

## Original Article

# A crosstalk triggered by hypoxia and maintained by MCP-1/miR-98/IL-6/p38 regulatory loop between human aortic smooth muscle cells and macrophages leads to aortic smooth muscle cells apoptosis via Stat1 activation

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**Abstract:** Hypoxia and inflammation are central characteristics of the abdominal aortic aneurysm (AAA), but the mechanisms for their relationship and actual role remain far from full understood. Here, we showed MCP-1 (monocyte chemoattractant protein-1) induced by hypoxia in primary human Aortic Smooth Muscle Cells (hASMCs) increased the chemotaxis of THP-1 macrophages and MCP-1 induced IL-6 expression in THP-1 cells via downregulating miR-98 which directly targets IL-6. In addition, IL-6 positively feedback regulated MCP-1 expression in hASMCs via p38 signal that is independent on hypoxia, and inhibition of p38 signal blocked the effect of IL-6 on MCP-1 expression regulation. Moreover, IL-6 exposure time-dependently induces hASMCs apoptosis via Stat1 activation. Collectively, our data provide compelling evidence on the association between hypoxia and inflammation triggered by hypoxia and then mediated by MCP-1/miR-98/IL-6/p38 regulatory loop, which leads to hASMCs apoptosis via Stat1 activation to contribute to AAA formation and progression.

**Keywords:** Abdominal aortic aneurysm, MCP-1, hypoxia, IL-6, macrophage

## Introduction

An abdominal aortic aneurysm (AAA) is a permanent localized dilatation of the abdominal aorta for adults aged > 65 years, which are often asymptomatic and are discovered as incidental findings in imaging investigation (such as abdominal ultrasonography or computerized tomography (CT) examination) or when the AAA ruptures leading to a medical emergency [1, 2]. Because no established pharmacological treatment is currently available to prevent AAA advancement and rupture [3], only 21% to 33% of patients with aortic rupture survive to surgery, with an additional 50% mortality following surgery [4]. Hallmarks of AAA pathogenesis include inflammation, vascular smooth muscle cell (VSMC) apoptosis, extracellular matrix (ECM) degradation and oxidative stress and so on [2].

Despite the frequency and associated morbidity and mortality of AAA, the specific cellular and molecular mechanisms that underlie AAA formation and progression have not been fully elucidated. But one of the candidates may be arterial wall hypoxia, which has been investigated in conditions such as atherosclerosis, intimal hyperplasia and myointimal hyperplasia [5]. Animal models of AAA have demonstrated low oxygen tension in the aortic media [6]. Studies have shown hypoxia exists in vivo in aneurysms of patients and low oxygen tensions within the aortic media are said to alter vascular smooth muscle cell function leading to aortic degradation and potential aneurysm formation, and that intraluminal thrombus as a common feature of AAA contributes to wall hypoxia and weakening [7, 8]. The global oxygen sensor HIF-1 $\alpha$  responds to low tissue oxygen by regulating hypoxia responsive genes that involved in a

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variety of signals to elicit a variety of functional responses in VSMCs to maintain vascular homeostasis [9].

AAA is histologically characterized by transmural infiltration of inflammatory cells [10], and data generated in mouse aneurysm models indicate that macrophage mediated inflammation is critical for the development and progression of aneurysm [11]. In AAA, macrophage are recruited prominently to the adventitia and media, where they secrete proteases that lead to matrix degradation, smooth muscle cells (SMCs) apoptosis, tissue weakening, and aortic enlargement [12]. Macrophage precursor recruitment to the adventitia during AngII-induced AAA formation was reported to be dependent on adventitial secretion of interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) and of CCR2 expression in monocytes [13]. Macrophage's cytotoxic function has been documented in models of cardiovascular diseases. In atherosclerosis, macrophages were found to induce SMCs apoptosis, thus contributing to plaque rupture [14, 15]. Macrophages were reported to induce cell apoptosis through secretion of Fas Ligand in a model of heart failure [16]. Although macrophages have been reported to induce cell death through several cytokines including TNF $\alpha$  and IL-1 $\beta$ , whether infiltrating macrophages directly contribute to SMC apoptosis remains to be explored.

Despite of the central role of hypoxia and macrophages infiltration in AAA formation and progression, the molecular mechanisms of and the relationship between hypoxia and macrophages remain unclear. Here, we found hypoxia triggered the MCP-1/miR-98/IL-6 regulatory loop mediated by p38 signal and lead to SMCs apoptosis by Stat1 activation to promote the AAA progression.

### Materials and methods

#### Cell culture

Primary human Aortic Smooth Muscle Cells (phASMCs) and growth kit were obtained from ATCC and cultured in vascular cell basal medium supplemented with 5 ng/ml rhFGF-basic, 5  $\mu$ g/ml Insulin, 50  $\mu$ g/ml Ascorbic Acid, 10 mM L-glutamine, 5 ng/ml rhEGF and 5% fetal bovine serum) in a humidified cell incubator with an

atmosphere of 5% CO<sub>2</sub> at 37°C. THP-1 cells and 293T cells were cultured in RPMI1640 or DMEM (Life Technologies), supplemented with 10% fetal bovine serum (Life Technologies) in a humidified cell incubator with an atmosphere of 5% CO<sub>2</sub> at 37°C.

