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# Heparan sulfate potentiates leukocyte adhesion on cardiac fibroblast by enhancing Vcam-1 and Icam-1 expression

Francisco Olivares-Silva[a], Rodolfo Landaeta[a], Pablo Aránguiz[a], Samir Bolivar[a,b], Claudio Humeres[a], Renatto Anfossi[a], Raúl Vivar[a], Pía Boza[a], Claudia Muñoz[a], Viviana Pardo-Jiménez[a], Concepción Peiró[c,d], Carlos F. Sánchez-Ferrer[c,d], Guillermo Díaz-Araya[a,e,*]

[a] Advanced Center for Chronic Diseases (ACCDiS), Faculty of Chemical & Pharmaceutical Sciences & Faculty of Medicine, University of Chile, Santiago, Chile
[b] Faculty of Chemistry and Pharmacy, Atlantic University, Barranquilla, Colombia
[c] Department of Pharmacology, Faculty of Medicine, Universidad Autónoma de Madrid, Madrid, Spain
[d] Instituto de Investigación Sanitaria Hospital Universitario La Paz (IdiPAZ), Madrid, Spain
[e] Department of Pharmacological and Toxicological Chemistry, Faculty of Chemical and Pharmaceutical Sciences, University of Chile, Santiago, Chile

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ABSTRACT

Cardiac fibroblasts (CF) act as sentinel cells responding to chemokines, cytokines and growth factors released in cardiac tissue in cardiac injury events, such as myocardial infarction (MI). Cardiac injury involves the release of various damage-associated molecular patterns (DAMPs) including heparan sulfate (HS), a constituent of the extracellular matrix (ECM), through the TLR4 receptor activation triggering a strong inflammatory response, inducing leukocytes recruitment. This latter cells are responsible of clearing cell debris and releasing cytokines that promote CF differentiation to myofibroblast (CMF), thus initiating scar formation.

CF were isolated from adult male rats and subsequently stimulated with HS or LPS, in the presence or absence of chemical inhibitors, to evaluate signaling pathways involved in ICAM-1 and VCAM-1 expression. siRNA against ICAM-1 and VCAM-1 were used to evaluate participation of these adhesion molecules on leukocytes recruitment.

HS through TLR4, PI3K/AKT and NF-KB increased ICAM-1 and VCAM-1 expression, which favored the adhesion of spleen mononuclear cells (SMC) and bone marrow granulocytes (PMN) to CF. These effects were prevented by siRNA against ICAM-1 and VCAM-1. Co-culture of CF with SMC increased α-SMA expression, skewing CF towards a pro-fibrotic phenotype, while CF pretreatment with HS partially reverted this effect.

*Conclusion:* These data show the dual role of HS during the initial stages of wound healing. Initially, HS enhance the pro-inflammatory role of CF increasing cytokines secretion; and later, by increasing protein adhesion molecules allows the adhesion of SMC on CF, which trigger CF-to-CMF differentiation.

## 1. Introduction

Cardiac fibroblasts (CF) maintain extracellular matrix (ECM) homeostasis in cardiac tissue, and respond to chemical signals from cellular and non-cellular origin, acting as sentinel cells of cardiac tissue [1]. CF respond to molecular patterns associated with pathogens (PAMPs) and molecular patterns associated with damage (DAMPs), secreting a wide variety of pro-inflammatory cytokines and chemokines [2]. This alters the function of resident cardiac cells by promoting the expression of adhesion molecules, among other mechanisms [3], but also the immune cells infiltration at the inflammatory focus subsequent to tissue damage. In this context, we have previously shown that upon stimulation with LPS or TGF-β CF secrete cytokines that can differentiate monocytes to a M1 or M2 phenotype, respectively [4] and finally promote the degradation of the ECM by metalloproteinases (MMPs) [5].

Toll-like receptors (TLRs) are a family of type I transmembrane receptors expressed in innate immune system cells, with first-line functions in defense against pathogens [6]. Within this family, TLR4 is able to respond to PAMPs, including bacterial lipopolysaccharide (LPS), bacterial flagellins and single-and double-stranded RNA. In addition, they respond to DAMPs such as heat shock proteins (HSP), high-

* Corresponding author at: Department of Chemical Pharmacology and Toxicology and Advanced Center for Chronic Diseases (ACCDiS), Faculty of Chemical & Pharmaceutical Sciences, University of Chile, 8380492 Santiago, Chile.
*E-mail address:* gadiaz@ciq.uchile.cl (G. Díaz-Araya).

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mobility group box-1 (HMGB1) and heparan sulfate (HS) [7]. These interactions trigger the expression of pro-inflammatory cytokines and the maturation of antigen-presenting innate immune system cells [8]. After ligand binding to TLR4, the MAPKs and transduction pathways are activated, initiating inflammatory cytokines expression [9]. To date, studies on the effects of TLR4 activation on CF are scarce. LPS, an exogenous ligand, has been shown to activate TLR4; however, studies with endogenous ligands released in a sterile inflammatory process, such as HS, have not been performed.

Heparan sulfate proteoglycans (HSPG) correspond to proteins with HS chains covalently attached to their structure [10]. Due to their location in the extracellular matrix, HSPG are directly involved in inflammatory processes. They have been reported to participate in cell adhesion through chemokine receptors activation, growth factor reservoir in the ECM by protein anchorage, leukocyte migration regulating chemokine gradients, and the modulation of angiogenic activity and cellular signaling [11]. In addition, the degrading activity of heparanases and MMPs, together with reactive oxygen species (ROS) [12] and leukocytes [13], have been shown to trigger HS cleavage, release and subsequently TLR4 activation [14], with consequences on the inflammatory process such as leukocyte activation [15] and cytokine secretion [7,16].

Cell adhesion molecules (CAMs) are surface proteins involved in modulating communication between a wide varieties of cell types. ICAM-1 and VCAM-1 are two members of the immunoglobulin superfamily (IgSF) that have critical implications in the recruitment and infiltration of inflammatory cells at the site of injury. VCAM-1 binds circulating lymphocytes and monocytes expressing α4β4 and α4β7 integrins, whereas ICAM-1 is the countereceptor for β2 integrins, such as LFA-1 and Mac-1 [17]. The interaction between ICAM-1 and integrins plays an important role in leukocyte recruitment and the onset of antigen-triggered immune responses. An increase in the expression of CAMs is temporally associated with the sequestering and infiltration of leukocytes in the myocardial tissue. In cardiac inflammation, resident cells and infiltrated leukocytes secrete cytokines capable of activating transcription of CAMs, thus increasing basal levels and perpetuating recruitment and transmigration [18]. In addition, it has been shown that DAMPs released in the area of cardiac damage increase the levels of CAMs and act similarly to cytokines [19]. When damage occurs in cardiac tissue, neutrophils are the first cells attracted to the site of injury, followed by monocytes and lymphocytes [20]. Neutrophils act as the first line of defense and they initiate an acute inflammatory response in order to phagocyte dead cells and facilitate tissue repair by releasing large amounts of ROS. They also secrete pro-inflammatory cytokines, MMPs and cathepsins capable of degrading ECM [21], and they activate other immune system cells by amplifying leukocyte recruitment [22].

Despite the above exposed observations, no studies have linked to date HS and CAMs in the context of CF. The aim of the present study was thus to explore how HS may functionally modulate CAMs expression and leukocyte adhesion by CF, and how this may ultimately impact on the phenotypic modulation of CF.

## 2. Materials and methods

### 2.1. Reagents

Fetal bovine serum (FBS), trypsin/EDTA, molecular weight standard and organic and inorganic compounds were purchased from Merck (Darmstadt, Germany). Reagents for chemiluminescence (ECL) were purchased from PerkinElmer Life Sciences (Boston, MA). Sterile plastic material was obtained from Corning Inc. (New York, NY). Primary anti-ICAM-1 antibody was purchased from R & D Systems (MN, USA). Anti-VCAM-1 primary antibody was purchased from Abcam (Cambridge, MA). Primary anti-GAPDH antibody and horseradish peroxidase-conjugated secondary antibodies (HRP) were obtained from Santa Cruz Biotechnology (CA, USA). Heparan bovine kidney sulfate, primary anti-α-SMA antibody, IMD-0354 and Ficoll-Histopaque® 1077 and 1119 were purchased from Sigma-Aldrich (St. Louis, MO). FluoroNunc™ microplates, Alexa Fluor® 555 secondary antibody and Opti-MEM® culture medium were purchased from Thermo Fisher Scientific (Waltham, MA). Lipofectamine® 2000 was obtained from Invitrogen (Carlsbad, CA). LPS-EB Ultrapure (*E. coli* O111: B4), TAK-242 and SB-431542 were purchased from InvivoGen (San Diego, CA). PD98059 was obtained from Gibco BRL (Carlsbad, CA). Ly294002 was purchased from Cayman Chemicals (Ann Arbor, MI). Dako was acquired from Agilent Technologies (Glostrub, Denmark).

