMC line and the monocytic cell line U937. Further studies employing human monocytes and primary aortic SMC will be carried out to strengthen the significance of our novel observations.

In conclusion, the new findings reported here are: (1) the interaction between SMC and monocytes up-regulates TNFα, IL-1β, IL-6, CX3CR1, MMP-2 and MMP-9 expression in each cell type; (2) activation of monocyte before cell interaction induces a further increase in TNFα and MMP-9 expression; (3) the expression of TNFα, CX3CR1 and MMP-9 is dependent on CX3CL1/CX3CR1 axis; (4) IL-1β and IL6 up-regulation is independent of CX3CL1/CX3CR1 pair; (5) CX3CL1/CX3CR1 binding triggers the activation of AP-1 transcription factor. The novel data extend the reported role of fractalkine and its receptor and suggest that within the plaque, the cross talk between monocytes and SMC amplifies the inflammatory response via CX3CL1–CX3CR1 axis that function as inductor of critical molecules for atheroma progression. Therefore, the CX3CL1–CX3CR1 pair may constitute a novel therapeutic target to interrupt/retard the inflammatory process associated to atherogenesis.

Supplementary materials related to this article can be found online at doi:10.1016/j.bbamcr.2011.08.009.

Acknowledgements

We are thankful to Gabriela Mesca for skilful assistance. This work was supported by the Romanian Ministry of Education, grant (41037/2007) and the Romanian Academy. The financial support of European Social Fund-"Cristofor I. Simionescu" Postdoctoral Fellowship Programme (ID POSDRU/89/1.5/S/55216), Sectorial Operational Programme Human Resources Development 2007–2013 to MC is gratefully acknowledged.

References

[1] P. Libby, Inflammation in atherosclerosis, Nature 420 (2002) 868–874.
[2] G.K. Hansson, P. Libby, U. Schonbeck, Z.Q. Yan, Innate and adaptive immunity in the pathogenesis of atherosclerosis, Circ. Res. 91 (2002) 281–291.
[3] C. Weber, A. Schober, A. Zernecke, Chemokines: key regulators of mononuclear cell recruitment in atherosclerotic vascular disease, Arterioscler. Thromb. Vasc. Biol. 24 (2004) 1997–2008.
[4] U. Muller-Werdan, H. Schumann, H. Loppnow, R. Fuchs, D. Darmer, J. Stadler, J. Holtz, K. Werdan, Endotoxin and tumor necrosis factor alpha exert a similar proinflammatory effect in neonatal rat cardiomyocytes, but have different cardiodepressant profiles, J. Mol. Cell. Cardiol. 30 (1998) 1027–1036.
[5] P. Libby, P.M. Ridker, A. Maseri, Inflammation and atherosclerosis, Circulation 105 (2002) 1135–1143.
[6] H. Ohta, H. Wada, T. Niwa, H. Kirii, N. Iwamoto, H. Fujii, K. Saito, K. Sekikawa, M. Seishima, Disruption of tumor necrosis factor-alpha gene diminishes the development of atherosclerosis in ApoE-deficient mice, Atherosclerosis 180 (2005) 11–17.
[7] R. Elhage, A. Maret, M.T. Pieraggi, J.C. Thiers, J.F. Arnal, F. Bayard, Differential effects of interleukin-1 receptor antagonist and tumor necrosis factor binding protein on fatty-streak formation in apolipoprotein E-deficient mice, Circulation 97 (1998) 242–244.
[8] R. Kleemann, S. Zadelaar, T. Kooistra, Cytokines and atherosclerosis: a comprehensive review of studies in mice, Cardiovasc. Res. 79 (2008) 360–376.
[9] S.A. Huber, P. Sakkinen, D. Conze, N. Hardin, R. Tracy, Interleukin-6 exacerbates early atherosclerosis in mice, Arterioscler. Thromb. Vasc. Biol. 19 (1999) 2364–2367.
[10] Z.S. Galis, G.K. Sukhova, R. Kranzhofer, S. Clark, P. Libby, Macrophage foam cells from experimental atheroma constitutively produce matrix-degrading proteinases, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 402–406.
[11] K. Morishige, H. Shimokawa, Y. Matsumoto, Y. Eto, T. Uwatoku, K. Abe, K. Sueishi, A. Takeshita, Overexpression of matrix metalloproteinase-9 promotes intravascular thrombus formation in porcine coronary arteries in vivo, Cardiovasc. Res. 57 (2003) 572–585.
[12] A.C. Doran, N. Meller, C.A. McNamara, Role of smooth muscle cells in the initiation and early progression of atherosclerosis, Arterioscler. Thromb. Vasc. Biol. 28 (2008) 812–819.
[13] Y. Zhu, Y. Hojo, U. Ikeda, M. Takahashi, K. Shimada, Interaction between monocytes and vascular smooth muscle cells enhances matrix metalloproteinase-1 production, J. Cardiovasc. Pharmacol. 36 (2000) 152–161.
[14] A.M. Fong, L.A. Robinson, D.A. Steeber, T.F. Tedder, O. Yoshie, T. Imai, D.D. Patel, Fractalkine and CX3CR1 mediate a novel mechanism of leukocyte capture, firm adhesion, and activation under physiologic flow, J. Exp. Med. 188 (1998) 1413–1419.
[15] A.D. Lucas, C. Bursill, T.J. Guzik, J. Sadowski, K.M. Channon, D.R. Greaves, Smooth muscle cells in human atherosclerotic plaques express the fractalkine receptor CX3CR1 and undergo chemotaxis to the CX3C chemokine fractalkine (CX3CL1), Circulation 108 (2003) 2498–2504.
[16] Q. Cai, L. Lanting, R. Natarajan, Growth factors induce monocyte binding to vascular smooth muscle cells: implications for monocyte retention in atherosclerosis, Am. J. Physiol. Cell Physiol. 287 (2004) C707–C714.
[17] J. Barlic, Y. Zhang, P.M. Murphy, Atherogenic lipids induce adhesion of human coronary artery smooth muscle cells to macrophages by up-regulating chemokine CX3CL1 on smooth muscle cells in a TNFalpha–NFkappaB-dependent manner, J. Biol. Chem. 282 (2007) 19167–19176.
[18] P. Lesnik, C.A. Haskell, I.F. Charo, Decreased atherosclerosis in CX3CR1−/− mice reveals a role for fractalkine in atherogenesis, J. Clin. Invest. 111 (2003) 333–340.
[19] D.H. McDermott, A.M. Fong, Q. Yang, J.M. Sechler, L.A. Cupples, M.N. Merrell, P.W. Wilson, R.B. D'Agostino, C.J. O'Donnell, D.D. Patel, P.M. Murphy, Chemokine receptor mutant CX3CR1-M280 has impaired adhesive function and correlates with protection from cardiovascular disease in humans, J. Clin. Invest. 111 (2003) 1241–1250.

