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Journal of Cellular Biochemistry 114:2273–2283 (2013)

Journal of Cellular Biochemistry

Monocytes and Smooth Muscle Cells Cross-Talk Activates STAT3 and Induces Resistin and Reactive Oxygen Species and Production

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ABSTRACT

During the early phase of atherosclerosis, monocytes attach to and migrate through the vessel wall where they activate and communicate with smooth muscle cells (SMC) affecting plaque progression by largely unknown mechanisms. Activation of STAT3 transcription factor is suggested to be critically involved in dedifferentiation, migration, and proliferation of SMC in the neointima formation after vascular injury. Monocytes-SMC cross-talk induces an inflammatory phenotype of the resident SMC, but the involvement of STAT3 in phenotype switching is not known. Resistin is a cytokine found in human atheroma associated to monocytes/macrophages with role in inflammation associated with cardiovascular disease. The aim of this study was to follow the effect of activated monocytes-SMC cross-talk on STAT3 activation and subsequent resistin and reactive oxygen species (ROS) production. Our results showed that the interaction of activated monocytes with SMC determines: (i) phosphorylation of STAT3 and reduction of SOCS3 expression in both cell types; (ii) intracellular ROS production dependent on NADPH oxidase (by increased Nox1 expression) and STAT3 activation in SMC; (iii) up-regulation of resistin expression in monocytes dependent on STAT3 activation. Furthermore, exposure of SMC to resistin induces ROS by increasing NADPH oxidase activity and the p22phox and Nox1 expression. In conclusion, the cross-talk between SMC and monocytes activates STAT3 transcription factor and lead to resistin up-regulation in monocytes and ROS production in SMC. Moreover, resistin increases the ROS levels in SMC. These data indicate that monocyte-SMC communication may represent an important factor for progression of the atherosclerotic lesion. J. Cell. Biochem. 114: 2273–2283, 2013.

KEY WORDS: SMMOTH MUSCLE CELLS; MONOCYTES; STAT3 TRANSCRIPTION FACTOR; RESISTIN; REACTIVE OXYGEN SPECIES

The initiation of atherosclerosis results from complex interactions of circulating factors and various cell types in the vessel wall, including endothelial cells, lymphocytes, monocytes, and smooth muscle cells (SMC) [Manduteanu and Simionescu, 2012]. Interaction of monocytes with cells of vascular wall allows these cells to transmigrate from the bloodstream into the intima where they differentiate into macrophages.

Monocytes and macrophages are innate immune cells that are central to the inflammatory response in the atherosclerotic plaque. These cells are the main producers of pro-inflammatory cytokines [Tabas, 2010; Woollard and Geissmann, 2010] during the response to different exogen or endogen agonists that are involved in atherosclerosis. The response of monocyte/macrophage as a result of their communication with surrounding cells in atherosclerotic lesion is also important in production of inflammatory cytokines. Thus, our recent results indicate that the direct contact between human aortic SMC and monocytes augments the inflammatory response in both cell types (increasing the expression of TNF-α, IL-1β, IL-6, CX3CR1, and MMP9) dependent by NF-kB and AP-1 transcription factors. Moreover, we identified that the cross-talk between monocytes and SMC, dependent on binding of fractalkine to its receptor (CX3CR1) induces some of these inflammatory molecules (TNF-α, CX3CR1, and MMP9) [Butoi et al., 2011]. Recent evidences indicate that fractalkine regulates inflammation through CX3CR1 and Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway [Huang et al., 2012]. JAK/STAT activation begins at the surface of the cell where the cytokine receptor engagement activates the associated JAK, which phosphor-

Grant sponsor: Romanian National Authority for Scientific Research, CNCS-UEFISCDI; Grant number: PN-II-ID-PCE-2011-3-0928; Grant sponsor: L'Oréal-UNESCO Awards 2011 "For women in science"; Grant number: LOR049.

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Manuscript Received: 7 February 2013; Manuscript Accepted: 12 April 2013

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 19 April 2013

DOI 10.1002/jcb.24571 • © 2013 Wiley Periodicals, Inc.

ylates the receptor cytoplasmic domain to allow recruitment and tyrosine phosphorylation of STAT. Activated STATs dimerize and translocate into the nucleus to activate specific gene expression of different inflammatory molecules. The effect of activated monocytes-SMC cross-talk on JAK/STAT pathway is not known.

Resistin is a cytokine found in human atheroma produced by monocytes/macrophages and may play an important role in inflammation associated with cardiovascular disease [Jung et al., 2006; Chen et al., 2010]. Plasma resistin levels have been associated with markers of inflammation such as TNF-α or IL-6, and resistin has been shown to be a predictive factor for coronary atherosclerosis in humans, independent of CRP [Burnett et al., 2005; Reilly et al., 2005; Yaturu et al., 2006]. Moreover, accumulating data suggest that resistin is involved in pathological processes leading to CVD including inflammation, thrombosis, angiogenesis, and endothelial and SMC dysfunctions. Thus, resistin was found to have a potential role in atherosclerosis by increasing proinflammatory mediators expression in vascular endothelial and SMC [Nagaev et al., 2006; Manduteanu et al., 2010; Gan et al., 2013], by promoting the vascular SMC proliferation [Calabro et al., 2004] and by inducing the oxidative stress in human coronary artery endothelial cells [Chen et al., 2010]. The increased reactive oxygen species (ROS) have a key role in vascular inflammation and atherogenesis [Madamanchi et al., 2005]. Thus, it have been shown that ROS function as important signaling molecules in the cardiovascular system and that resistin enhances intracellular ROS levels in endothelial cells [Chen et al., 2010]. In SMC, ROS mediate different pathophysiological processes, such as growth, migration, apoptosis, and secretion of inflammatory cytokines [Taniyama and Griendling, 2003]. Vascular SMC contain several sources of ROS, among which NADPH oxidase is the most important [Ushio-Fukai et al., 1996]. The NADPH oxidases (Nox) are multiprotein complexes of various subunits depending on the cell type. The enzyme complex, originally described in phagocytes, consists of two membrane-bound subunits: the p22phox subunit bound to the catalytic subunit gp91 (Nox1—in non-phagocytes; Nox2—in phagocytes) and potentially three cytosolic subunits, Rac1 (in non-phagocytes) or Rac2 (in phagocytes), p47phox, and p67phox, which are recruited upon activation to the membrane-bound Nox/p22phox complex. Up-regulation of p22phox or/and Nox1 subunits is critical for NADPH oxidase activation and ROS generation in vascular SMC [Ushio-Fukai et al., 1996; Hordijk, 2006]. The effect of resistin on modulation of ROS levels in SMC is not known.

