

Hypoxia modifies the polarization of macrophages and their inflammatory microenvironment, and inhibits malignant behavior in cancer cells

XIXIAN KE, CHENG CHEN, YONGXIANG SONG, QINGYONG CAI, JIAN LI, YANG TANG,
XU HAN, WENDONG QU, ANPING CHEN, HUI WANG, GANG XU* and DAXING LIU*

Department of Cardiothoracic Surgery, Affiliated Hospital of Zunyi Medical University, Zunyi, Guizhou 563000, P.R. China

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Abstract. Macrophages are a heterogeneous group of phagocytes that play critical roles in inflammation, infection and tumor growth. Macrophages respond to different environmental factors and are thereby polarized into specialized functional subsets. Although hypoxia is an important environmental factor, its impact on human macrophage polarization and subsequent modification of the inflammatory microenvironment have not been fully established. The present study aimed to elucidate the effect of hypoxia exposure on the ability of human macrophages to polarize into the classically activated (pro-inflammatory) M1, and the alternatively activated (anti-inflammatory) M2 phenotypes. The effect on the inflammatory microenvironment and the subsequent modification of A549 lung carcinoma cells was also investigated. The presented data show that hypoxia promoted macrophage polarization towards the M2 phenotype, and modified the inflammatory microenvironment by decreasing the release of proinflammatory cytokines. Modification of the microenvironment by proinflammatory M1 macrophages under hypoxia reversed the inhibition of malignant behaviors within the proinflammatory microenvironment. Furthermore, it was identified p38 signaling (a major contributor to the response to reactive oxygen species generated by hypoxic stress), but not hypoxia-induced factor, as a key regulator of macrophages under hypoxia. Taken together, the data suggest that hypoxia affects the inflammatory microenvironment by modifying the polarization of macrophages, and thus, reversing

the inhibitory effects of a proinflammatory microenvironment on the malignant behaviors of several types of cancer cell.

Introduction

Macrophages are a class of immune cells residing in all tissues (1). Macrophages are broadly classified as M1 pro-inflammatory, or M2 anti-inflammatory macrophages (2,3). Tumor-associated macrophages (TAMs) were first identified 30 years ago (4). Observations indicated that TAMs accumulated around tumors and were primarily derived from monocytes (5,6). The two polarization states of macrophages were also observed in TAMs; M1 macrophages were found in the early stages of neoplasia or in vascularized areas, whilst M2 macrophages were observed during tumor progression, and were indicative of poor prognosis (2,7-10).

Hypoxia immobilizes macrophages such that they accumulate in hypoxic regions (11). As hypoxia is a common feature of most solid tumors, TAMs are observed in hypoxic regions within a variety of tumor types (12). High concentrations of chemokines such as hypoxia-inducible factor (HIF)-1, HIF-2 and endothelin-2 are secreted from hypoxic tissues, which subsequently attract macrophages (11,13). Additionally, macrophages in hypoxic environments express higher levels of growth and angiogenic factors, including vascular endothelial growth factor (VEGF), glucose transporter-1 and tumor necrosis factor alpha (TNF- α), compared with macrophages in normoxic environments (11,14,15). Overall, hypoxia attracts higher numbers of M2 or M2-like TAMs and promotes the polarization of M1 to M2 macrophages (13,16).

Lung carcinoma is one of the most common and fatal carcinomas worldwide, and its incidence is increasing annually (17,18). The poor prognosis of patients with lung carcinoma has prompted numerous studies focused either on the development of therapeutic strategies, or aimed to further the understanding of lung cancer biology. Hypoxia is an important factor in the modification of the tumor microenvironment, and thus plays a pivotal role in all stages of carcinoma development, including tumorigenesis, progression, angiogenesis and metastasis (19,20). The effect of hypoxia on lung cancer has been widely investigated; Meng *et al* (21) reported that high levels of hypoxia were

Correspondence to: Dr Daxing Liu, Department of Cardiothoracic Surgery, Affiliated Hospital of Zunyi Medical University, 149 Dalian Road, Huichuan, Zunyi, Guizhou 563000, P.R. China
E-mail: daxing1970@163.com

*Contributed equally

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positively associated with both higher cancer grades at diagnosis and poor prognosis. It has also been demonstrated that the expression level of HIF-1 α is positively correlated with poor prognosis and the expression of various genes in lung cancer, including epidermal growth factor receptor, matrix metalloproteinase-9 and p53 (22,23). However, little is known about the effects of the hypoxia-modified microenvironment on lung cancer cells, and the subsequent effects on malignant behaviors in lung cancer.