#### Real-time PCR for mature miRNAs and mRNA

Total RNA was extracted with a Trizol protocol, and cDNAs from the mRNAs were synthesized with the Super-Script first-strand synthesis system (Fermentas Life Science). Real-time PCR was carried out according to the standard protocol on ABI 7500 with SYBR Green detection (Applied Biosystems). GAPDH was used as an internal control and the qRT-PCR was repeated three times. The primers for GAPDH were: forward primer 5'-GAAAGCCTGCCGTTGACTAA-3', reverse primer 5'-GCATCACCCGAGGAGA-AAT-3'; for MCP-1 were: forward primer 5'-GATCTCAGTGCAGAGGCTCG-3', reverse primer 5'-TTTGCTTGCCAGGTGGTCC-3'; for IL-6 were: forward primer 5'-TGCAATAACCACCCCTGACC-3', reverse primer 5'-GTGCCCATGCTACATTGCC-3'. MiRNAs from cultured cells isolated and purified with miRNA isolation system (OMEGA Bio-Tek). cDNA was generated with the miScript II RT Kit (QIAGEN) and the quantitative real-time PCR (qRT-PCR) was done by using the miScript SYBR Green PCR Kit (QIAGEN) following the manufacturer's instructions. The miRNA sequence-specific qRT-PCR primers for miR-98 and endogenous control RNU6 were purchased from QIAGEN, and the qRT-PCR analysis was carried out using ABI 7500 Real-Time PCR System (Applied Biosystems). The gene expression threshold cycle (CT) values of miRNAs were calculated by normalizing with internal control RNU6 and relative quantization values were calculated.

#### Western blot

Total proteins were extracted from corresponding cells using the RIPA buffer (Pierce) in the presence of Protease Inhibitor Cocktail (Pierce). The protein concentration of the lysates was measured using a BCA Protein Assay Kit (Pierce). Equivalent amounts of protein were resolved and mixed with 5 $\times$  Lane Marker Reducing Sample Buffer (Pierce), electrophoresed in a 10% SDS-acrylamide gel and transferred onto Immobilon-P Transfer Membrane

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(Millipore). The membranes were blocked with 5% non-fat milk in Tris-buffered saline and then incubated with primary antibodies followed by secondary antibody. The signal was detected using an ECL detection system (Millipore). The HIF-1 $\alpha$ , MCP-1, IL-6, p38, p-p38 and pStat1 antibodies were from Santa Cruz Biotechnology and pMK2 antibody was from NOVUS, and  $\beta$ -Actin antibody was from Cell Signaling Technology. HRP-conjugated secondary antibody was from Thermo.

### ELISA assay

Cells were cultured in 35-mm dishes at a density of  $5 \times 10^4$  cells/dishes for 12 hours, and then replaced with corresponding treatments for 36 hours. After cultured in fresh medium for 24 hours, the culture medium was collected and 0.2 ml medium was used to analyze MCP-1 and IL-6 protein production using ELISA kit from RD Systems according to the manufacturer's instructions.

### Chemotaxis assay

Chemotaxis of THP-1 cells was assessed using the transwell Assay Kit (BD Biosciences) according to the manufacturer's instructions. Briefly, THP-1  $3 \times 10^4$  cells in 300  $\mu$ l serum-free medium were added to the upper chamber. Then, 0.5 ml of conditioned medium was added to the lower chamber as a chemoattractant. Cells were incubated for 24 h at 37°C, and then non-chemotaxis cells were removed with cotton swabs. Cells that migrated to the bottom of the membrane were fixed with pre-cold methanol and stained with 2% Giemsa solution. Stained cells were visualized under a microscope. To minimize the bias, at least three randomly selected fields with 100 $\times$  magnification were counted, and the average number was taken.

### Transfection of miRNA mimics, inhibitor and siRNAs

miR-98 mimics and relative control were purchased from Ambion.  $5 \times 10^5$  THP-1 Cells were seeded onto 6-well plates the day before transfection. The transfection was carried out using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's procedure. The mimics and control were used at a final concentration of 100 nM. At 48 h post-transfection, follow-up experiments were performed. The siRNAs tar-

geting p38, Stat1 and control were purchased from Santa Cruz Biotechnology. The transfection of 50 nM, 100 nM and 200 nM p38 siRNAs or control was performed as above, 48 h later, p38 was determined by western blot, and the experiment was repeated four times. The transfection of 200 nM Stat1 siRNAs was performed as above.