### 2.2. Animals

Adult male Sprague-Dawley rats were obtained from the animal breeding facility of the Faculty of Chemical and Pharmaceutical Sciences of the University of Chile. The animals were kept in cages (with light/dark cycles of 12 h) and free access to food and water. All studies were developed in compliance with the NIH Guide for the Care and Use of Laboratory Animals, updated in 2011 (http://grants.nih.gov/grants/olaw/Guide-for-the-Care-and-Use-of-Laboratory-Animals), and experimental protocols were approved by the Faculty Ethics Committee.

### 2.3. Cardiac fibroblasts isolation

CF were isolated from male Sprague-Dawley rats (6–8 weeks old) using enzymatic digestion, as previously described [9]. Briefly, the rats were anesthetized with a 2:1 intraperitoneal injection of ketamine/xylazine and hearts were extracted in an aseptic environment. The atria were removed and the ventricles were cut into small pieces (1–2 mm), incubated with type II collagenase and then centrifuged at 1000 RPM for 10 min. The resulting pellet was resuspended in 10 ml of DMEM/F12 supplemented with 10% FBS and antibiotics (100 μg/ml streptomycin, 100 IU/ml penicillin, and 0.25 μg/ml amphotericin B) and cultured in a 5% $CO_2$ atmosphere at 37 °C until confluence (5 days). The purity of the CF population was checked by positive staining with several markers. Staining was positive for vimentin (Santa Cruz Biotechnology, CA), and negative for sarcomeric actin and desmin (Sigma-Aldrich, MO).

### 2.4. Isolation of SMC and PMN

To obtain SMC (spleen mononuclear cells, mainly lymphocytes and monocytes), rat spleens were dissected from the abdominal cavity [4] and filtered through a 50 μm cell strainer until obtaining a homogeneous suspension. To deplete granulocytes and erythrocytes, the suspension was layered on 7 ml Ficoll-Histopaque® (1.077 g/ml) and centrifuged at room temperature for 30 min and 400G. The SMC rich interface was received and washed twice with 1 × PBS, pH 7.4. To lyse the remaining erythrocytes, it was resuspended in pellet in 0.85 M $NH_4Cl$ for 5 min to be washed again with PBS. Finally, the purified SMCs were centrifuged and resuspended in PBS + 3% FBS for further experiments. A similar methodology was used to isolate PMN (polymorphonuclear cells, mainly neutrophils) with some modifications [23]. Rat tibia and femur were removed to wash the medullary cavity with a 25 G syringe and filter the bone marrow extract through a sieve. The suspension was placed in 7 ml of Ficoll-Histopaque 1.119 g/ml and 1.077 g/ml (1:1) and centrifuged at room temperature for 30 min and 300 G. The interface between both Ficoll-Histopaque® was received and washed twice with PBS. Finally, the pellet was resuspended in PBS + 3% FBS and reserved for use.

### 2.5. Western blot (WB)

Equivalent amounts of protein were separated by SDS-PAGE.

Proteins were transferred to nitrocellulose membranes and blocked with fat-free milk (5% w/v). The membranes were incubated with the corresponding antibodies (ICAM-1 1:1000, VCAM-1 1:5000, α-SMA 1:1000, pro-IL-1β 1:1000, GAPDH 1:1000) overnight at 4 °C. The secondary antibody (1:1000) was incubated for 2 h at room temperature. ECL was used for immunodetection. Protein levels were determined by densitometry using ImageJ (Bethesda, MD) and normalized to the corresponding level of GAPDH.

### 2.6. Transfection with siRNA against ICAM-1 and VCAM-1

CF were seeded in 96-well plates to 70% confluence. After 24 h, mixtures of Lipofectamine® 2000 and specific siRNA adjusted to 100 nM were prepared in Opti-MEM®. These mixtures were diluted 5 × to 1 × (20 nM) prior to co-incubation with CF for 16 h at 37 °C. After the time, the medium was changed to DMEM/F12 for another 24 h. Scramble siRNA (MISSION®, Sigma-Aldrich) was used as the control. The sequences used were: ICAM-1: 5'-GCCUCAGCACGUACCUCUAdTdT-3 '(sense); 5'-UAGAGGUACGUGCUGAGGCdTdT-3 '(antisense). VCAM-1: 5'-AAUGCAACUCUCACCUUAAdTdT-3 '(sense); 5'-UUAAGGUGAGAGUUGCAUUdTdT-3 '(antisense); Scramble siRNA: 5'-UUCUCCGAACGUGUCUCGUdTdT-3 '(sense); 5'-ACGUGACACGUUCGGAGAAdTdT-3 '(antisense).

### 2.7. In vitro adhesion assay

In vitro cell adhesion assays were performed using a commercial kit (Vybrant Cell Adhesion kit; Molecular Probes, Eugene, OR) according to the manufacturer's instructions. SMC or PMN ($5 \times 10^6$) were resuspended in 1 ml of DMEM/F12 and labeled with calcein AM (5 μM) for 30 min at 37 °C. The labeled SMCs or PMNs ($5 \times 10^6$/ml, 200 μl/well) were added to FluoroNunc™ microplates with a monolayer of $2 \times 10^4$ CF/well previously treated with HS or LPS for 24 h. The conditioned culture medium was completely removed before the leukocytes were added. Total fluorescence of the labeled cells was determined using a fluorescence spectrometer (excitation 470 nm, emission 517 nm) equipped with a microplate reader. After co-incubating for 2 h at 37 °C, the unbound cells were removed by washing four times with DMEM/F12. Finally, 200 μl of PBS was added to each well and the fluorescence was measured concomitantly.

### 2.8. Immunocytochemistry

CF were seeded on coverslips and DMEM/F12 in presence/absence of HS for 24 h. Subsequently, they were co-incubated with SMC for 72 h. After this time, they were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.1% Triton X-100 and blocked with 3% BSA for 30 min. They were then incubated with primary antibody against α-SMA overnight at 4 °C and with Alexa Fluor® 555 secondary antibody for 2 h at room temperature. The nucleus was labeled with Hoechst (blue). Finally, the coverslips were mounted on slides using Dako and the images were obtained by confocal microscopy.

### 2.9. Violet crystal staining

CF were seeded in DMEM-F12 and presence/absence of LPS and HS for 24 h. They were then co-incubated with SMC and PMN for 2 h, washed with PBS and incubated with violet crystal (5 mg/ml, 10% MeOH) for 20 min. Finally, CF were extensively washed and dried at room temperature. The images were obtained by light microscopy.

### 2.10. TNF-α and TGF-β1 analysis

CF were seeded in DMEM-F12 and presence/absence of HS or LPS for 24 h. They were then co-incubated with/without SMC for 24 h, and the culture medium was collected, centrifuged (5000 rpm for 10 min, then TNF-α and TGF-β levels were quantified by ELISA following manufacturer protocol (Quantikine Rat TNF-alpha Immunoassay, R&D, MN, USA; Rat TGF-beta 1 ELISA Kit ab119558, Eugene, USA).

### 2.11. Statistic analysis

All data are presented as the mean ± SD of at least three independent experiments. The differences between parameters were evaluated by two-way ANOVA for each variable. Tukey's test was used to compare the effects of different conditions on the parameters. The level of significance was set at $p < 0.05$. GraphPad Prism 6.0 software (La Jolla, CA) was used for statistical analyzes.