Considering the influence that hypoxia exerts on TAMs, the present study was undertaken to assess the hypothesis that hypoxia influences the malignant behaviors of several types of cancer cell (including lung cancer cells) through the modification of the microenvironment.

Materials and methods

Cell culture and macrophage polarization. Human myeloid leukemia THP-1 cells were obtained from the American Type Culture Collection and maintained in RPMI 1640 medium (Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS) and 1% of penicillin-streptomycin (Gibco; Thermo Fisher Scientific, Inc.), and cultured at 37°C with 5% CO₂. To obtain macrophages with an M ϕ phenotype, THP-1 cells were differentiated by incubation with 10 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich; Merck KGaA) for 24 h at 37°C. Polarization towards the M1 phenotype was subsequently induced by culturing M ϕ cells with 100 ng/ml lipopolysaccharide (LPS, Sigma-Aldrich; Merck KGaA) and 20 ng/ml Interferon- γ (IFN- γ) for a further 48 h; polarization towards the M2 phenotype was induced by culturing M ϕ cells with 20 ng/ml interleukin-4 (IL-4).

The human non-small cell lung cancer cell line A549 and the human liver cancer cell line HepG2 were cultured in Dulbecco's Modified Eagles Medium (Gibco; Thermo Fisher Scientific, Inc.). The HeLa human cervical cancer cell line and the MCF-7 breast cancer cell line (American Type Culture Collection) were cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.). All cell cultures were supplemented with 10% FBS and 1% penicillin-streptomycin.

To identify the effect of hypoxia on the polarization of macrophages, stimulation was conducted in a Galaxy 14S incubator with oxygen control (New Brunswick Scientific; Eppendorf) containing 1% O₂, 5% CO₂ and 94% N₂, with or without 5 μ M SB203580, an inhibitor of p38 MAPK. After 24 and 48 h incubation, supernatants and cells were collected for further analysis.

Western blotting. For each sample, $\sim 1 \times 10^6$ cells were resuspended in 400 μ l RIPA buffer (Sigma-Aldrich; Merck KGaA) and lysed using the SoniConvert™ sonicator (DocSense, Chengdu, China). The lysate was quantified using a bicinchoninic acid assay kit (Sigma Aldrich; Merck KGaA) following the manufacturers' instructions. A total of 20 μ g protein was fractionated on a 4-10% SDS-PAGE gel and transferred to a PVDF membrane (EMD Millipore). The membrane was then blocked for 30 min at room temperature in blocking buffer [5% silk milk, 2.5% normal goat serum (Sigma-Aldrich; Merck KGaA), 0.025% Tween 20 in PBS] and probed for the proteins of interest. The primary antibodies used were

listed as follows: Anti-IL-1 β (cat. no. ab8320), anti-TNF- α (cat. no. ab6671), anti-Human Leukocyte Antigen (HLA)-DR (cat. no. 92511), anti-thymus and activation regulated chemokine (TARC) (cat. no. ab182793), anti-CD163 (cat. no. ab182422), anti-p38 (cat. no. ab170099), anti-p38 (phospho Y182; cat. no. ab47363), anti-HIF-1 α (cat. no. ab1) and anti- β -actin a (cat. no. ab8227). The goat anti-rabbit secondary antibody was then employed (cat. no. ab7090). All antibodies were purchased from Abcam. The primary antibodies were diluted to 1:2,000 and incubated with the membrane at room temperature for 1 h. The secondary antibody was diluted to 1:10,000 and the membrane was incubated at room temperature for 1 h. The blots were then quantified using SuperSignal West Dura ECL substrate (EMD Millipore).