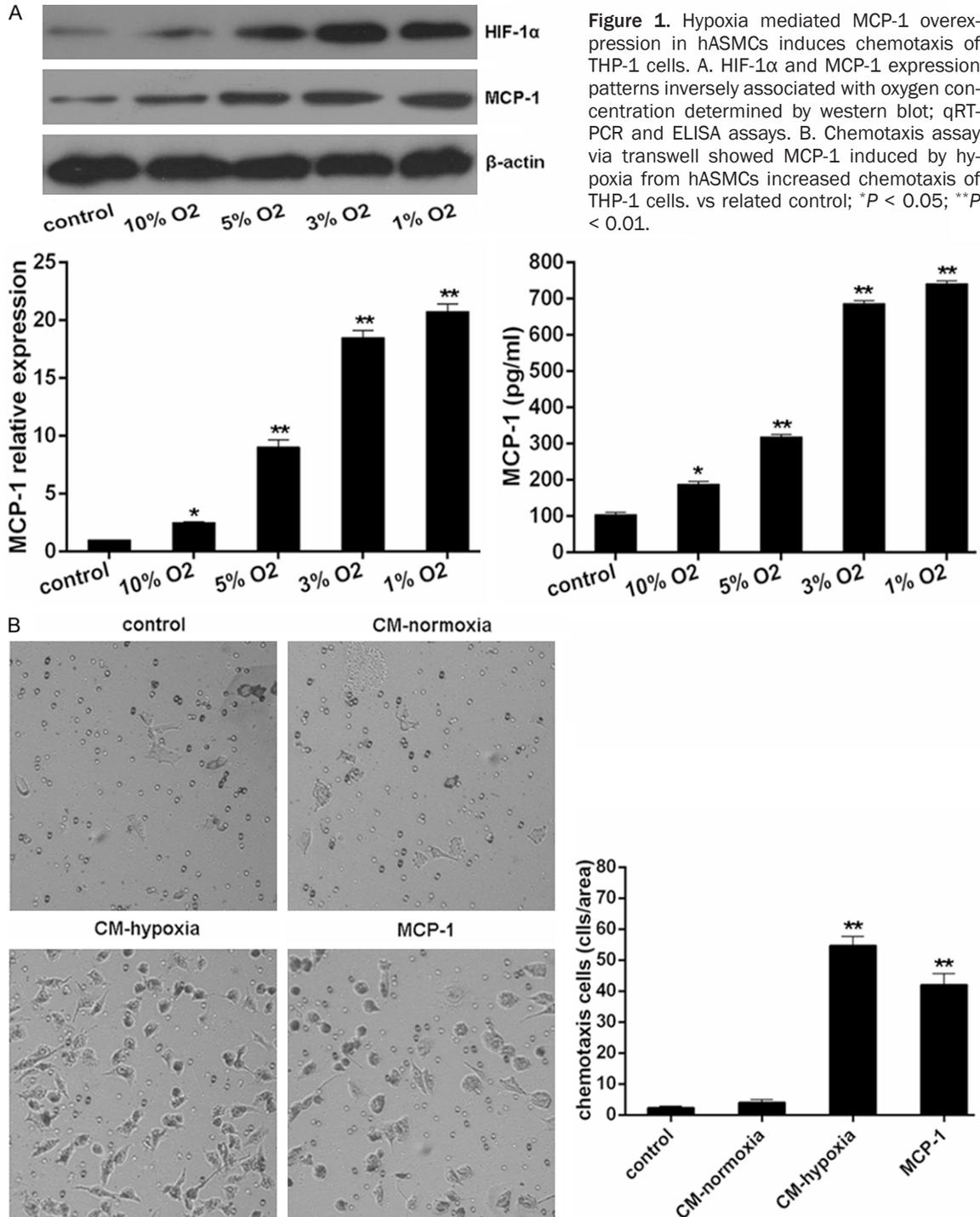
### Luciferase reporter assay

Two single strands of the wild type 3'UTR with miR-98 binding site and two single strands of the mutant type with 7 bases deleted in the miR-98 binding site (as mutant control), of IL-6 were synthesized with restriction sites for SpeI and HindIII located at both ends of the oligonucleotides for further cloning. The single strands DNA sequences were following: the wild type 3'UTR of IL-6 (sense: 5'-CTAGT TCAGAGCCAGATCATTCTTGAAAGTGTAGGCTTACCTCAAATAAATGGCTAACTTATACATATTTTTAAA-3'; antisense: 5'-AGCTT TAAAAATATGTATAAGTTAGCCATTATTATTGAGGTAAGCCTACACTTTCCAAGAAATGATCTGGCTCTGAA-3') and the mutated type 3'UTR of IL-6 (sense: 5'-CTAGT TCAGAGCCAGATCATTCTTGAAAGTGTAGGCT-----AATAAATGGCTAACTTATACATATTTTTAAA-3'; antisense: 5'-AGCTT TAAAAATATGTATAAGTTAGCCATTATT-----AGCCTACACTTTCCAAGAAATGATCTGGCTCTGAA-3'). The corresponding sense and antisense strands were annealed and subsequently cloned into pMir-Report plasmid downstream of firefly luciferase reporter gene. 293T Cells were seeded in 96 well-plates and co-transfected with pMir-Report luciferase vector, pRL-TK Renilla luciferase vector and miR-98 mimics or control. 48 h later the luciferase activities were determined using a Dual-Luciferase Reporter Assay System (Promega) where the Renilla luciferase activity was used as internal control and the firefly luciferase activity was calculated as the mean  $\pm$  SD after being normalized by Renilla luciferase activity.