## 3. Results

### 3.1. HS induces ICAM-1, VCAM-1, TNF-α and pro-IL-1β expression levels in CF through TLR4 activation

ICAM-1 and VCAM-1 proteins play a key role in the adhesion and activation of leukocytes in inflammatory processes, whereas TNF-α and pro-IL-1β (the immature form of IL-1β) are strong pro-inflammatory cytokines. Thus, the expression of both CAMs, TNF-α and pro-IL-1β were studied using an endogenous pro-inflammatory stimulus such as HS, whereas LPS was used as an exogenous stimulus. As shown in Fig. 1A and C, both HS (10 μg/ml) and LPS (1 μg/ml) for 24 h, increased ICAM-1 and VCAM-1 expression levels after 24 h, with this effect being more pronounced for the LPS stimulus. To evaluate TLR4 participation on ICAM-1 and VCAM-1 expression induced by HS, CF were pre-treated with TAK-242 (4 μM, TLR4 inhibitor) for 1 h and then stimulated with HS (10 μg/ml) for 24 h. Fig. 1B and D show that the increase in ICAM-1 and VCAM-1 induced by HS was completely prevented by the pre-treatment with TAK-242, while TAK-242 by itself did not modify adhesion proteins levels. As shown in Fig. 1E, both HS (10 μg/ml) and LPS (1 μg/ml) for 8 h, increased pro-IL-1β expression levels, whereas in Fig. 1F, both HS (10 μg/ml) and LPS (1 μg/ml) for 24 h increased TNF-α secretion levels, although both effects were more pronounced for the LPS stimuli.

### 3.2. HS increases ICAM-1 and VCAM-1 expression in CF through PI3K/Akt and NF-κB transduction pathways

The participation of NF-KB, PI3K/Akt and ERK1/2 signaling pathways in ICAM-1 and VCAM-1 expression by HS were evaluated. CF were pre-treated with 1 μM IMD-0354 (NF-KB inhibitor), 10 μM LY294002 (PI3K/Akt inhibitor) and 10 μM PD98059 (ERK1/2 inhibitor) respectively, all of them during 1 h and then stimulated with HS (10 μg/ml) for 24 h. The results indicate that pre-treatment with IMD-0354 and LY294002 decreased the ICAM-1 (Fig. 2A and B) and VCAM-1 (Fig. 2D and E) expression induced by HS. IMD-0354 and LY294002 by themselves did not affect the control CAMs expression. On the contrary, PD98059 pre-treatment did not prevent the induction of ICAM-1 (Fig. 2C) and VCAM-1 (Fig. 2F) by HS, suggesting that the ERK1/2 pathway did not participate in CAMs induction by HS in CF. PD98059 alone had no effect on control ICAM-1 and VCAM-1 levels.

### 3.3. HS increases SMC and PMN adhesion to CF

To determine the effect of HS and LPS on SMC and PMN adhesion to CF, adhesion assays on pre-stimulated CF with HS (10 μg/ml) and LPS (1 μg/ml) for 24 h were performed. After time, the conditioned culture medium was completely removed and replaced with fresh medium and the adhesion test was then performed according to described methodology. The results indicate that HS, as well as LPS, generate a significant increase of PMN (Fig. 3A) and SMC (Fig. 3B) adhesion on CF monolayer, being HS and LPS effects statistically significant regarding the control condition. This effect on PMN and SMC adhesion was clearly

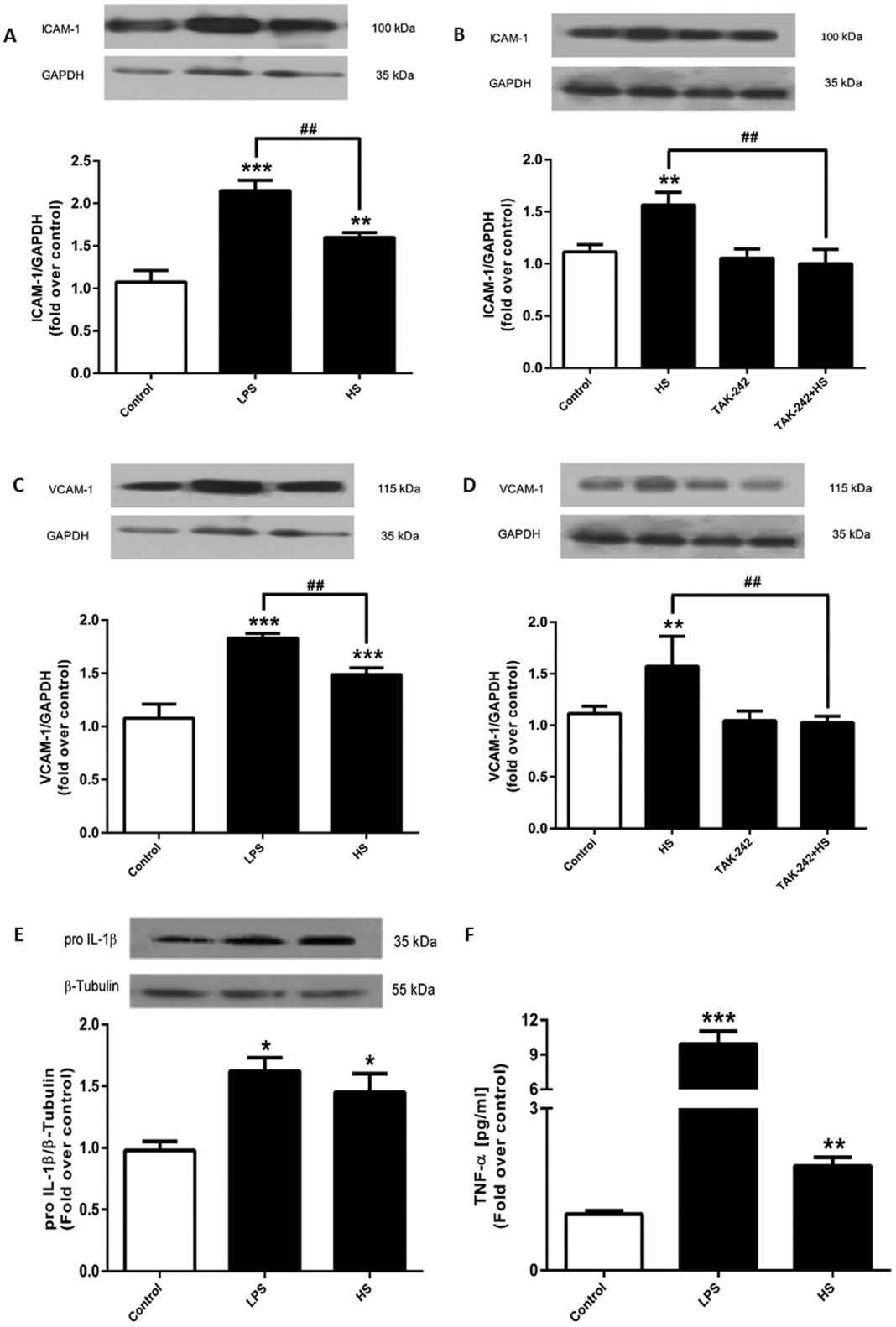


**Fig. 1.** ICAM-1 and VCAM-1 protein levels induced by HS through TLR4 receptor. CF were treated either with LPS (1 μg/ml) or HS (10 μg/ml) for the indicated time in the presence or absence of the TLR4 inhibitor TAK-242 (4 μM). ICAM-1 (A and B), VCAM-1 (C and D) protein levels were analyzed. Representative immunoblots of ICAM-1, VCAM-1 and GAPDH as loading control are shown in the upper panel of each graph. CF were treated either with LPS (1 μg/ml) or HS (10 μg/ml) for 8 h for pro-Il-1β, 24 h for TNF-α. proIL-1β protein (E) and secreted TNF-α (F) were analyzed. The results represent mean value ± SD of at least 3 independent experiments (**$p < 0.01$ and ***$p < 0.001$ vs control, ##$p < 0.01$ vs HS).