As previously mentioned, in humans, resistin is mainly expressed by monocytes/macrophages and we and others have found that activation of monocytes with LPS, IL-6, TNF-α, or high glucose levels increases the expression of resistin in human monocytes [Kunnari et al., 2009; Broch et al., 2010; Stan et al., 2011]. However, the sources and the detailed mechanisms of resistin induction in atherosclerotic lesions and resistin-mediated effects on vascular cells are largely unknown.

In the present study, we hypothesized that the communication of LPS-activated monocytes with SMC may activate the JAK/STAT3 pathway, leading to oxidative stress in SMC and resistin up-regulation in monocytes, which may further contributes to SMC phenotype modulation by specific mechanisms.

We have demonstrated for the first time that the communication between activated-monocytes and SMC induces the activation of STAT3 transcription factor in both cell types and increases the intracellular ROS in SMC and resistin expression in monocytes. Moreover, we have found that resistin affects SMC by inducing ROS production through activation of NADPH oxidase and STAT3. This study provides new insights into the mechanisms by which monocyte-SMC cross-talk affects both cells behavior, and indentifies the cell cross-talk as a source of resistin production that further induces ROS in SMC.

MATERIALS AND METHODS

CHEMICALS

SiRNAs (STAT1/STAT3/scrambled) and siRNA transfection reagent Hiperfect were obtained from Santa Cruz Biotechnology. CD14 MicroBeads were from Miltenyi Biotech. Human resistin enzyme-linked immunosorbent assays were from R&D Systems. RT-PCR reagents and enzymes were from Invitrogen.

Monoclonal antibodies, anti-human resistin were from Santa Cruz Biotechnology, anti-pSTAT3 from Abcam, anti-actin from Sigma–Aldrich and anti-SOCS3 from R&D Systems. The FITC labeled anti-human CX3CR1 was from MBL (Biozol, Germany). The secondary antibodies and all the other reagents were from Sigma Aldrich Chemie GmbH (Germany).

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 immuno-histochemistry experiments revealed that they are positive for smooth muscle alpha-actin, and for vinculin, negative for von Willebrand factor [Tirziu et al., 1999], and display functional store-operated channels responsive for capacitative calcium entry [Raicu and Florea, 2001; Popov and Shepherd, 2004]. SMC were cultured in DMEM as described [Dragomir et al., 2008]. 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.

Human fresh monocytes were isolated from blood of healthy donors from the Blood Transfusion Center Bucharest (as described in the Supplementary Material).

The investigation was carried out according to the principles outlined in the Declaration of Helsinki [Association, 1997]. The Ethics Committee of the Institute of Cellular Biology and Pathology "Nicolae Simionescu," Bucharest, approved the protocol.

EXPERIMENTAL DESIGN: INCUBATION OF LPS-ACTIVATED MONOCYTES WITH SMC FOLLOWED BY CELL SEPARATION

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 (as described previously [Butoi et al., 2011]). 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 CD14MicroBeads and a magnetic cell sorting (MACS) separator (Miltenyi Biotech) according to the manufacturer's instructions. For the experiments when cells were stained with fluorofors for the ROS quantification (2′,7′-dichlorofluorescein diacetate or dihydroethidium) the cell separation was done by pipeting the adherent monocytes in PBS until they detached. The complete removal of adherent monocytes was monitorized under the microscope.

To search for the signaling mechanisms involved in the induction of resistin and ROS by cell cross-talk, SMC and LPS-activated monocytes were co-cultured in presence or absence of STAT3 inhibitor, S3I-201 (20 μmol/L) or ROS inhibitors: apocynin (Ay, 100 μmol/L)—inhibitor of NADPH oxidase, allopurinol (All, 50 μmol/L)—inhibitor of xanthine oxidase and rotenone (Rot, 50 μmol/L)—inhibitor of mitochondrial electron transport chain. The effect of resistin on intracellular ROS was evaluated by exposure of SMC to resistin (100 ng/ml) in the presence or absence of the above-mentioned inhibitors.

DETECTION OF REACTIVE OXYGEN SPECIES

After 18 h of monocytes-SMC interactions, the 2′,7′-dichlorofluorescein diacetate (DCFH-DA, 10 μM)—a fluorogenic dye that measures hydroxyl, peroxyl and other ROS activity within the cell-, was added to the co-cultured cells. After 30-min incubation, the cells were separated, washed, re-suspended in PBS, and counted using a TECAN monochromator or by flow cytometry (Beckman Coulter Gallios). For flow cytometry experiments, 10,000 cells were acquired for each sample. Data were analyzed with Summit v4.3 software (Dako).

MEASUREMENT OF THE INTRACELLULAR PRODUCTION OF SUPEROXIDE ANION WITH DIHYDROETHIDIUM

The whole intracellular production of superoxide anion (O_2^-) was measured with dihydroethidium (DHE), which is a compound that can diffuse through cell membranes, rapidly oxidized in hydroethidine (HE) under the action of O_2^-. The 20 μM DHE was added to the cells and incubate for 30 min at 37°C. The cells were washed with PBS and the fluorescent signal was measured with a TECAN plate reader at excitation 535 nm and emission 610 nm. The DHE fluorescent signal was normalized to the DNA fluorescent units of the same well (as measured using Hoechst). The data are expressed as the average DHE fluorescent units per DNA fluorescent units.

NADPH OXIDASE ASSAY

The NADPH oxidase activity in cell homogenates was determined using NADPH assay kit (Sigma–Aldrich), according to the manufacturer's instructions, or the lucigenin-enhanced chemiluminescence assay as previously described [Ungvari et al., 2003]. Briefly, the reaction carried out in a total volume of 150 μl containing 5 mmol/L phosphate buffer, 1 mmol/L EGTA, pH 7.0, 5 μmol/L lucigenin, and 100 μmol/L NADPH, was started by addition of cell homogenate (100 μg protein); the light emission was recorded using a microplate luminometer (Berthold). The NADPH oxidase activity was expressed as relative light units (RLU) per microgram of total protein.

WESTERN BLOT

The protein expression of resistin, SOCS, phospho-STAT3, total STAT3, and actin were assessed in the total extract of SMC or monocytes obtained by homogenizing the cells in Laemmli electrophoresis buffer as described [Dragomir et al., 2008]. The signals were visualized using SuperSignal West Pico chemiluminescent substrate and quantified by densitometry employing gel analyzer system Luminiscent image analyzer LAS 4000 (Fujifilm) and Image reader LAS 4000 software.

TRANSFECTION OF SMALL-INTERFERING RNA (SIRNA)

The siRNA (STAT1/STAT3/scrambled) was transfected into cells using siRNA transfection reagent Hiperfect® (Qiagen) according to the manufacturer's protocol. Twenty-four hours after transfection, the cells were harvested and analyzed. After transfection, the interaction between SMC and monocytes, followed by cell separation, were performed as described.