### Statistical analysis

All data are expressed as means  $\pm$  standard deviation from three independent experiments. Statistical analyses were performed using SPSS16.0 software (SPSS, Chicago, IL). The differences between groups were analyzed using Student's t-test with only two groups or one-way analysis of variance (ANOVA) when more than two groups were compared. *P* values less than 0.05 were considered statistically significant.

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**Figure 1.** Hypoxia mediated MCP-1 overexpression in hASMCs induces chemotaxis of THP-1 cells. A. HIF-1 $\alpha$  and MCP-1 expression patterns inversely associated with oxygen concentration determined by western blot; qRT-PCR and ELISA assays. B. Chemotaxis assay via transwell showed MCP-1 induced by hypoxia from hASMCs increased chemotaxis of THP-1 cells. vs related control; \* $P < 0.05$ ; \*\* $P < 0.01$ .

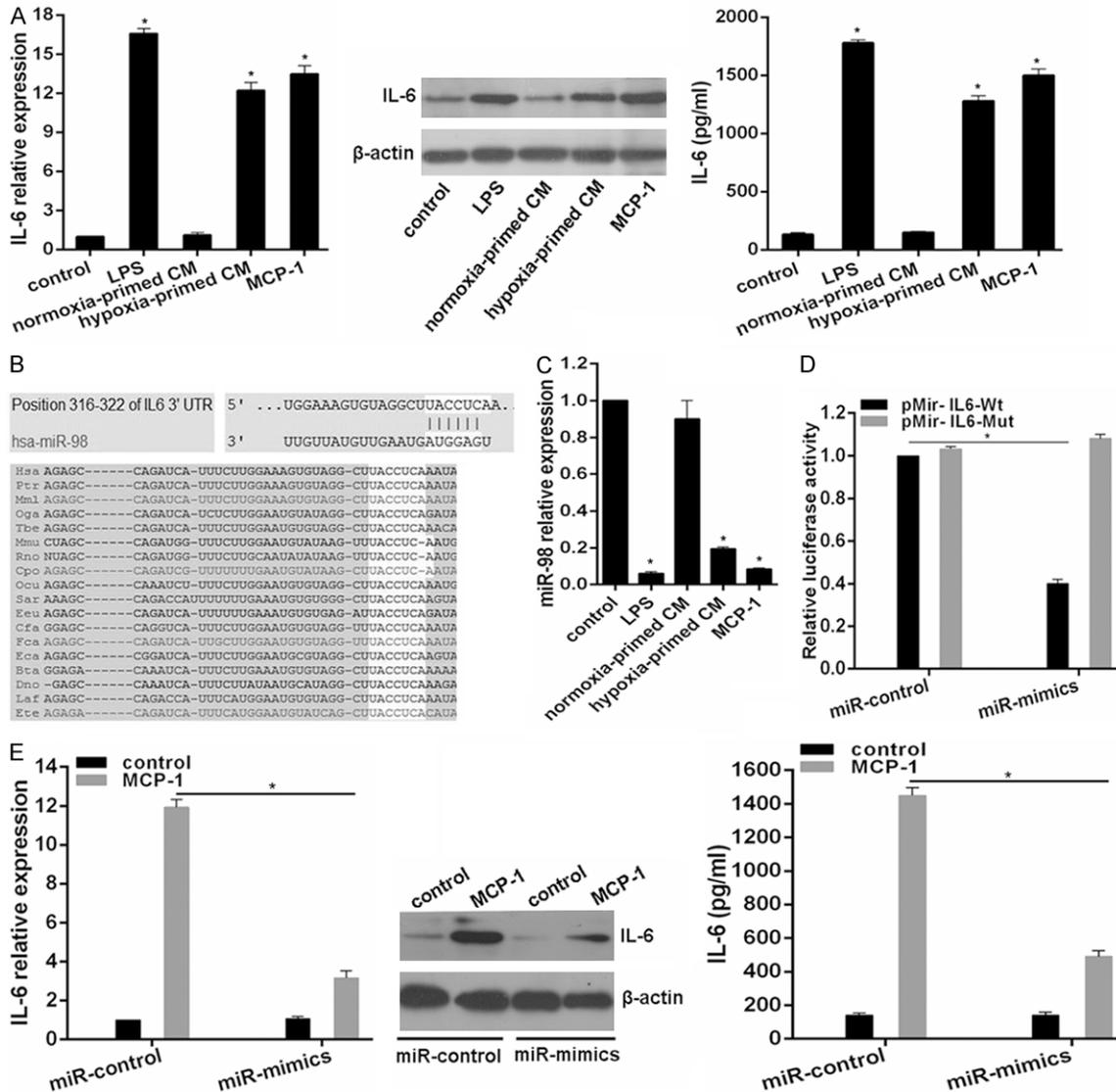
### Results

#### *Hypoxia increases chemotaxis of THP-1 cells via inducing MCP-1 expression in hASMCs*

Hypoxia is an essential feature of AAA, to investigate the role and mechanisms of hypoxia in AAA, primary Human Aortic Smooth Muscle

Cells (hASMCs) were exposed to different oxygen level for 24 h. Then the oxygen sensor HIF-1 $\alpha$  protein level and MCP-1 expression in mRNA and protein levels were examined. As shown in **Figure 1A**, hypoxia at 3% oxygen significantly induced HIF-1 $\alpha$  protein level accompanied with increase of MCP-1mRNA and protein levels in cells and culture media. So 3% oxygen was

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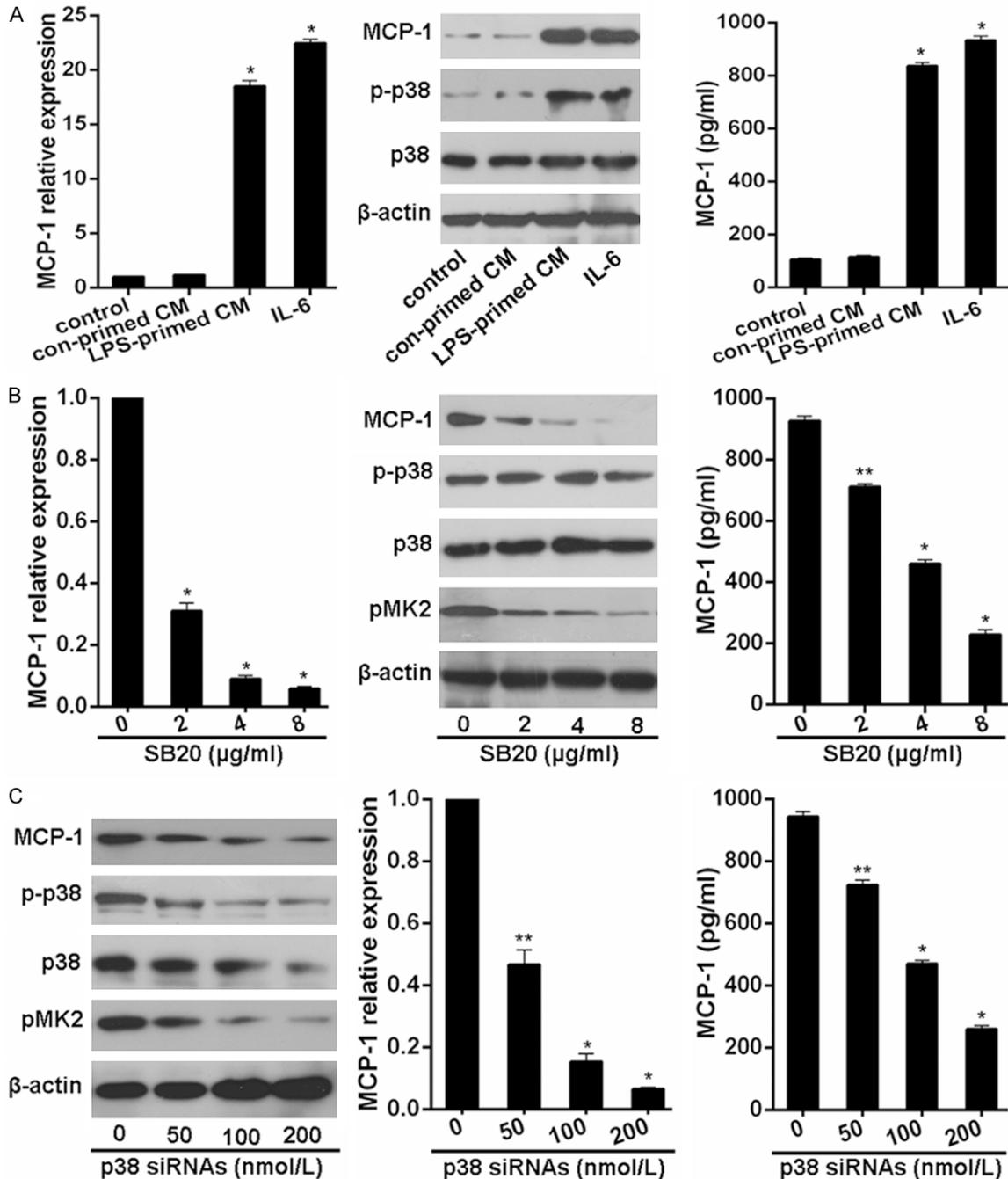