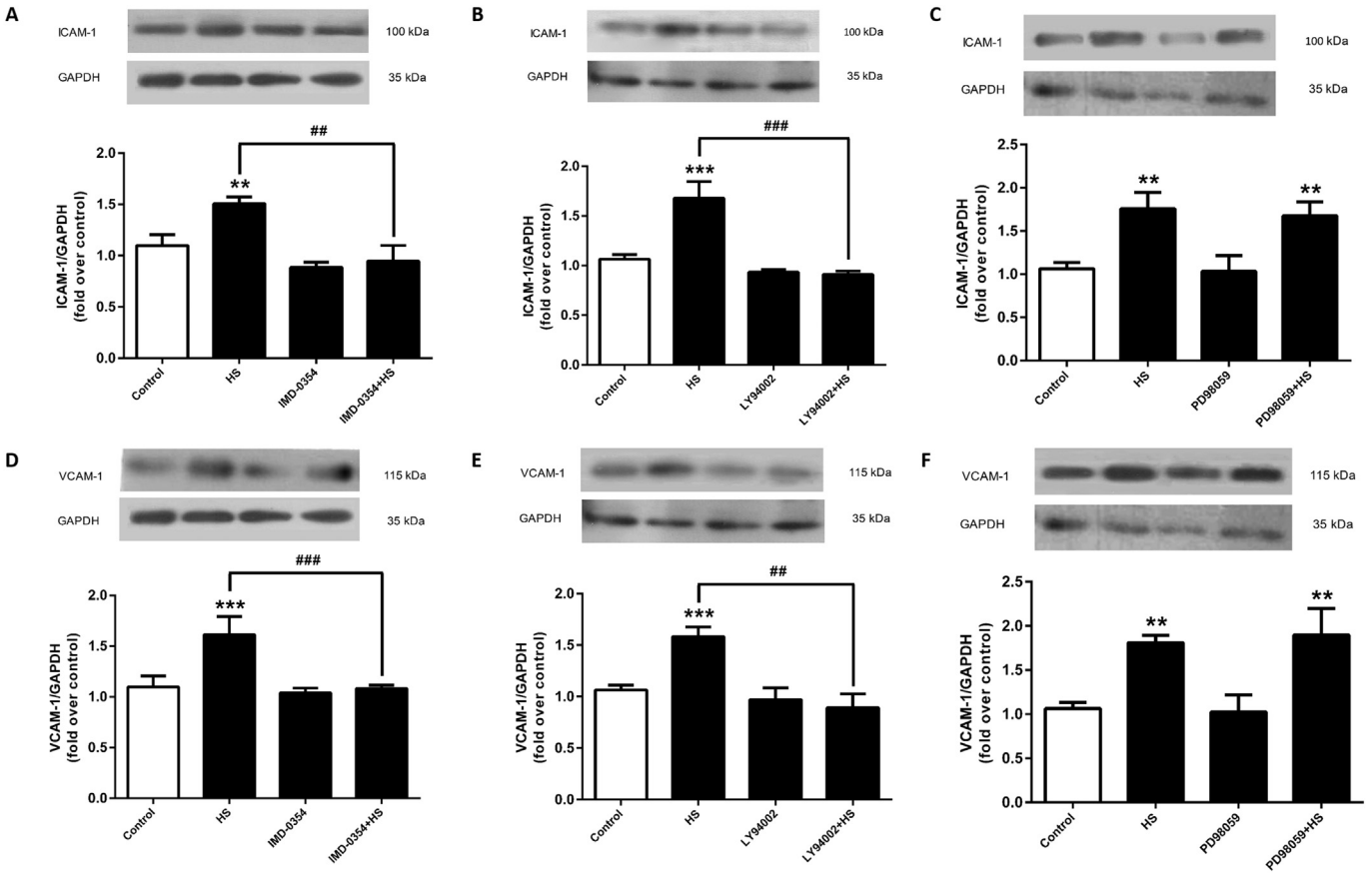


**Fig. 2.** ICAM-1 and VCAM-1 protein levels induced by HS through PI3K/Akt and NF-KB transduction pathways. CF were stimulated either with LPS (1 μg/ml) or HS (10 μg/ml) for 24 h in the presence or absence of the NK-κB inhibitor IMD-0354 (1 μM), the PI3K/Akt inhibitor LY294002 (10 μM) or the ERK 1/2 inhibitor PD98059 (10 μM); ICAM-1 (A-C) and VCAM-1 (D-F) protein levels were analyzed. Representative western blot of ICAM-1, VCAM-1 and GAPDH as loading control are displayed in the upper panel of each graph. The results represents mean value ± SD of at least 3 independent experiments (**p < 0.01 and ***p < 0.001 vs control, ##p < 0.01 and ###p < 0.001 vs HS).

observed in the images obtained by staining with violet crystal (Fig. 3C). It is observed that the non-stimulating CFs have less PMNs and SMCs attached, compared to those stimulated with HS and LPS, which are consistent with the results obtained previously.

### 3.4. TLR4, PI3K/Akt and NF-κB mediate the increased PMN and SMC adhesion to CF induced by HS

Since TLR4, PI3K/Akt and NF-KB were involved in CAMs expression induced by HS and LPS, we next assessed whether the blockade of these transduction pathways could also interfere with the increased PMN and SMC adhesion to CF. CF were pre-treated with the inhibitors TAK-242, LY294002, IMD-0354 and PD98059 for 1 h, and subsequently stimulated with HS (10 μg/ml) for 24 h. The Fig. 4A and B show that the increased adhesion of PMN and SMC elicited by HS was indeed prevented by all three drugs. The inhibition of the ERK1/2 pathway had no effect on the adhesion responses induced by HS. These results support the participation of TLR4, PI3K/Akt and NF-KB signaling pathways on PMN and SMC adhesion to CF.

### 3.5. ICAM-1 and VCAM-1 mediates the adhesion of SMC and PMN to CF induced by HS

To corroborate that ICAM-1 and VCAM-1 mediate the adhesion of PMN and SMC adhesion to CF in response to HS, knockdown experiments of these proteins were performed using siRNA. CF were pre-treated with siRNA against ICAM-1 and VCAM-1 according to the previously described methodology, and then stimulated with HS (10 μg/ml) for 24 h. Afterwards, CF were co-incubated with PMN or SMC according to the adhesion assay protocol. The results show that ICAM-1 or VCAM-1 knockdown decreased PMN (Fig. 5A) and SMC (Fig. 5B) adhesion down to control levels. These results thus demonstrate that PMN and SMC adhesion to CF in response to HS is ICAM-1 and VCAM-1-dependent.

### 3.6. HS decreases α-SMA expression in CF

Previously we have shown that LPS decreases α-SMA expression, which is a marker to CF differentiation to CMF [24]. We then assessed whether HS could modify α-SMA expression, as well as the participation of TLR4, and NF-KB, PI3K/Akt and ERK1/2 signaling pathways in this process. Fig. 6A to C show that the pre-treatment for 1 h with TAK-242, IMD-0354 or LY294002 prevented the decrease in α-SMA expression observed 72 h after the exposure of CF to HS (10 μg/ml). TAK-242, IMD-0354 and LY294002 by themselves did not affect α-SMA expression with respect to the control levels. ERK 1/2 was not involved in the reduced α-SMA expression by HS, since the ERK 1/2 inhibitor PD98059 did not modify the effect of HS (Fig. 6D). PD98059 did not modify either control ICAM-1 or VCAM-1 levels.

### 3.7. PMN and SMC adhesion induced by HS increase α-SMA expression in CF

Although we observed a positive effect of HS on SMC and PMN adhesion to CF, it remained to be determined whether this interaction had an effect on CF phenotype or structural protein expression levels. CF were stimulated with HS (10 μg/ml) for 24 h, and the adhesion of PMN and SMC was determined at a longer time of 72 h. After this time,