REAL-TIME PCR

Total cellular RNA was isolated from cells using GenElute® Mammalian Total RNA kit (Sigma). First-strand cDNA synthesis was performed employing 1 μg of total RNA and MMLV reverse transcriptase, according to the manufacturer's protocol (Invitrogen). Assessment of resistin, p22phox and Nox1 mRNA expression was done by amplification of cDNA using a LightCycler 480 Real Time PCR System (Roche) and SYBR Green I chemistry. The primer sequences are shown in Table I. Optimized amplification conditions were 0.2 μM of each primer, 2.5 mM $MgCl_2$, annealing at 60°C and extension at 72°C for 40 cycles. Actin gene was used as internal control. The relative quantification was done by comparative CT method and expressed as arbitrary units.

ELISA ASSAY

The resistin protein levels released in the conditioned medium from control cells (monocytes or SMC) and from co-cultured cells were quantified using an ELISA assay kit (R&D systems, UK), according to the manufacturer's instructions.

TABLE I. Sequences and GenBank® Accession Number of Oligonucleotide Primers Used for Real Time PCR

Gene	GenBank® accession number	Sequences of oligonucleotide primers	Predicted size (bp)
Resistin	NM_020415.3	5′-ctgttggtgtctagcaagacc-3′ 3′-ccaatgctgcttattgccctaaa-5′	104
p22phox	NM_000101	5′-cccagtggtactttggtgcc-3′ 5′-gcggtcatgtacttctgtccc-3′	124
Nox1	NM_013955	5′-cacaagaaaaatccttgggtcaa-3′ 3′-gacagcagattgcgacacaca-5′	110
Actin	NM_001101	5′-catgtacgttgctatccaggc-3′ 3′-ctccttaatgtcacgcacgat-5′	250

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.

RESULTS

THE INTERACTION BETWEEN LPS-ACTIVATED MONOCYTES WITH SMC INDUCES STAT3 PHOSPHORYLATION AND DECREASES THE SOCS3 PROTEIN EXPRESSION IN BOTH CELL TYPE

Since our previous data showed that binding of fractalkine to its receptor (CX3CR1) induces the over-expression of pro-atherosclerotic mediators by monocytes–SMC interaction [Butoi et al., 2011], and recently it has been shown that fractalkine up-regulates inflammation through CX3CR1 and JAK2/STAT3 pathway [Huang et al., 2012], we searched for the effect of cell cross-talk on STAT3 activation in both, monocytes and SMC. The expression of phosphorylated and total form of STAT3 in SMC and monocytes was assessed before and after cells interaction followed by individual cell separation (as described in the experimental design).We found that prior to their contact, SMC and monocytes do not express the activated form of STAT3 (Fig. 1A and B, lanes So and Mo, respectively). Activation of monocytes or SMC with LPS (100 ng/ml) did not activate STAT3 (Fig. 1B, lane Mlps and Supplementary Fig. 1C, respectively). Conversely, upon monocyte–SMC interaction (18 h), the fosfo-STAT3 was significantly increased in both, interacted SMC and monocytes (Fig. 1A and B, lanes Si and Mi, respectively).

It is known that suppressors of cytokine signaling (SOCS) proteins are pivotal in negative regulation of JAK/STAT [Wormald and Hilton, 2004]. Since our data indicated that the STAT3 signaling is activated by cell cross-talk, to further understand the effect of cross-talk on JAK/STAT pathway modulation, we questioned if SOCS3 expression is modulated by cell interaction. We found that after 18 h of cell communication, when STAT3 was increased (Fig. 1A and B), SOCS3 protein expression was significantly decreased in both, SMC (Fig. 1C) and monocytes (Fig. 1D). Exposure of monocytes or SMC to LPS without further interaction with SMC did not significantly affect SOCS3

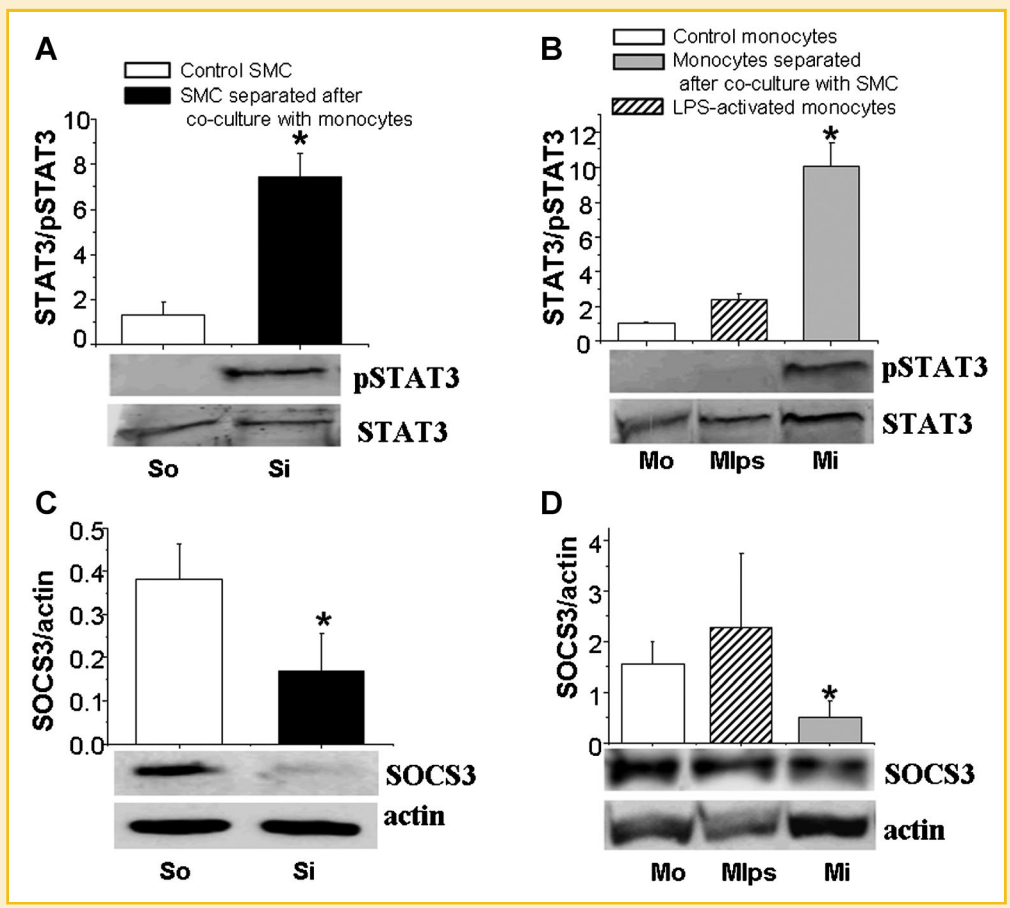

Fig. 1. Cross-talk between activated monocyte and SMC induces activation of STAT3. A,B: Evaluation of phospho-STAT3 (Western blot) in SMC (A) and monocytes (B) before (white columns) and after co-culture of activated monocyte with SMC. The cell interaction activates STAT3 in both, monocytes (Mi, gray column) and SMC (Si, black column) separated after 18 hour of co-culture. C,D: Interaction of SMC with LPS-activated monocytes decreases SOCS3 protein expression in both cells type (Si and Mi). n = 3, $^{*}P < 0.05$ interacted cells (Si, Mi) versus control cells (So, Mo).

expression (Fig. 1D, lane Mlps and Supplementary Fig. 1 D, respectively).