**Figure 2.** MCP-1 induces IL-6 expression via downregulating miR-98 expression. A. Hypoxia primed CM and MCP-1 induced IL-6 expression in THP-1 cells detected by qRT-PCR; western blot and ELISA assays; and CM from LPS treatment was set as positive control; vs control;  $*P < 0.01$ . B. Schematic of the putative binding sites of miR-98 in 3'-UTR of IL-6 is presented; which is broadly conserved among vertebrates. C. miR-98 expression pattern detected by qRT-PCR; vs control;  $*P < 0.01$ . D. Luciferase reporter assay in 293T cells cotransfected with miR-miR-98 mimics; a luciferase reporter containing wild-type IL6 3'-UTR or a mutant version; and a renilla luciferase reporter for normalization. The mean of the results from cells transfected with pMir-IL6-Wt and miR-control was set as 1;  $*P < 0.01$ . E. IL-6 mRNA and protein levels induced by MCP-1 under ectopic miR-98 overexpression in THP-1 cells and culture media were determined with qRT-PCR; Western blot and ELISA; respectively;  $*P < 0.01$ .

applied as hypoxia in followed experiments. To verify whether hypoxia induced MCP-1 contributes to macrophages chemotaxis, chemotaxis assay was performed on THP-1 cells. Conditioned media (CM) from hASMCs under hypoxia culture and 100 ng/ml MCP-1 induced robust chemotaxis of THP-1 cells (**Figure 1B**), implicating hypoxia maybe triggered macrophages chemotaxis via induction of MCP-1 from hASMCs.

### MCP-1 induces IL-6 expression via downregulating miR-98 expression in THP-1 cells

Interleukin 6 (IL-6) as an important inflammatory mediator is a multifunctional cytokine that involved in immune responses, cell survival, apoptosis and proliferation. Here, we found IL-6 mRNA and secretory protein levels in THP-1 cells increased after incubation with CM from hypoxia-primed hASMCs and LPS stimulation