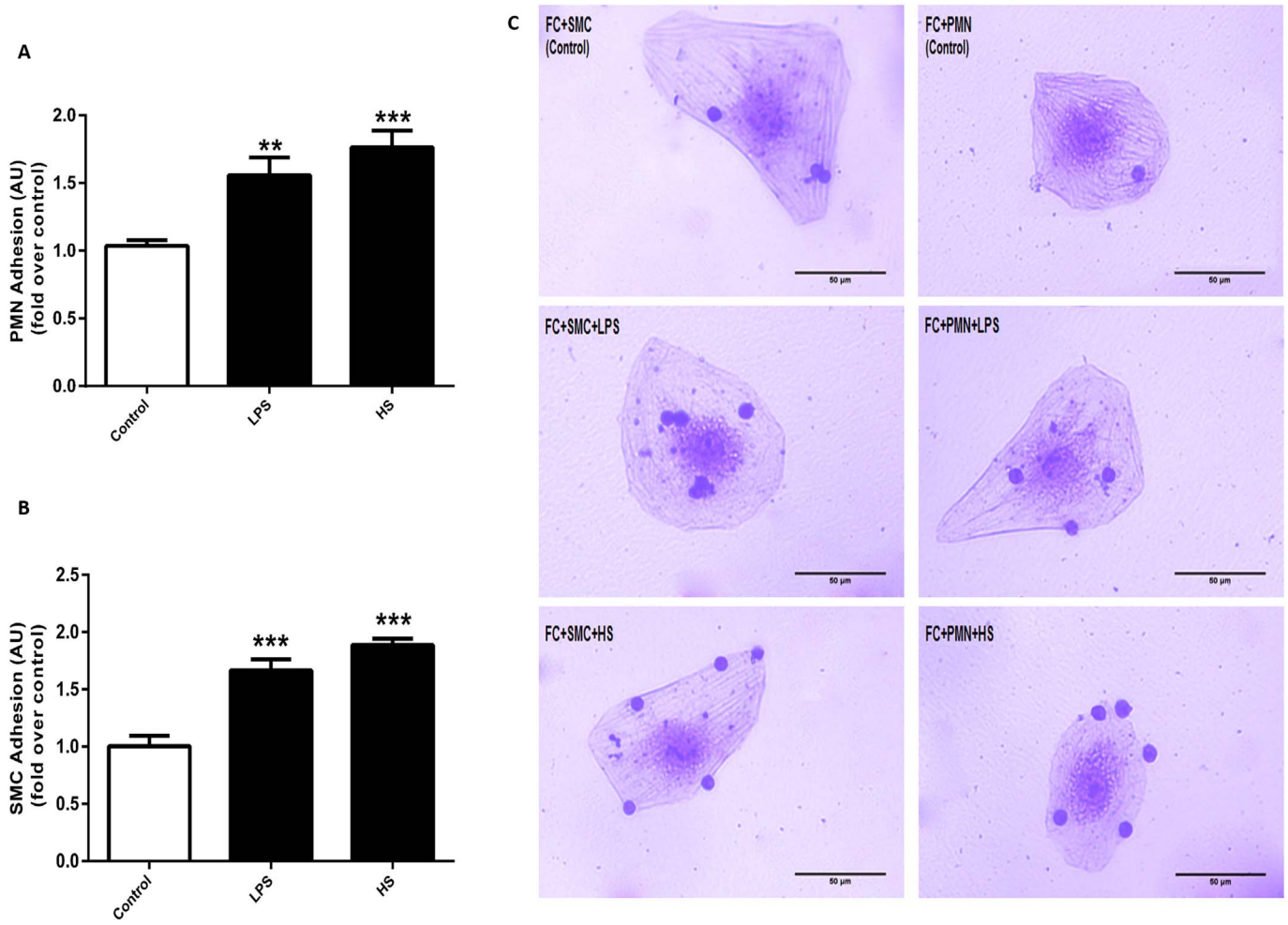


**Fig. 3.** PMN and SMC adhesion promoted by HS. Fluorescent-labeled PMN (A) and SMC (B) were added to confluent monolayers of CF that were previously treated with LPS (1 µg/ml) or HS (10 µg/ml) for 24 h. After 2 h, PMN and SMC adhesion was measured as described in Materials and methods. The results represent mean value ± SD of at least 3 independent experiments (**p < 0.01 and ***p < 0.001 vs control, #p < 0.05 vs LPS). Illustrative violet crystal stain images for each of the conditions analyzed are shown in (C).

CF were washed with PBS to remove the largest amount of adhered PMN and SMC and α-SMA expression levels were examined in CF monolayer by WB. Fig. 7A shows that PMN co-incubation with CF resulted in increased α-SMA expression levels. Moreover, HS decreased α-SMA expression, but this effect that was prevented by the co-incubation with PMN. Similar results were found when CF were co-incubated with SMC instead of PMN (Fig. 7B).

Finally, because the co-incubation between SMC and CF promoted α-SMA expression, immunofluorescence experiments were performed to determine whether the over-expression of α-SMA correlated with changes in the assembly of this proteins to form the stress fibers. The results show that CF displayed low expression levels and assembly of α-

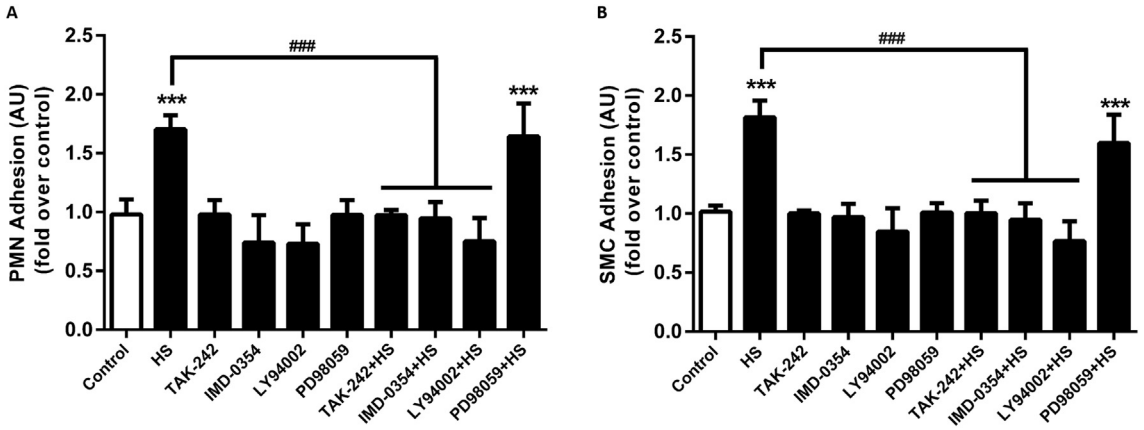


**Fig. 4.** HS-elicited PMN and SMC adhesion mediated by TLR4, PI3K/Akt and NF-κB. Calcein-stained PMN (A) and SMC (B) were co-incubated with confluent monolayers of CF that were previously stimulated with HS (10 µg/ml) for the indicated time and in presence or absence of TAK-242 (4 µM), IMD-0354 (1 µM), LY294002 (10 µM) or PD98059 (10 µM). Afterwards, PMN and SMC adhesion was determined by fluorescence. The results represent mean value ± SD of at least 3 independent experiments (***p < 0.001 vs control, ###p < 0.001 vs HS).

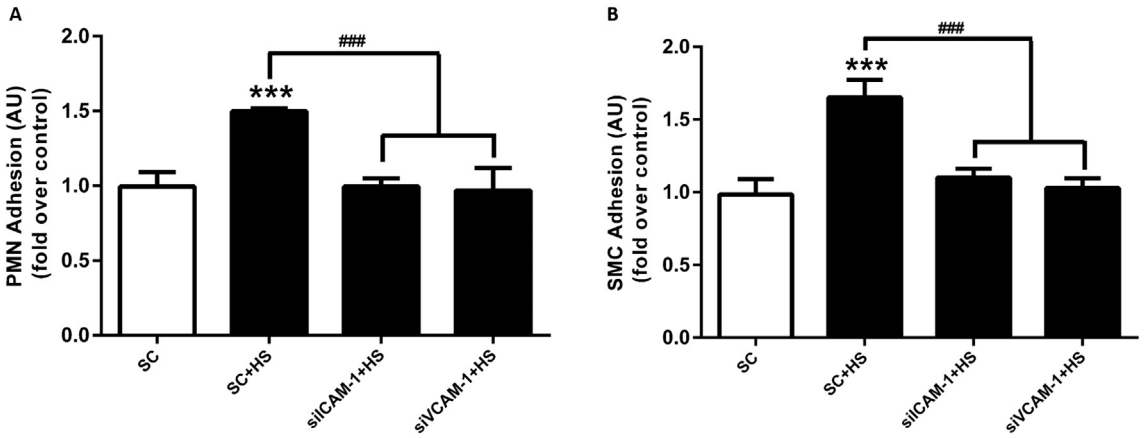


**Fig. 5.** HS-triggered PMN and SMC adhesion dependent of ICAM-1 and VCAM-1. Fluorescent-labeled PMN (A) and SMC (B) were added to confluent monolayers of CF that were previously transfected with scrambled (SC), ICAM-1 or VCAM-1 siRNA to assess the participation of these proteins in leukocyte adhesion, and then treated with HS (10 μg/ml) for 24 h. After 2 h, PMN and SMC adhesion was measured as described in Materials and methods (***p < 0.001 vs control, ###p < 0.001 vs SC + HS).