THE INTERACTION BETWEEN LPS-ACTIVATED MONOCYTES WITH SMC INCREASED THE INTRACELLULAR REACTIVE OXYGEN SPECIES IN SMC

Recently we have obtained that monocytes-SMC cross-talk induces the expression of inflammatory molecules in both cell types. Moreover, we have observed that upon interaction, the intracellular ROS levels are increased in SMC and decreased in monocytes [Butoi et al., 2011]. Here, we followed the effect of cross-talk between activated-monocyte and SMC on ROS production and further investigated the pathway involved in ROS modulation. We have obtained that, like in monocyte-SMC cross-talk, co-culture of activated monocytes with SMC has the same effect on ROS production: increased in SMC and decreased in monocytes. Since the cell cross-talk activated STAT3 in SMC (Fig. 1A), we further investigated if STAT3 activation is involved in the increased ROS production. Moreover, the possible ROS source was investigated using specific inhibitors. Thus, LPS-activated monocytes were added to confluent SMC in presence or absence of a specific inhibitor of STAT3 (S3I-201), or inhibitors for different sources of ROS: allopurinol (All)—inhibitor of xanthine oxidase, apocynin (Ay)—inhibitor of NADPH oxidase and rotenone—inhibitor of mitochondrial electron transport chain. The intracellular ROS levels were quantified using DCFH-DA (flow cytometry) and anion superoxide (O_2^-) using dihidroetidium (fluorimetry). The results showed that co-culture of LPS-activated monocytes with SMC increased ROS levels in SMC (Fig. 2A) and decreased ROS levels in monocytes (Fig. 2B). Interestingly, the interaction of LPS-activated monocytes with SMC in the presence of S3I-201 or apocynin, significantly reduced the ROS and O_2^- production in SMC (Fig. 2A and C), and had no effect on ROS

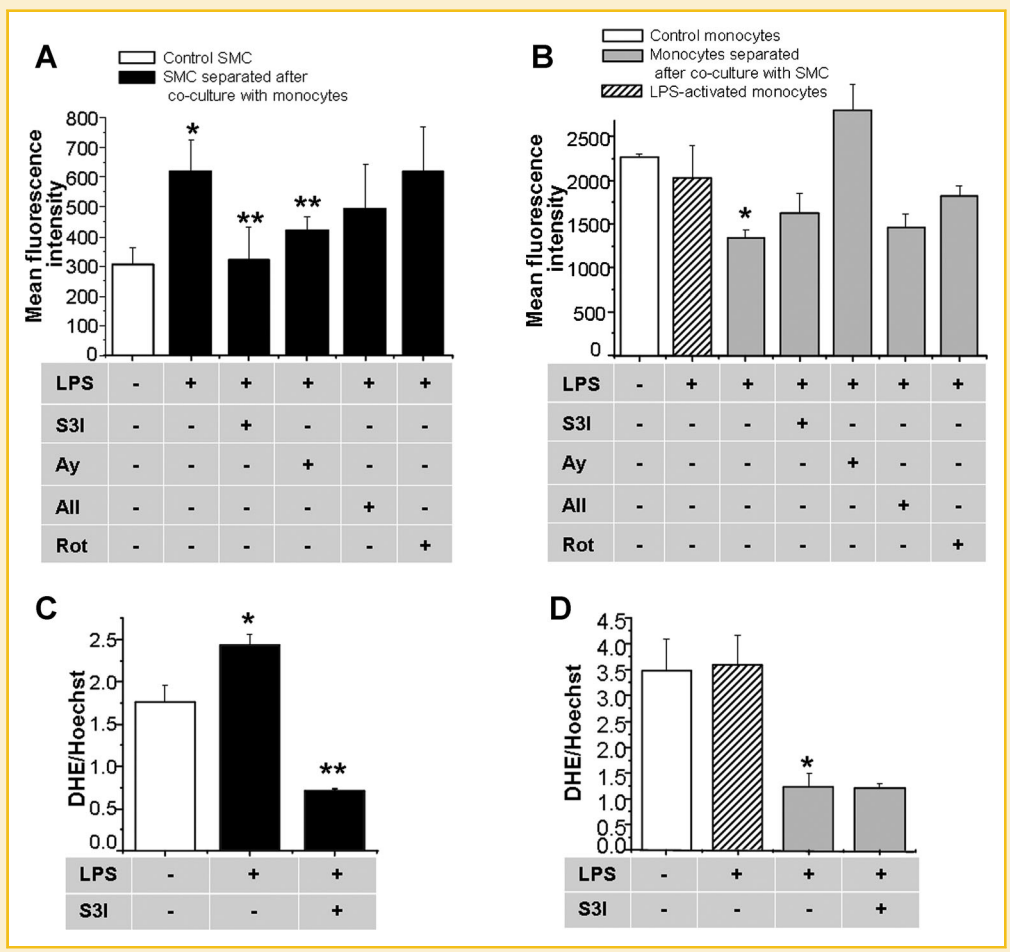

Fig. 2. The effect of activated monocyte—SMC interaction on intracellular level of reactive oxygen species (ROS) in both cell types. A: The cell cross-talk increases ROS in SMC separated after co-culture. Note that STAT3 inhibitor (S3I) and apocynin (Ay) but not allopurinol (All) and rotenone (Rot) reduces ROS levels in interacted SMC. n = 4 $^*P < 0.05$ interacted SMC (black columns) versus control SMC (white column); $^{**}P < 0.05$ interacted SMC (Si) versus Si treated with inhibitors (S3I and Ay). B: ROS are reduced in monocytes separated after their co-culture with SMC (gray columns) compared with control monocytes (white column). $^*P < 0.05$ monocytes separated after co-culture versus control monocytes. C,D: Quantification of anion superoxide (O^{2-}) using dihydroethidium. C: SMC separated after interaction with monocytes exhibit increased $O2^-$ levels and S3I suppresses O^{2-}. D: Intracellular $O2^-$ levels in monocytes separated after co-culture with SMC are reduced compared with control; n = 4, $^*P < 0.05$ co-cultured cells versus control cells; $^{**}P < 0.05$ interacted SMC (Si) versus Si treated with STAT3 inhibitor.

production in monocytes (Fig. 2B and D) suggesting the specific involvement of STAT3 pathway and NADPH oxidase in ROS production in SMC.