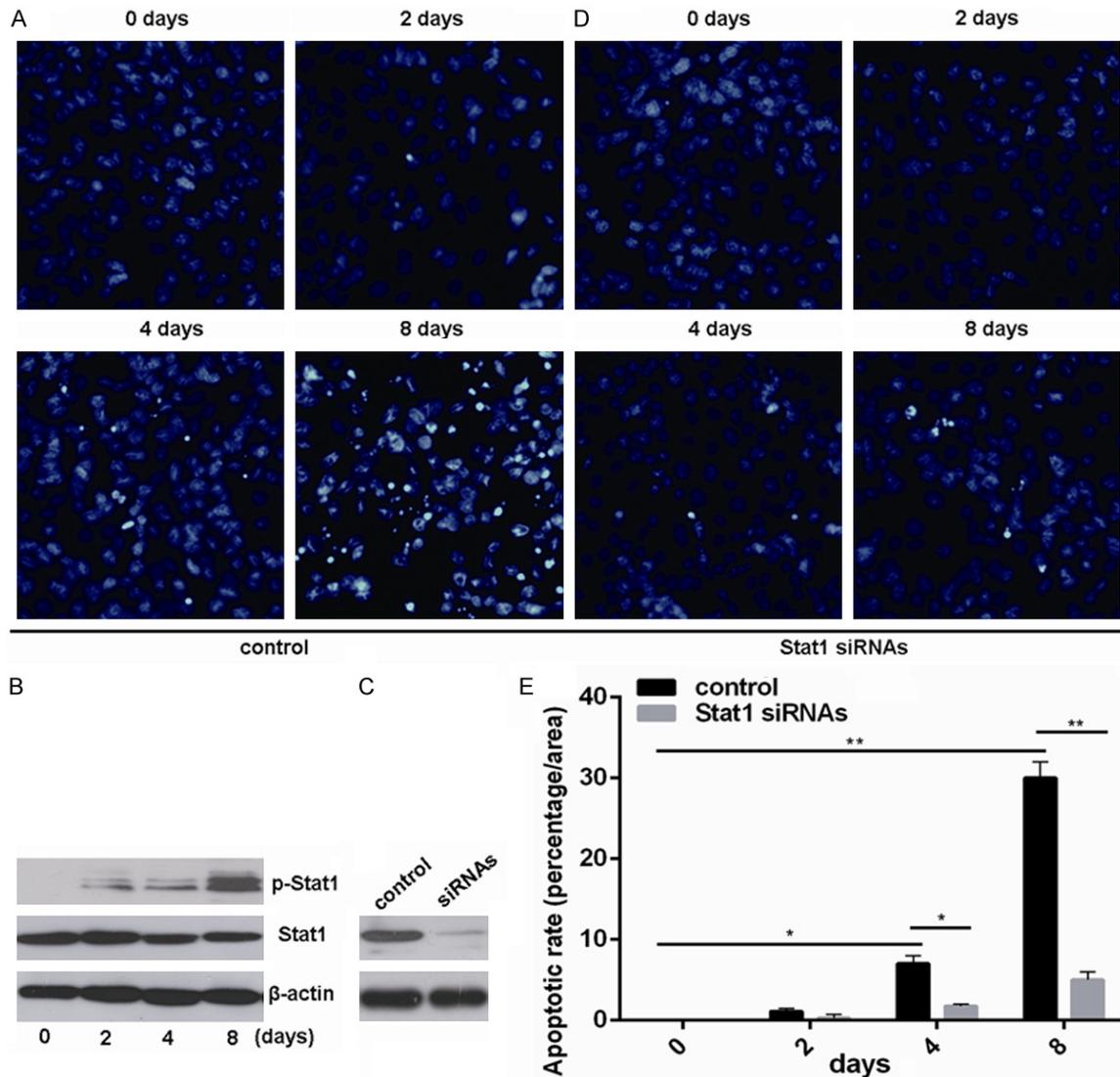


**Figure 3.** p38 mediated the expression induction of MCP-1 by IL-6 in hASMCs. A. IL-6 and CM from LPS activated THP-1 cells induced MCP-1 expression and p38 activation determined by qRT-PCR; Western blot and ELISA assays. B. p38 signal inhibitor impaired IL-6 induced MCP-1 expression in PHASMCs dose-dependently. C. p38 siRNAs knockdown p38 and blocked IL-6 induced MCP-1 expression in PHASMCs dose-dependently. Vs related control; \* $P < 0.01$ ; \*\* $P < 0.01$ .

(as positive control) and 100 ng/ml MCP-1 treatments (Figure 2A). To explore the mechanisms of MCP-1 induced IL-6 upregulation, we focused on the miRNA mediated expression regulation. Potential miRNAs that will target

IL-6 were predicted using public database-TargetScan (<http://www.targetscan.org>) and miR-98 with critically conserved binding site was selected for further expression and function confirmation (Figure 2B). Expectedly, miR-98

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**Figure 4.** IL-6 induced hASMCs apoptosis via Stat1 activation. A and D. The apoptosis was determined by hoechst staining evaluated by fluorescence microscopy. B and C. Related proteins levels were detected by Western blot. E. The apoptotic rate was calculated. \* $P < 0.05$ ; \*\* $P < 0.01$ .

expression was markedly downregulated in THP-1 cells after incubated with CM from hypoxia-primed and LPS activated PHASMCs and 100 ng/ml MCP-1 treatment, which was inversely associated with IL-6 expression pattern (**Figure 2C**). To determine whether miR-98 directly targets IL-6, the luciferase reporter vectors with the putative IL-6 3'-UTR target site for miR-98 downstream of the luciferase gene (pMir- IL6-Wt, set as wild-type) and mutant version with a deletion of 6 bp in the seed region (pMir- IL6-Mut) were constructed. As shown in **Figure 2D**, miR-98 mimics significantly reduced luciferase activity of the vector with the wild-

type IL-6 3'-UTR in 293T cells, but the mutant version abrogated the suppressive ability of miR-98. To investigate whether miR-98 is involved in MCP-1 induced IL-6 expression, THP-1 cells firstly were transfected with miR-98 mimics, and then the IL-6 expression was measured after 100 ng/ml MCP-1 treatment. As shown, overexpressed miR-98 obviously impaired MCP-1 induced IL-6 expression in THP-1 cells (**Figure 2E**). These results strongly suggest downregulation of miR-98 led to IL-6 overexpression via direct binding to putative binding site in the IL-6 3'-UTR region during CM or MCP-1 induction in THP-1 cells.

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### *IL-6 feedback induce MCP-1 expression via p38 MAPK in hASMCs*

As shown above, MCP-1 increased chemotaxis of THP-1 cells and induced IL-6 expression in THP-1 cells, here we demonstrated that CM from LPS activated THP-1 cells and 20 ng/ml IL-6 treatment upregulated MCP-1 mRNA and protein expression in hASMCs, and p38 signal was obviously activated represented as increase of phosphorylated p38 (p-p38) level (**Figure 3A**). p38 signal specific inhibitor SB203580 (SB20) treatment blocked 10 ng/ml IL-6 induced MCP-1 mRNA and protein upregulation in a dose-dependent manner in hASMCs, accompanied with the decreased level of phosphorylated MAPKPK-2 (pMK2), a downstream kinase of p38, but not p-p38 (**Figure 3B**). Because SB20 does not inhibit phosphorylation of p38 but inhibits phosphorylation of its downstream kinase MK2, siRNAs specific for p38 were applied to investigate the direct effect of p38 signal on IL-6 induced MCP-1 expression. We found siRNAs significantly inhibited the expression of MCP-1, p-p38, p38 and pMK2 in hASMCs incubated with 20 ng/ml IL-6 in a dose-dependent manner (**Figure 3C**). These results suggested IL-6 from macrophages feedback induces MCP-1 expression via p38 MAPK signal in hASMCs.