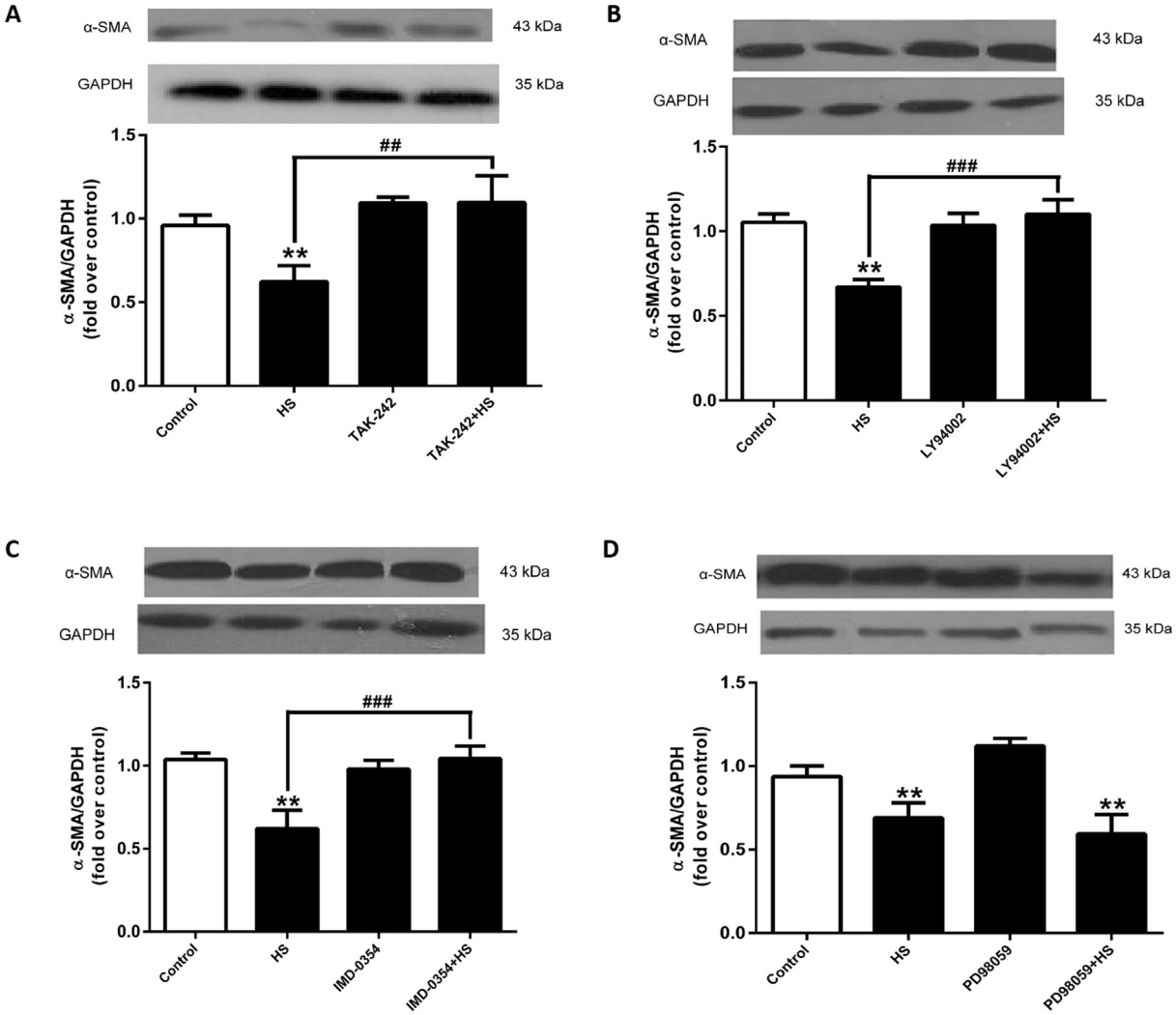


**Fig. 6.** α-SMA protein levels decreased by HS through TLR4, PI3K/Akt and NF-κB. CF were stimulated either with HS (10 μg/ml) for the indicated time and in presence or absence of TAK-242 (4 μM), IMD-0354 (1 μM), LY294002 (10 μM) or PD98059 (10 μM) and then α-SMA expression was analyzed. Representative western blot of α-SMA and GAPDH as loading control are displayed in the upper panel of each graph. The results represent mean value ± SD of at least 3 independent experiments (**p < 0.01 vs control, ##p < 0.01 and ###p < 0.001 vs HS).

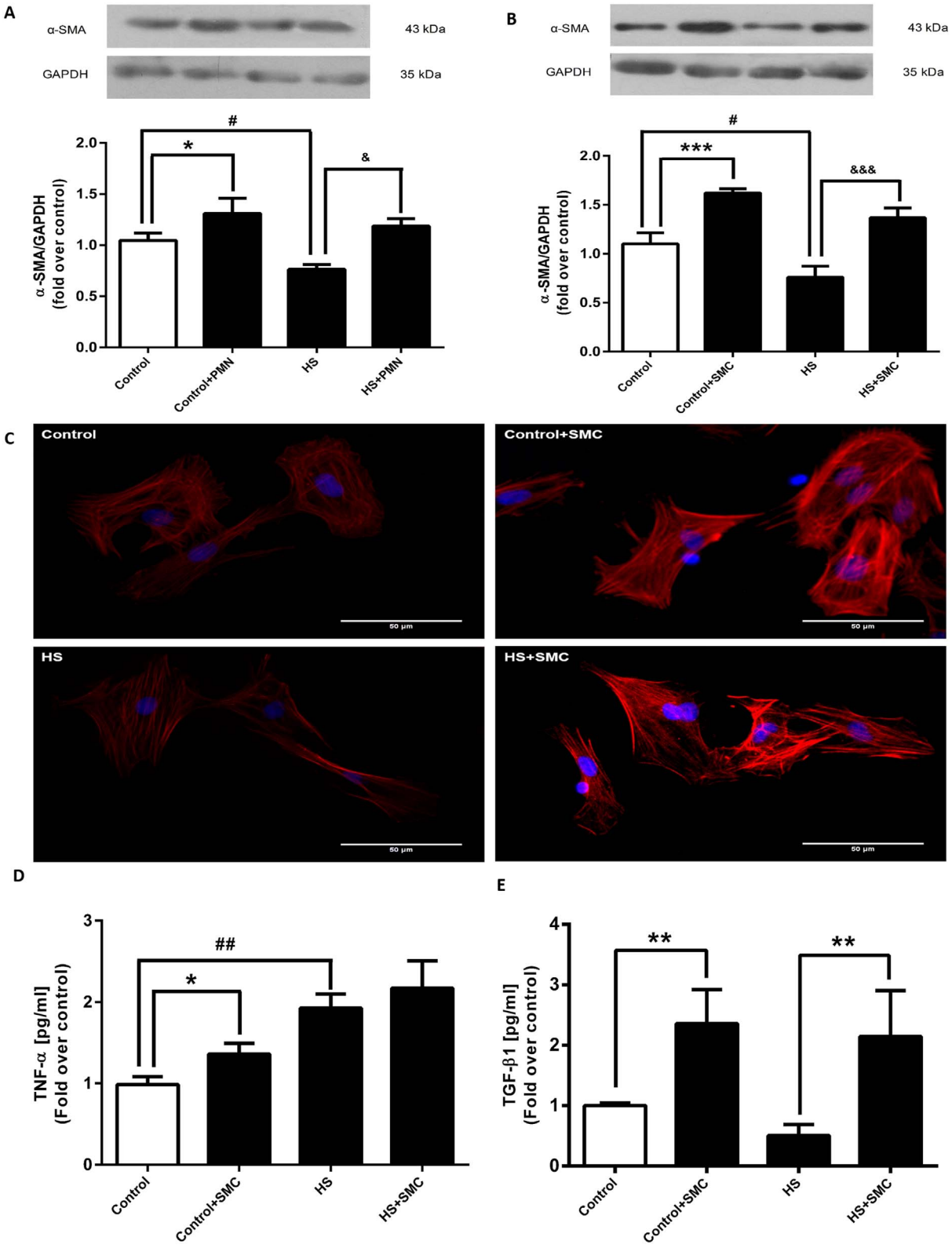


**Fig. 7.** HS-induced PMN and SMC adhesion increased α-SMA assembly. Calcein-labeled PMN (A) and SMC (B) were added to confluent monolayers of CF that were previously stimulated with HS (10 μg/ml) for 24 h. After 72 h, adhered PMN and SMC were removed and α-SMA protein levels were analyzed by immunoblot. Representative western blot of α-SMA and GAPDH as loading control are displayed in the upper panel of each graph. The results represent mean value ± SD of at least 3 independent experiments (*$p < 0.05$ and ***$p < 0.001$ vs control, &$p < 0.05$ and &&&$p < 0.001$ vs control, #$p < 0.05$ vs control). CF were stimulated with HS (10 μg/ml) for 24 h, the adhesion of SMC was performed for 24 h, then the CF culture medium was collected and ELISA assays for TGF-β (C) and TNF-α (D) were performed. Illustrative immunofluorescence using anti-α-SMA stain is shown for each condition in (B); white arrows indicate a SMC in contact with a CF.