THE NADPH OXIDASE ACTIVITY IS INCREASED BY CELL CROSS-TALK IN SMC

Among the potential sources of ROS in human SMC, the NADPH oxidase appears to be especially important for redox signaling. Our results revealed that, as compared with control cells, SMC separated after co-culture with LPS-activated monocytes express increased NADPH oxidase activity (Fig. 3A, Si—black column) and the inhibition of STAT3 activation (cells were co-cultured in the presence of S3I-201) reduces the oxidase activity (Fig. 3B). The NADPH oxidase activity was not significantly reduced in monocytes separated after their interaction with SMC (Fig. 3A, Mi—gray column). To further investigate the mechanism involved in induction of NADPH oxidase activity in SMC, the effect of cell cross-talk on regulatory subunits of NADPH oxidase in SMC was assessed. The real-time PCR experiments revealed that the expression of p22phox mRNA was not affected (Fig. 3D) but, Nox1mRNA was significantly increased in SMC by cell cross-talk (Fig. 3C). Moreover, transient transfection experiments that knockdown the expression of STAT3 in SMC, showed that this transcription factor is involved in modulation of Nox1 induced by cell cross-talk (Fig. 3C). The NADPH subunities, p22phox and Nox2, were not affected by cell cross-talk in separated monocytes (data not shown).

THE DIRECT COMMUNICATION OF LPS-ACTIVATED MONOCYTES WITH SMC INDUCES GENE AND PROTEIN EXPRESSION OF RESISTIN BY STAT3 ACTIVATION

It is known that in humans, resistin is produced by monocytes/macrophages [Jung et al., 2006] and that human SMC do not produce resistin. Thus, we assessed the expression of resistin in U937 monocytes, before and after their interaction with SMC. We found that activation of U937 monocytes with LPS for 6 h (without further co-culture with SMC) did not affect resistin gene and protein expression (Fig. 4A and B, hachured columns). Interestingly, upon interaction of LPS-activated monocytes with SMC, the expression of resistin was significantly increased at both, gene (Fig. 4A) and protein levels (Fig. 4B).

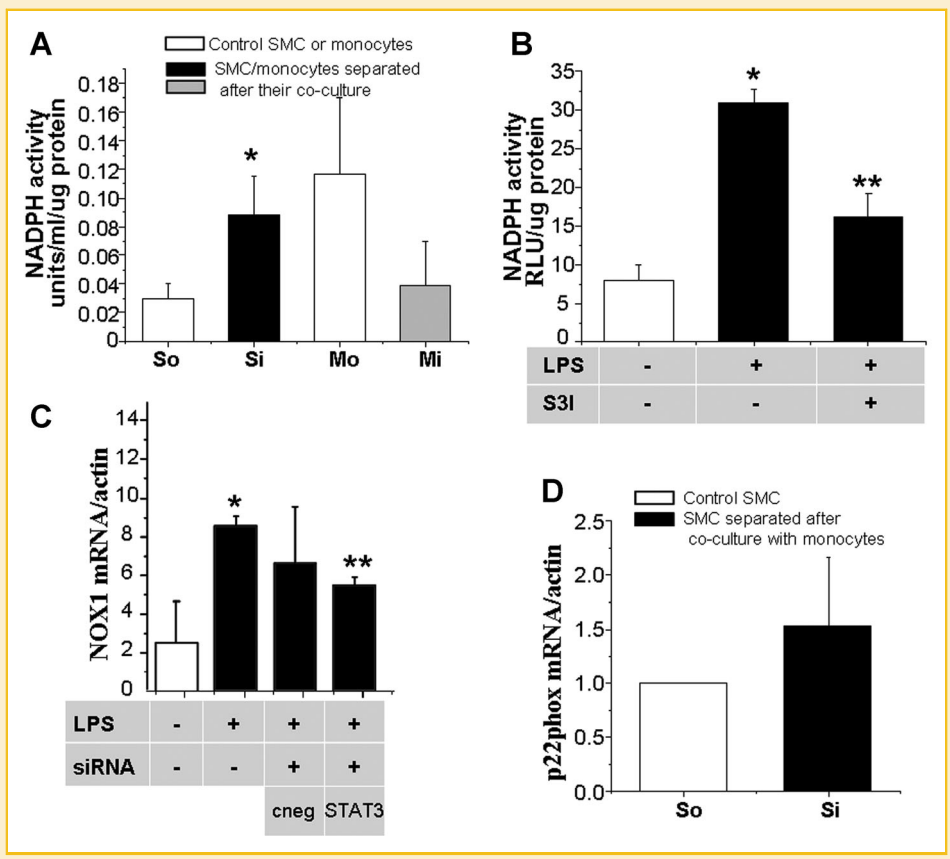

Fig. 3. Interaction between monocytes and SMC modulates NADPH oxidase activity and NOX1 expression. A: Interacted SMC (Si) exhibit increased NADPH oxidase activity compared with control SMC (So). In contrast, monocytes separated after co-culture (Mi) exhibit reduced NADPH oxidase activity. n = 3, $^{*}P < 0.05$ interacted SMC (Si) versu control SMC (So). B: The STAT3 inhibitor (S3I) decreases the NADPH activity induced by cell interaction in SMC; n = 3, $^{**}P < 0.05$ interacted SMC versus interacted SMC treated with S3I inhibitor. C: Gene expression of Nox1 is increased in interacted SMC versus control cells. The silencing of STAT3 binding sites by transfection with specific siRNA before cell interaction significantly decreases mRNA Nox1 expression. n = 4 $^{*}P < 0.05$ interacted SMC versus control SMC; $^{**}P < 0.05$ interacted SMC versus transfected (STAT3 siRNA) and next interacted SMC. D: Gene expression of p22phox—in control SMC (white column, So) and in SMC separated after co-culture with monocytes (black column, Si); n = 5.