### *IL-6 contributes to apoptosis of hASMCs by activating Stat1*

To assess whether overexpressed IL-6 from activated macrophages has biological effects beyond MCP-1 expression induction, apoptosis was determined by hoechst staining in hASMCs in the presence of 20 ng/ml IL-6 at different time points. As shown, the apoptotic rate remarkably increased in hASMCs cells time-dependently in the presence of 20 ng/ml IL-6 (**Figure 4A and 4D**). In regard to the mechanism, we found IL-6 treatment significantly led to Stat1 activation represented as p-Stat1 level increase time-dependently examined by western blot (**Figure 4B**). To investigate whether Stat1 signal was involved in IL-6 induced hASMCs apoptosis, siRNAs specific for Stat1 were applied. We found siRNAs significantly inhibited Stat1 expression and blocked IL-6 induced hASMCs apoptosis (**Figure 4C-E**). These results suggest IL-6 from activated macrophages will induce hASMCs apoptosis time-dependently.

## Discussion

Hypoxia and inflammation are two major characteristics of AAA, but the cellular biological role between them and mechanisms responsible for their relationship remains unclear. In the present study, we showed hypoxia promoted HIF-1 $\alpha$  protein accumulation and induced MCP-1 expression in mRNA and protein levels with secretion increase in human Aortic Smooth Muscle Cells (hASMCs). To assess whether MCP-1 from hASMCs induced by hypoxia had direct biological effect on inflammation, we performed chemotaxis assay and found conditioned medium from hypoxia primed hASMCs or MCP-1 alone effectively induced THP-1 macrophages chemotaxis. Beyond chemotaxis induction of THP-1 cells, MCP-1 also upregulated IL-6 expression via inhibiting miR-98 expression in THP-1 cells.

MCP-1 has been implicated in the pathogenesis of several cardiovascular diseases including AAA. Elevated MCP-1 mRNA and protein expression has been consistently detected in aneurysmal aortic tissues of human patients as well as animal models [17-19]. Blocking MCP-1 signal via a series of methods successfully inhibits AAA development [13, 20, 21]. Ectopic administration of MCP-1 to the arterial wall of PKC $\gamma$ -mice, which has an aneurysm-resistant phenotype, was sufficient to restore local inflammatory response and AAA development [22]. Recently, a report using a SMC/RAW264.7 macrophage co-culture system, showed MCP-1 primed mouse macrophage RAWs caused a significantly higher level of apoptosis in SMCs as compared to control macrophages [23]. Here, we found human recombinant MCP-1 induced IL-6 expression in human macrophages THP-1 via downregulating miR-98 and confirmed IL-6 is a direct target of miR-98 upon MCP-1 stimulus.

microRNAs (miRNAs) have emerged as an established class of well-conserved, short non-coding RNAs (19-25nt) that play major roles in various biological processes including cell-cycle regulation, cell differentiation, apoptosis, development, angiogenesis and metabolism, by controlling stability and translation of mRNAs in a sequence-specific manner [24]. miRNAs have also been implicated in the etiology of a variety of human disease, such as cancer, metabolic diseases, cardiovascular diseases, neuro-degenerative diseases, viral infection, and

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many others [25]. MiR-29 is likely to be a crucial regulator of aortic aneurysm disease by modulating several genes and pathways responsible for ECM composition, dynamics, and disease contributing impairment [26]. In murine AAA experiment from Maegdefessel et al, miR-29b was significantly downregulated at three different time points during murine AAA development and progression [27]. Here, our data implicated miRNAs involved in AAA maybe from infiltrating macrophages, such as miR-98. To assess whether THP-1 cells have feedback effect on hSMCs via IL-6, we found IL-6 not only positively feedback regulated MCP-1 expression, but also led to apoptosis increase of hSMCs to educed the cytotoxic effect of macrophages. IL-6 is a multifunctional cytokine that is important for immune responses, cell survival, apoptosis and proliferation [28]. Our investigation demonstrated IL-6 mediated MCP-1 upregulation in hSMCs via p38 signal activation independent on hypoxia, which is consistent with the results in myeloma cells [29], and induced hSMCs apoptosis via Stat1 activation, which is confirmed by report from Regis et al. [30].

In summary, we revealed the molecular base for the relationship between hypoxia primed hSMCs and macrophages. Hypoxia induced MCP-1 expression and secretion in hSMCs and mediated macrophage chemotaxis. Hypoxia triggers the regulatory loop of MCP-1/miR-98/IL-6, and the signal loop maintains the molecular crosstalk and leads to hSMCs apoptosis. Our findings implicate valuable clues for exploring the mechanisms of AAA formation and progression.

### Disclosure of conflict of interest

None.

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