SMA in stress fibers (Fig. 7C). On the other hand, and similarly to what was observed by WB, HS further decreased α-SMA expression levels and assembly (less red staining and less fibers). However, the co-culture with SMC led to increased α-SMA expression levels and assembly on stress fibers in CF both under control and HS-stimulated conditions.

### 3.8. Adherent SMC trigger an increase on TGF-β1 and TNF-α secretion from CF and SMC

As mentioned before, SMC interaction with CF triggered changes on CF phenotype which could be dependent of cytokines or growth factor secreted from CF as well as from SMC. With this aim, CF were stimulated with HS (10 μg/ml) for 24 h, the adhesion of SMC was performed for 24 h, and after this time the CF culture medium was collected, centrifuged to separate from non-adherent SMC, and TGF-β1 and TNF-α secretion levels were quantified. In Fig. 7D, the results show that HS increases TNF-α secretion levels from CF. Moreover, in CF with adherent SMC the TNF-α levels were also increased; however, although TNF-α secretion levels were higher in CF/SMC HS-treated cells, they did not were statistically different from CF/HS-treated. In Fig. 7E, the results show that HS decreases TGF-β secretion levels from CF. Nonetheless, the interaction between SMC and CF increased TGF-β secretion levels. Finally, in CF HS-treated with adherent SMC the TGF-β levels were also increased, and higher than CF/HS-treated.

## 4. Discussion

In the present work, we investigated the impact of HS on ICAM-1 and VCAM-1 expression levels in CF, as well as the transduction pathways involved, and the consequent alterations on PMN and SMC adhesion to CF. Finally, we analyzed the consequences of this CF/PMN or SMC physical interaction on the phenotypic features of CF.

### 4.1. ICAM-1, VCAM-1, TNF-α and pro-IL-1β expression in CF are induced by HS

In our previous finding we has showed that LPS increased TNF-α secretion and pro-IL-1β levels on CF, which are TLR4 dependent [4,9]. Here we showed that HS also increases both pro-inflammatory cytokines, highlighting the pro-inflammatory role of CF. In the heart, it has been shown that chronic degradation of ECM leads to a continuous generation of soluble HSPGs, which could activate innate immune system receptors such as TLR4. This would in turn trigger a chronic inflammatory response, generating adhesion protein synthesis, leukocyte recruitment and cardiac remodeling [25]. In CF, ICAM-1 and VCAM-1 are constitutively expressed, but during inflammatory cardiac events an increased CAMs expression has been associated with infiltration and activation of leukocytes at the site of injury. An overexpression of CAMs has been also reported in cardiac fibroblasts in response to different stimuli such as LPS, IL-1, TGF-β, phorbol esters, among others [26]. The present results demonstrate that HS and LPS can promote ICAM-1 and VCAM-1 expression in CF, similarly to what has been observed in endothelial cells stimulated with LPS [27]. Although it has been shown that shedded syndecan-4 in response to LPS increases CAMs expression on neonatal CF [25], the present study demonstrates the effects of HS chains by themselves through the TLR4 receptor on adult CF, previously found without participation on the expression of ICAM-1 and VCAM-1.

Our results further show a TLR4 dependence for CAMs increase, which has been also observed in other cell types, such as synovial [28], endothelial [27], and renal cells [29]. TLR4 is widely expressed in the heart and its expression is induced during myocardial infarction [30]. Indeed, KO mice for TLR4 ($TLR4^{-/-}$) have lower injury size and inflammatory parameters than WT, making clear the involvement of these receptors in the inflammatory response in the heart [31]. On the other hand, our results point out that the PI3K/Akt/NF-KB transduction pathways play a critical role in inducing ICAM-1 and VCAM1 expression. PI3K/AKT inhibition abolished the proinflammatory effects of HS accordingly with other studies in alveolar epithelial cells [32] and in LPS-stimulated mouse hearts [33]. On the other hand, it has been shown that NF-κB controls the production of cytokines and inflammation-related proteins [34], which is in agreement with our findings showing that inhibition of NF-KB abrogated the ICAM-1 and VCAM-1 expression induced by HS. These observations are also consistent with previous findings from our group, where LPS elicited an increase on ICAM-1 and VCAM-1 expression [4] and activation of the NF-KB pathway [9] as well as in human pericyte studies [35] and in endothelial cells [27]. However, NF-KB is activated by other stimuli, such as IL-1β and TNF-α, which are released by CF as a consequence of TLR4 stimulation [4,3], and may have some impact on the CAMs regulation. Finally, the present study indicates that inhibition of the ERK1/2 pathway has no effect on ICAM-1 and VCAM-1 expression induced by HS in CF. However, these data are opposed to other reports using endothelial cells [36] and mammary cancer cell lines [37] stimulated with LPS, where ERK1/2 inhibition decreased CAMs expression. These discrepancies, that have already been reviewed in other publications [38], may be due to the different species studied (human cells vs. rat cells) and/or the different signaling pathways that are activated in every cell type. It is noteworthy mentioning that even an increase in VCAM-1 expression has been reported upon ERK1/2 inhibition in TNF-α stimulated gingival fibroblasts, which highlights the importance of cytokine balance and signaling diversity in regulating the expression of CAMs [39].

### 4.2. Increased adhesion of PMN and SMC to CF is induced by HS

In the presence of tissue damage to the myocardium necrosis of cardiomyocytes and other cell types of cardiac tissue, an inflammatory cascade takes place that serves both to clean the damaged area of dead cells as well as to repair and healing the damaged tissue. This way, cardiac post-tissue damage repair is intimately related to the inflammatory response. In this context, the interaction between fibroblasts and leukocytes has been studied in cells of different origins, such as lung fibroblasts [40], renal fibroblasts [41] and gingival fibroblasts [42]. These interactions between tissue cells (in this case fibroblasts), and cells of the innate immune system (leukocytes), modulate the degree of inflammation in these tissues and may determine the level of fibrosis that will be reached in that tissue as a result of the repair process. It is widely documented that within the first few hours post-MI, the influx of leukocytes to the cardiac tissue plays a crucial role in remodeling [26]. Therefore, the study of CF-leukocyte interactions, triggered by HS, may shed new light on interventions to modulate these inflammatory events. The present results indicate that after CF stimulation with HS, the SMC adhesion increases, similarly to LPS treatment. These results have congruence with other findings in intestinal fibroblasts [43] and synovial [44], which were able to adhere to a greater number of PBMCs after LPS stimulation. Concerning PMNs, an increased adhesion of these cells to CF stimulated with HS and LPS was also observed. Studies on fibroblasts derived from periodontal ligament [45] and lung [46] evidence a greater neutrophils adhesion when stimulating the fibroblasts with LPS, thus highlighting the importance of TLR4 in the initial stages of the inflammatory process associated with pathogens. Moreover, we show in this study for the first time that TLR4 mediates the increase in PMN and SMC adhesion to CF stimulated with HS, thus providing new information about the role of these receptors in mediating the stimulation of CF with an endogenous agent such as HS in the context of sterile inflammation in cardiac tissue. Similar to ICAM-1 and VCAM-1 expression, not only the inhibition of TLR4 but also the blockade of PI3K/Akt and NF-KB signaling pathways resulted in a decrease in leukocyte adhesion, in accordance with other studies that show the involvement of these pathways in adhesion molecules expression and the subsequent interaction and adhesion of PMN and