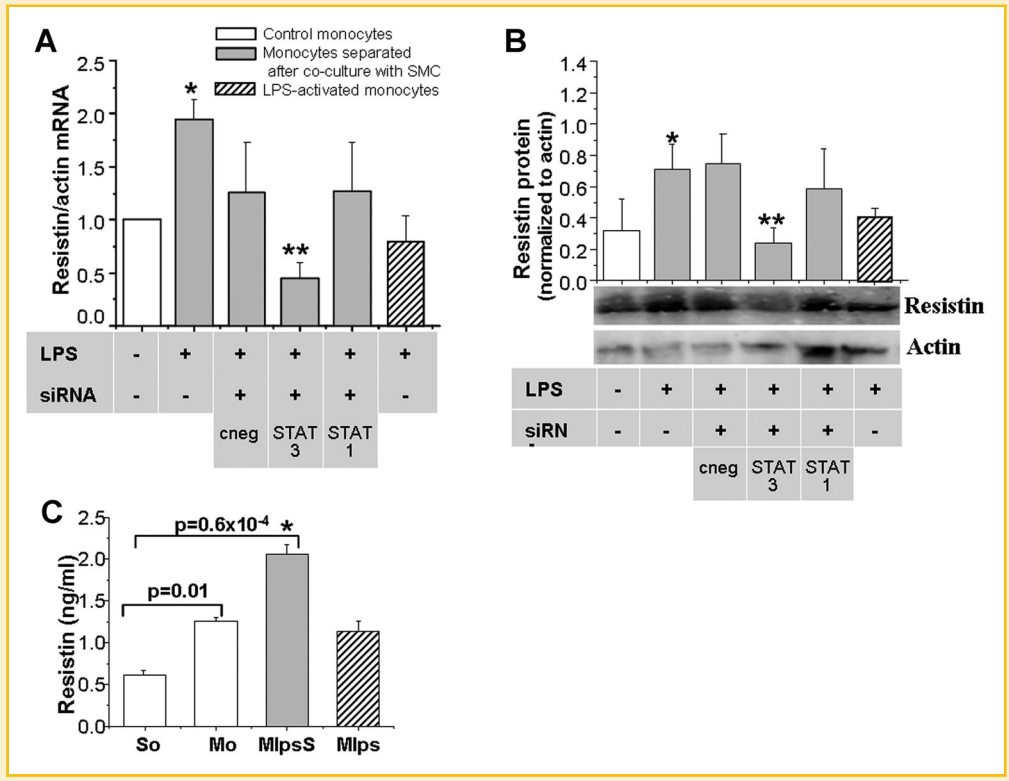

Fig. 4. Monocyte—SMC cross-talk increases resistin expression in monocytes. Resistin gene (A) and protein (B) expression was analyzed in control monocytes (white column), monocytes activated with LPS (hachured column) or LPS-activated monocytes separated after their co-culture with SMC (gray columns). Silencing STAT3 binding sites by transfection with siRNA before cell interaction significantly decreases resistin gene and protein expression. STAT1 siRNA or negative control siRNA did not affect the resistin expression. $^{*}P < 0.05$ interacted LPS-activated monocytes versus control monocytes; $^{**}P < 0.05$ interacted monocytes versus transfected and next interacted cells. C: The co-culture of SMC with LPS-activated monocytes increases resistin secretion in conditioned media (MlpsS) compared with Mo or So. Activation of monocytes with LPS (without further co-culture with SMC) do not affect the resistin secretion (Mlps). n = 3, $^{*}P < 0.01$ interacted LPS-activated monocytes versus control cells (So, Mo).

To assess if monocyte–SMC interaction induces the release of resistin protein, we determined the presence of resistin in the conditioned medium from control monocytes and SMC, monocytes activated with LPS and from co-culture of LPS-activated monocytes with SMC, using an ELISA assay (R&D system). As shown in Figure 4C, the resistin level released in the conditioned media of co-cultured cells (lane MlpsS) was significantly increased compared to resistin level in the culture media of control SMC (So), control monocytes (Mo), or monocytes activated with LPS (Mlps, Fig. 4C).

Since recently it has been shown that STAT1/3 signaling pathway is involved in resistin production [Kim et al., 2013], we investigated the modulation of resistin expression upon monocyte-SMC interaction by both STAT1 and STAT3 transcription factors. Thus, before cell co-culture, the endogenous expression of STAT1 and STAT3 was blocked with target-specific siRNA in both cell types. After cell separation we assessed resistin gene and protein expression in control cells and interacted cells. STAT3 knockdown strongly decreased resistin mRNA (Fig. 4A) and protein (Fig. 4B) expression, while monocyte transfection with negative control (scramble) or STAT1 siRNA had no effect on resistin expression, induced by cell cross-talk (Fig. 4A and B). These data indicate that STAT3 activation in monocytes is specifically involved in the resistin modulation by monocyte-SMC cross-talk.

RESISTIN INCREASES INTRACELLULAR ROS LEVELS IN SMC BY INCREASING THE NADPH OXIDASE ACTIVITY

Previously, it has been demonstrated that resistin increased the intracellular levels of ROS in human coronary artery endothelial cells [Chen et al., 2010]. Here, we evaluated the effect of resistin on ROS levels in human aortic SMC and the involvement of NADPH oxidase in this process. SMC were exposed to resistin (R, 100 ng/ml) for 24 h and intracellular total ROS and O_2^- were evaluated. We found that activation of SMC with resistin increased the intracellular O_2^- and total ROS levels (as determined by DHE and DCFH-DA, respectively). Resistin-induced ROS production was reduced by the NADPH oxidase inhibitor apocynin (100 μM) and the inhibitor of xanthine oxidase allopurinol (50 μM), but not by the inhibitor of the mitochondrial electron transport chain complex I rotenone (50 μM) (Fig. 5A and B). Since recently we have obtained that the exposure of SMC to resistin activates STAT3 [Gan et al., 2013] and the ROS and O_2^- production in SMC induced by interaction of SMC with monocytes was decreased by the inhibitor of STAT3 (S3I-201, Fig. 2A and C), we followed the effect of S3I-201 on ROS induced by resistin. The results showed that the intracellular ROS and O_2^- levels are reduced by STAT3 inhibitor, underling the involvement of STAT3 in ROS production in SMC. Moreover, the NADPH oxidase activity was increased by resistin (Fig. 5C). Searching for the effect of resistin on the regulatory subunits