[20] E. Dragomir, I. Manduteanu, M. Calin, A.M. Gan, D. Stan, R.R. Koenen, C. Weber, M. Simionescu, High glucose conditions induce upregulation of fractalkine and monocyte chemotactic protein-1 in human smooth muscle cells, Thromb. Haemost. 100 (2008) 1155–1165.
[21] W.M. Association, Declaration of Helsinki. Recommendations guiding physicians in biomedical research involving human subjects, Cardiovasc. Res. 35 (1997) 2–3.
[22] D. Tirziu, V.V. Jinga, G. Serban, M. Simionescu, The effects of low density lipoproteins modified by incubation with chondroitin 6-sulfate on human aortic smooth muscle cells, Atherosclerosis 147 (1999) 155–166.
[23] C.E., D. Popov, P.R. Shepherd, High glucose-induced alterations in human aortic smooth muscle cells: phenotypic changes and signal transduction mediators, Proc. Rom. Acad. B 6 (2004) 47–51.
[24] M. Raicu, S. Florea, Deleterious effects of nifedipine on smooth muscle cells implies alterations of intracellular calcium signaling, Fundam. Clin. Pharmacol. 15 (2001) 387–392.
[25] M.W. Pfaffl, A new mathematical model for relative quantification in real-time RT-PCR, Nucleic Acids Res. 29 (2001) e45.
[26] B. Chandrasekar, S. Mummidi, R.P. Perla, S. Bysani, N.O. Dulin, F. Liu, P.C. Melby, Fractalkine (CX3CL1) stimulated by nuclear factor kappaB (NF-kappaB)-dependent inflammatory signals induces aortic smooth muscle cell proliferation through an autocrine pathway, Biochem. J. 373 (2003) 547–558.
[27] J. Barlic, Y. Zhang, J.F. Foley, P.M. Murphy, Oxidized lipid-driven chemokine receptor switch, CCR2 to CX3CR1, mediates adhesion of human macrophages to coronary artery smooth muscle cells through a peroxisome proliferator-activated receptor gamma-dependent pathway, Circulation 114 (2006) 807–819.
[28] A.C. Newby, Metalloproteinase expression in monocytes and macrophages and its relationship to atherosclerotic plaque instability, Arterioscler. Thromb. Vasc. Biol. 28 (2008) 2108–2114.
[29] B.D. Shames, C.H. Selzman, E.J. Pulido, X. Meng, D.R. Meldrum, R.C. McIntyre Jr., A.H. Harken, A. Banerjee, LPS-induced NF-kappaB activation and TNF-alpha release in human monocytes are protein tyrosine kinase dependent and protein kinase C independent, J. Surg. Res. 83 (1999) 69–74.
[30] L. Chen, A. Frister, S. Wang, A. Ludwig, H. Behr, S. Pippig, B. Li, A. Simm, B. Hofmann, C. Pilowski, S. Koch, M. Buerke, S. Rose-John, K. Werdan, H. Loppnow, Interaction of vascular smooth muscle cells and monocytes by soluble factors synergistically enhances IL-6 and MCP-1 production, Am. J. Physiol. Heart Circ. Physiol. 296 (2009) H987–H996.
[31] H. Mangge, H. Hubmann, S. Pilz, K. Schauenstein, W. Renner, W. Marz, Beyond cholesterol—inflammatory cytokines, the key mediators in atherosclerosis, Clin. Chem. Lab. Med. 42 (2004) 467–474.