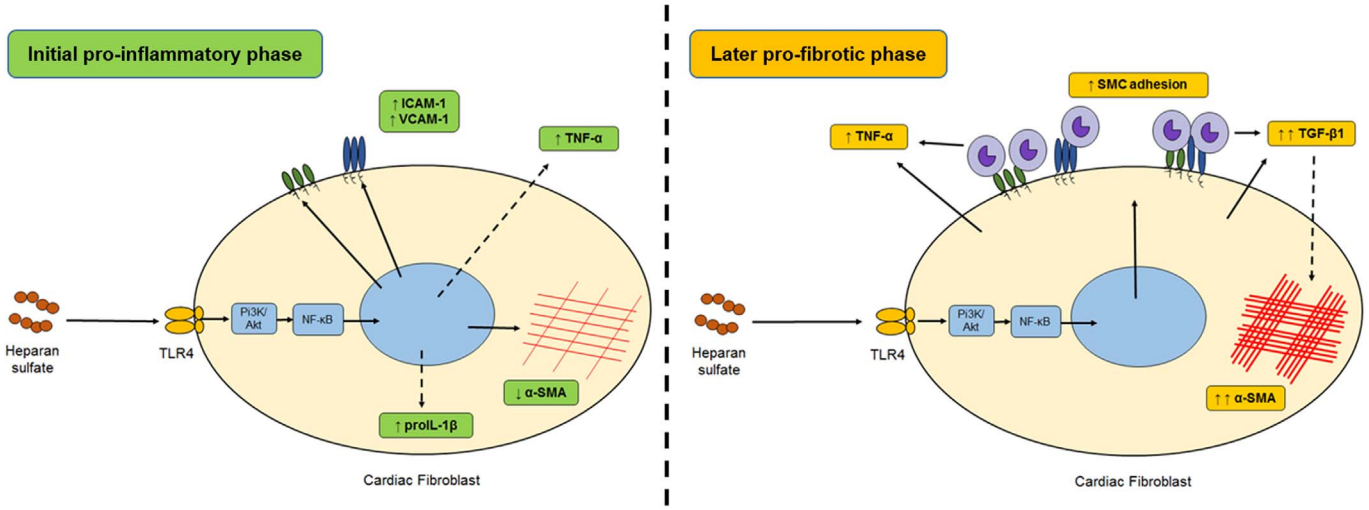


**Fig. 8.** Schematic representation of HS effects on CF. Soluble HS chains activates TLR4 receptor on CF, and through PI3K/AKT/NF-κB pathway increases pro-IL-1β protein levels, TNF-α secretion and ICAM-1/VCAM-1 expression levels, promoting a pro-inflammatory phenotype, which is parallel to decrease on α-SMA expression and assembly. In a later phase, the HS-induced CAM levels enhances SMC adhesion promoting TNF-α and TGF-β secretion from CF and SMC. Notably, TGF-β increases α-SMA expression and assembly and skews the CF to a pro-fibrotic phenotype.

PBMC to cardiac [47] and pulmonary endothelium [48], respectively. Since ligand binding of TLR4 triggers the downstream activation of PI3K/AKT and NF-KB, the blockade receptor of either the receptor or downstream transduction pathways will cause a decrease in CAMs expression and significantly impact on leukocytes adhesion.

A key role for ICAM-1 and VCAM-1 in mediating PMN and SMC adhesion to CF was further strengthened by CAMs knockdown experiments. In this regard, the participation of both ICAM-1 and VCAM-1 in the adhesion of monocytes to dermal and gingival fibroblasts has been previously reported [42,49], while ICAM-1 mediates the adhesion of neutrophils to CF [26]. However, the role of VCAM-1 in the adhesion of neutrophils to CF has been only explored in endothelial cells [50]; thus, the present study delivers new information on this adhesion molecule in mediating neutrophils adhesion. The decrease in adhesion to CF with silenced ICAM-1 and VCAM-1 was only partially blocked since only a 60–70% silencing was achieved with the siRNA used [4]. Overall, the adhesion processed were dependent on the expression of adhesion molecules on CF, although the mechanisms that induce leukocyte activation remain to be better defined.

### *4.3. Decreased expression of α-SMA in CF associated with PMN and SMC, induced by HS*

After a cardiac tissue injury, CF experience phenotypic differentiation to CMF, which is characterized by increased α-SMA expression and its assembly on stress fibers, thus providing contractile properties necessary to carry out the process of tissue contraction and repair [51]. Here, we have shown that the stimulation of CF with HS induces a lower expression of α-SMA. We have previously demonstrated that LPS also reduces α-SMA expression, thus reverting the CMF to CF phenotype [24]. This in turn indicates the ability of TLR4 activation to reverse a fibrotic process through a mechanism dependent on SMAD3 inactivation and SMAD7 activation, respectively, as proteins that control TGF-β-mediated α-SMA expression. Such a decrease in α-SMA has also been reported in CMF that overexpress syndecan-4, which reduces MI progression and leukocyte infiltration [52].

On the other hand, CF co-incubation with PMN or SMC showed an increase in α-SMA levels. Similar results have been obtained in CF co-cultured with monocytes [53] or dermal fibroblasts co-cultured with B lymphocytes [54]. However, other studies display opposite findings, with monocytes exerting an anti-fibrotic effect on lung tissue [55], which highlights different behaviors among tissues and the type of leukocyte involved. Our results showed that in CF stimulated with HS and then co-incubated with PMN or SMC there was an increase in α-SMA expression, although this increase was lower than that observed in CF co-cultured with PMN or SMC but not exposed previously to HS. In this regard, it would be expected that the HS stimulus, by increasing the adhesion of PMN and SMC, would trigger a more marked increase in α-SMA expression; however, there are two opposing stimuli involved. Thus, HS alone reduces the expression of α-SMA in CF and does not allow the differentiation to CMF, thus maintaining a more pro-inflammatory profile. On the other hand, CF co-incubation with PMN or SMC increases the expression of α-SMA. Therefore, the combined effect of HS on CF and the subsequent co-incubation with PMN or SMC will result in an overall more marked expression of α-SMA, where the effect of CF co-incubation with immune system cells predominates. In this context, it has been shown that both PMN and SMC release TGF-β (the main growth factor involved in CF to CMF differentiation). Our results, showed that TGF-β1 levels secreted to the culture medium, from either SMC or/and CF, were increased as consequence of SMC adhesion to CF. Therefore, our findings suggest that the interaction between CF and PMN or SMC trigger the secretion of this growth factor and thus activate TGF-β receptors on CF to induce α-SMA expression, its subsequent assembly and the differentiation to the CMF phenotype. This observation is in agreement with other reports where co-culture of skin fibroblasts from systemic sclerosis patients with neutrophils [56] result in pro-fibrotic actions of neutrophils through the release of ROS, MMPs and cathepsin [57]. This alters the composition of ECM, also by indirectly activating cell elements such as macrophages [22]. Regarding the differentiation of CF to CMF, we have previously demonstrated that CF and CMF secrete higher amounts of TGF-β2 than TGF-β1 [24], and that, on both CF and CMF, LPS did not affect TGF-β expression levels. Therefore, our results suggest that the increased expression of α-SMA in CF is rather regulated by the adhesion of PMN or SMC, and not by the TGF-β secretion by CF themselves.

Finally, we study whether SMC adhesion on CF modify pro-inflammatory cytokines secretion. Previously, we had showed that CF did not secrete IL-1β unless that inflammasome complex can be activated by ATP [4]. However, here we show that TNF-α secretion levels from SMC or/and CF were increased by SMC-CF interaction; however, we did not observe and additional increase on CF HS-treated with adherent SMC. Therefore, we conclude that HS triggers and initial proinflammatory effect on CF, but later the profibrotic one is activated.

In summary, collectively these results show for the first time the

dual role of HS, increasing PMN and SMC adhesion to CF, but reducing at a time the expression of α-SMA. This apparently antagonistic function would make sense and could depend on the time-lapse after tissue damage. Thus, during the onset of post-MI inflammation, it is important for leukocytes to reach the injury zone and to adhere to CF to clean cell debris and release MMPs that degrade ECM to better allow their infiltration to the entire area of damage tissue; at a later stage, however, the CF interaction between PMN or SMC would trigger the secretion of factors that differentiate CF into a pro-fibrotic phenotype such as CMF, thus favoring an early scar response. Therefore, it is important at a first stage to maintain a balance between proinflammatory and profibrotic signals that confer a greater stability to the lesion and favor healing. However, the excessive interaction between CF and PMN or SMC could favor a chronicity of the fibrotic response, then leading to an adverse remodeling of the cardiac tissue (Fig. 8).

## 5. Limitations

The main limitation of this study is that our experiments were carried out in vitro. We know that the results obtained in vitro provide interesting information about the effects of HS on CF differentiation and interaction with leukocytes; however, they are far to demonstrate the role of HS on tissue homeostasis and wound healing, thus in vivo trials would be useful to confirm these findings.

## Disclosures

The authors declare that they have no conflict of interest.

## Transparency document

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