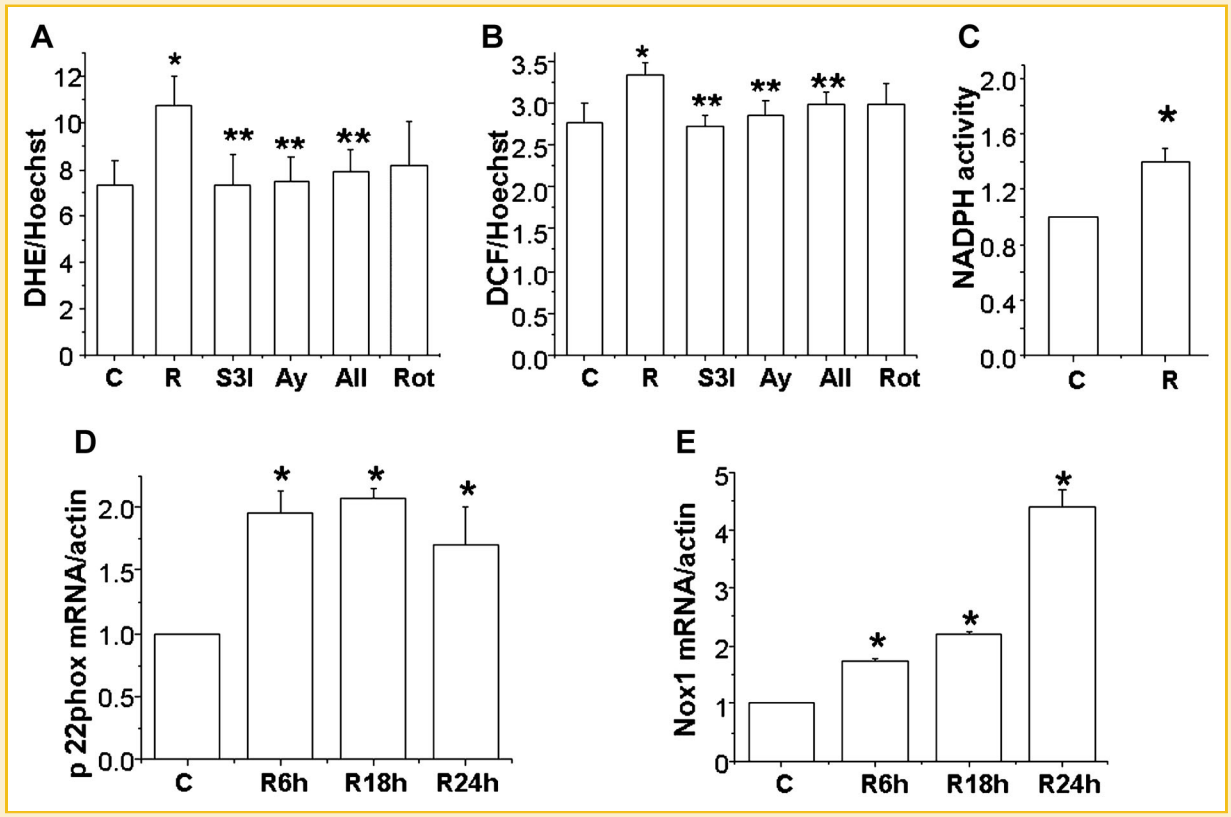

Fig. 5. The effect of resistin on the intracellular level of anion superoxide (A) and reactive oxygen species (B) in SMC. SMC were exposed to resistin (R, 100 ng/ml, 24 h) in presence or absence of STAT3 inhibitor (S3I), apocynin (Ay), rotenone (Rot), or allopurinol (All). (C) non-stimulated control cells; $^{*}P < 0.05$ significantly different from control; $^{**}P < 0.05$ inhibitors versus SMC. C: The NADPH oxidase activity evaluated in control SMC (C) or resistin-activated SMC. Modulation of NADPH oxidase subunities p22phox (D) and Nox1 (E) gene expression in SMC activated with resistin for different time periods. $^{*}P < 0.05$ significantly different from control; ($n = 3$).

of NADPH oxidase expression, we found that both, p22phox (Fig. 5D) and Nox1 (Fig. 5E) gene expression are induced by resistin, starting with 6 h and until 24 h of SMC activation.

DISCUSSION

SMC and macrophages represent major cellular components in vascular disorders such as atherosclerosis. Recent data indicate that the cross-talk between monocytes/macrophages and SMC affects the behavior of both cell types, resulting in the development and progression of atherosclerosis [Zhu et al., 2000; Doran et al., 2008; Butoi et al., 2011]. Activation of STAT3 transcription factor is suggested to be critically involved in switching of SMC phenotype from "contractile" to "synthetic" [Marrero, 2005; Daniel et al., 2012]. Addressing the monocyte/macrophage-dependent mechanisms of SMC activation, or vice versa, may provide important insight into the mechanisms and new therapeutic strategies for various atherosclerotic vascular diseases.

In the present study, we found that the direct interaction between LPS-activated monocytes and SMC modulates STAT3 transcription factor activity by increasing STAT3 phosphorylation and decreasing SOCS3 expression, leading to increased resistin expression in monocytes and intracellular ROS in SMC. In addition, we present evidence that the oxidative stress induced by resistin in SMC is dependent on STAT3 and NADPH oxidase activation.

It is known that JAK/STAT signaling pathway is a critical regulator of inflammatory processes in various cells of the vessel wall. A variety of receptor ligands involving cytokines, interferons, and neurohumoral factors as well as the mechanical stress and oxidative stress activate JAK/STAT signaling pathway and thereby modify the synthesis and release of pro-inflammatory mediators [Grote et al., 2005]. Our experiments demonstrated for the first time, that the cross-talk of activated monocytes with SMC is also an inducer of STAT3 activation. Interestingly, in SMC STAT3 is transiently induced at 15 and 30 min (Supplementary Fig. 1A), as a direct effect of cell interaction, and further induced at 18 h of cell interaction, probably as an indirect effect. In monocytes, we found that STAT3 is time-dependent induced by activated monocytes–SMC cross-talk, starting with 15 min (Supplementary Fig. 1B), and still increased at 18 h. The increased STAT3 phosphorylation at 18 h of cell cross-talk found in this study may be an indirect effect of inflammatory molecules that are known as STATs activators (i.e., IL-6 and TNF-α) produced upon cell interaction [Butoi et al., 2011]. Moreover, even resistin released by cell interaction may activate STAT3, since we recently demonstrated that the resistin induces STAT3 activation in SMC [Gan et al., 2013]. SOCS proteins were identified as negative-feedback

loops to inhibit JAK/STAT pathway signal propagation [Yoshimura et al., 2007]. Thus, the negative regulation of JAK/STAT by the SOCS proteins is crucial for maintaining a well-balanced JAK/STAT signaling [Terrell et al., 2006]. The cross-talk of activated monocytes with SMC for 18 h decreased the SOCS3 expression, which drawn in parallel with the increase in STAT3 phosphorylation, underling the activation state of the cells. It was demonstrated that SOCS1/SOCS3 inhibition, leading to sustained cytokine activation of STAT, contributes to the progression of chronic inflammatory diseases [Wong et al., 2006], whereas SOCS3 expression suppressed inflammation in mouse arthritis [Yoshimura et al., 2007]. We found that at shorter time of cell interaction the SOCS3 protein was rapid and time-dependent reduced in monocytes (inversely with STAT3 phosphorylation), and un-modified in SMC (Supplementary Fig. 1A and B), suggesting different regulatory mechanisms in SMC compared with monocytes. In this respect, it was found that SOCS can also be induced by other signaling pathways, independent of JAK/STAT signaling [Cittadini et al., 2012].