[32] Z. Li, T. Song, G.Z. Liu, L.Y. Liu, Inhibitory effects of cariporide on LPC-induced expression of ICAM-1 and adhesion of monocytes to smooth muscle cells in vitro, Acta Pharmacol. Sin. 27 (2006) 1326–1332.
[33] A.C. Newby, Matrix metalloproteinases regulate migration, proliferation, and death of vascular smooth muscle cells by degrading matrix and non-matrix substrates, Cardiovasc. Res. 69 (2006) 614–624.
[34] A. Cho, J. Graves, M.A. Reidy, Mitogen-activated protein kinases mediate matrix metalloproteinase-9 expression in vascular smooth muscle cells, Arterioscler. Thromb. Vasc. Biol. 20 (2000) 2527–2532.
[35] D.R. Greaves, T. Hakkinen, A.D. Lucas, K. Liddiard, E. Jones, C.M. Quinn, J. Senaratne, F.R. Green, K. Tyson, J. Boyle, C. Shanahan, P.L. Weissberg, S. Gordon, S. Yla-Hertualla, Linked chromosome 16q13 chemokines, macrophage-derived chemokine, fractalkine, and thymus- and activation-regulated chemokine, are expressed in human atherosclerotic lesions, Arterioscler. Thromb. Vasc. Biol. 21 (2001) 923–929.
[36] Q. Cai, L. Lanting, R. Natarajan, Interaction of monocytes with vascular smooth muscle cells regulates monocyte survival and differentiation through distinct pathways, Arterioscler. Thromb. Vasc. Biol. 24 (2004) 2263–2270.
[37] A. Ludwig, T. Berkhout, K. Moores, P. Groot, G. Chapman, Fractalkine is expressed by smooth muscle cells in response to IFN-gamma and TNF-alpha and is modulated by metalloproteinase activity, J. Immunol. 168 (2002) 604–612.
[38] M.P. de Winther, E. Kanters, G. Kraal, M.H. Hofker, Nuclear factor kappaB signaling in atherogenesis, Arterioscler. Thromb. Vasc. Biol. 25 (2005) 904–914.
[39] S.R. Green, K.H. Han, Y. Chen, F. Almazan, I.F. Charo, Y.I. Miller, O. Quehenberger, The CC chemokine MCP-1 stimulates surface expression of CX3CR1 and enhances the adhesion of monocytes to fractalkine/CX3CL1 via p38 MAPK, J. Immunol. 176 (2006) 7412–7420.
[40] Y. Saitoh, K. Koizumi, H. Sakurai, T. Minami, I. Saiki, RANKL-induced down-regulation of CX3CR1 via PI3K/Akt signaling pathway suppresses Fractalkine/CX3CL1-induced cellular responses in RAW264.7 cells, Biochem. Biophys. Res. Commun. 364 (2007) 417–422.
[41] B. Cambien, M. Pomeranz, M.A. Millet, B. Rossi, A. Schmid-Alliana, Signal transduction involved in MCP-1-mediated monocytic transendothelial migration, Blood 97 (2001) 359–366.
[42] W. Zhu, J.S. Downey, J. Gu, F. Di Padova, H. Gram, J. Han, Regulation of TNF expression by multiple mitogen-activated protein kinase pathways, J. Immunol. 164 (2000) 6349–6358.
[43] A.J. Whitmarsh, R.J. Davis, Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways, J. Mol. Med. 74 (1996) 589–607.
[44] N. Sizemore, S. Leung, G.R. Stark, Activation of phosphatidylinositol 3-kinase in response to interleukin-1 leads to phosphorylation and activation of the NF-kappaB p65/RelA subunit, Mol. Cell. Biol. 19 (1999) 4798–4805.