Although abnormalities in JAK-STAT signaling pathway are implicated in vascular SMC dysfunction [Grote et al., 2005], little is known regarding the interference of JAK-STAT signaling with the production of ROS. Our previous studies revealed that the cross-talk between SMC and monocytes induced increased ROS levels in SMC [Butoi et al., 2011]. Here, we found that ROS are also induced in SMC as a result of the interaction between SMC and LPS-activated monocytes and that STAT3 activation is involved in this process. Since STAT3 was found to be activated rapidly by cell interaction (15 and 30 min), and inhibition of STAT3 activation reduced ROS, we may suppose that the activation of JAK/STAT pathway is involved in ROS production. Thus, the ROS production can be another effect of STAT3 activation leading to SMC dysfunction. Moreover, inhibitors of NADPH oxidase reduced the ROS generated by cell cross talk. Previously, it was shown that superoxide anions generated by NADPH oxidase are critically involved in JAK/STAT activation [Schieffer et al., 2000]. All these data assume that there is an autocrine pathway of ROS production and STAT3 activation in SMC. Interestingly, in monocytes we found that ROS are reduced by cell cross-talk. This result, together with increased STAT3 phosphorylation (that previously was found essential for maintaining macrophage survival [Liu et al., 2003]) can sustain the previously data showing that vascular SMC/monocyte interactions lead to antiapoptotic effects and increased CD36 expression in monocytes [Cai et al., 2004], leading thus to macrophage differentiation.

In addition to involvement of JAK/STAT in migration and proliferation of SMC there is know that this signaling pathways controls important inflammatory processes in vascular cells [Grote et al., 2005]. Increasing evidences indicate resistin as an inflammatory molecule with important roles in atherosclerosis and cardiovascular diseases [Jung et al., 2006]; however, the molecular mechanism underlying the increase of resistin expression in inflammatory conditions remains unclear. Previously it was shown that TNF-α and high glucose concentrations increase resistin levels in monocytes/macrophages by different mechanisms involving JNK and MAPKs pathways and NF-kB and AP-1 transcription factors [Shyu et al., 2009; Stan et al., 2011]. Moreover, STAT3 transcription factor was found to be involved in resistin production in rat vascular smooth muscle [Wang et al., 2009]. Since recently we have shown that monocyte-SMC cross-talk induces pro-inflammatory molecules, here we investigated if cell cross-talk increases resistin expression in monocytes. Our data indicate that the interaction between LPS-activated monocytes and SMC increases the gene and protein expression of resistin in separated monocytes after co-culture, compared with control monocytes. Activation of monocytes with LPS did not increase gene or protein expression of resistin compared with control. Moreover, resistin was significantly increased in LPS-activated monocytes separated after interaction with SMC, compared with resistin expression in monocytes activated with LPS (without further co-culture), demonstrating that the increased expression of resistin is specifically due to the cell cross-talk. There are conflicting results regarding the effect of LPS on resistin in U937 monocytes. Thus, it has been shown that LPS does not affect resistin expression in U937 at 4 h [Yang et al., 2003], while other data indicate that LPS up-regulates resistin in neutrophils and in U937 cells at 4 and 24 h [Kunnari et al., 2009]. The induction of resistin expression by cell cross-talk was mediated by STAT3 pathway since the knockdown of STAT3 by specific siRNA inhibited the induction of resistin.

Interestingly, our preliminary data indicate that the cross-talk between human freshly isolated monocytes with SMC increased the resistin expression (Supplementary Fig. 2A) while p22phox or Nox2 expressions were not affected in human monocytes (Supplementary Fig. 2B and C). Moreover, as in the experiments with U937 cells, the gene expression of Nox1 in SMC (separated after co-culture with human monocytes) was increased and p22phox expression was not significantly affected (Supplementary Fig. 2D and E). These new data may suggest that human monocytes display similar cross-talk features as the U937 cell line and further experiments will clarify this matter.

In vitro studies revealed that resistin affects not only monocytes themselves but also their vascular counterparts, endothelial cells and SMC. Thus, it was found that resistin induces ROS in endothelial cells [Manduteanu et al., 2009; Chen et al., 2010] and promotes vascular SMC migration and proliferation [Calabro et al., 2004; Jiang et al., 2009]. In the current study we found that resistin increased the intracellular ROS levels in SMC. By inducing the NADPH oxidase activity, resistin has an impact on ROS production, which is likely one of the mechanism for vascular cells dysfunction. Understanding the molecular mechanisms of resistin action may help to develop new and effective strategies to control the detrimental effects of resistin on the vascular system. Thus our results underlined that the inhibitors of NADPH oxidase, of xantin oxidase and of STAT3 reduced the ROS produced by resistin in SMC.

Taken together, our present results indicate that the cross-talk of activated monocytes with SMC activates the STAT3 in both cell types and increases resistin expression in monocytes and the intracellular level of ROS in SMC. Moreover, exposure of SMC to resistin increases the ROS dependent by NADPH oxidase and STAT3 activation.

Our previous results showing that the monocyte-SMC interactions increase the expression of TNF-α, IL6, IL-1β, CX3CR1, and MMP9, together with the present results demonstrating that cross-talk between activated monocytes and SMC activates STAT3 in both cells leading to ROS production in SMC and up-regulation of resistin in monocytes, suggest that the cell cross-talk could lead to SMC

dysfunction and be an important factor in the progression of atherosclerotic lesions, and may indicate important targets for anti-inflammatory therapy in atherosclerosis.

ACKNOWLEDGMENTS

We are thankful to Gabriela Mesca for skilful assistance. This work was supported by a grant from the Romanian National Authority for Scientific Research, CNCS-UEFISCDI, project PN-II-ID-PCE-2011-3-0928, by L'Oréal-UNESCO Awards 2011 "For women in science", by CARDIOPRO Project ID: 143ERDF co-financed investment in RTDI for Competitiveness and by the Romanian Academy.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

Supplementary Figure 1. Dynamic response of phospho-STAT3 and SOCS3 in SMC (A) and monocytes (B) at shorter time period of interactions (15, 30, 60 min) between SMC and LPS-activated monocytes. Representative blot of two independent experiments. The effect of LPS on pSTAT3 (C) and SOCS (D) protein expression. Total STAT3, and SOCS were evaluated by Western Blot experiments in control SMC (So) and LPS activated SMC (Slps). Exposure of SMC with LPS do not affect pSTAT3 or SOCS3 expression, $n = 3$.

Supplementary Figure 2. The effect of human monocyte–SMC interaction on resistin, p22phox (A and C), Nox1 (B) and resistin (D) gene expression. Human freshly isolated monocytes were exposed to 100ng/ml LPS and co-cultured with SMC for 18 h. Then, the interacted monocytes (Mi) were separated from SMC (Si) and p22phox, Nox1 and resistin mRNA was quantified in control human monocytes (Mo) control SMC (So) and separated monocytes and SMC. White columns: control monocytes and SMC; gray and black columns: separated monocytes and SMC, respectively, after their co-culture. Interactions of human monocytes with SMC increase the Nox1 gene expression in SMC and resistin in monocytes. $^{*}P < 0.05$, co-cultured versus control cells.