Flow cytometry. Macrophages were harvested by trypsinization, washed with PBS and resuspended in staining buffer containing 2% goat serum (Sigma-Aldrich; Merck KGaA) and 0.5 mM EDTA in PBS. The cells were then stained with PE-conjugated antibodies against CD86 (cat. no. 374205; clone, BU63, BioLegend, Inc.) or CD206 (cat. no. 321105; clone, 15-2, BioLegend, Inc.). After 3 min staining in darkness at room temperature, the cells were washed twice using staining buffer and analyzed using a 3-laser Navios flow cytometer (Beckman Coulter, Inc.). FlowJo software was used for data analysis (version. 1.6.0).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from the macrophages using TRIzol® reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol; 1 μ g total RNA was employed for reverse transcription using a cDNA Synthesis Kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. qPCR was then performed using a SYBR® Green PCR Master Mix (Thermo Fisher Scientific, Inc.) on an ABI7500 system (Applied Biosystems; Thermo Fisher Scientific Inc.). Expression levels were normalized to that of β -actin and the fold-change in expression was obtained using the 2^{- $\Delta\Delta C_q$} method (24). The primer sequences were as follows: β -actin forward, 5'-CATGTACGTTGCTATCCAGGC-3', and reverse, 5'-CTCCTTAATGTCACGCACGAT-3'; IL-1 β forward, 5'-ATGATGGCTATTACAGTGGCAA-3', and reverse, 5'-GTCGGAGATTCGTAGCTGGA-3'; TNF- α forward, 5'-AACAGAGAGGATTTCGTTTCCG-3', and reverse, 5'-TTTGACCTGAGGGTAAGACTTCT-3'; VEGF forward, 5'-GTCGAGGAAGAGAGAGACGG-3', and reverse, 5'-GTCTGTCTGTCTGTCCGTCA-3'; HLA-DR forward, 5'-TGGTTTCTATCCAGGCAGCA-3', and reverse, 5'-TTCAGACCGTGCTCTCCATT-3'; CCL17 forward, 5'-AGGTCTTGAAGCCTCCTCAC-3', and reverse, 5'-AGTTCAGACAAGGGGATGGG-3'; CD163 forward, 5'-GAGCAGCACATGGGAGATTG-3', and reverse, 5'-ACCTCCTCCATTTACCAGGC-3'.

ELISA. The expression levels of the assayed proteins were determined using the following corresponding ELISA kits according to the manufacturer's protocol [Multisciences (Lianke) Biotech Co., Ltd]: IL-1 β (cat. no. 70-EK101B), TNF- α (cat. no. 70-EK182), VEGF [including the predominant isoforms, VEGF₁₈₉, VEGF₁₆₅, VEGF₁₄₅ and VEGF₁₂₁ (cat. no. 70-EK183)] and CCL17 (cat. no. 70-ek1115).

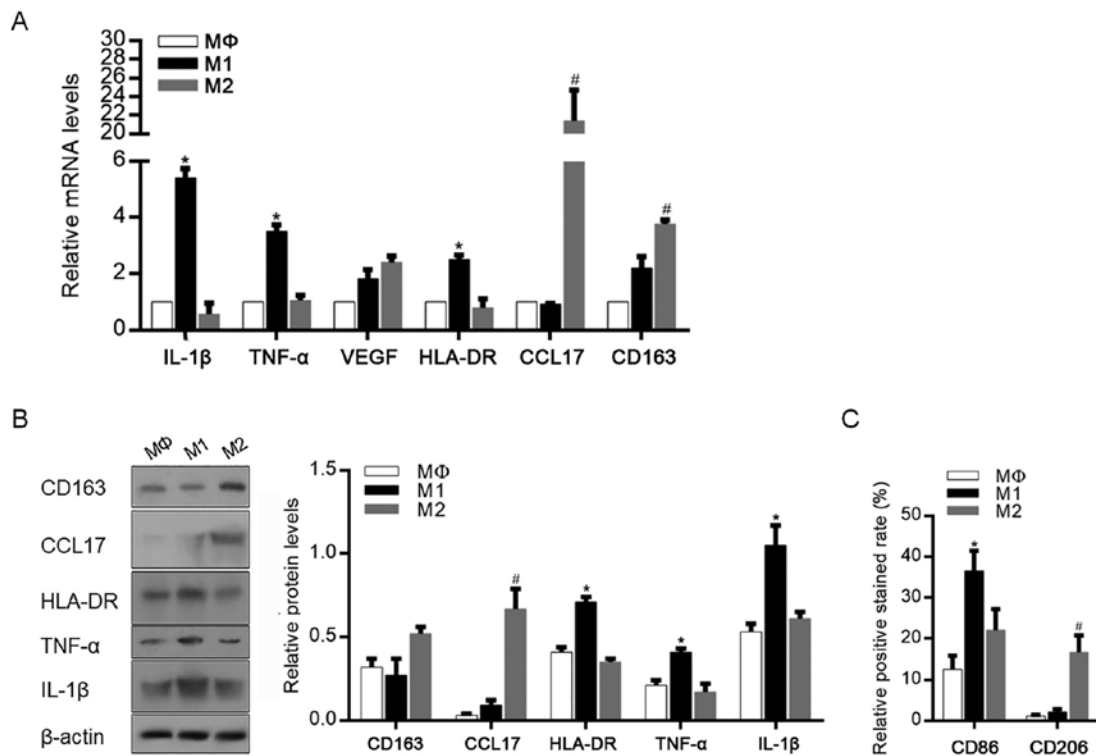


Figure 1. Identification of M1 and M2 macrophage phenotypes after differentiation. (A) mRNA levels of IL-1 β , TNF- α , VEGF, HLA-DR, CCL17 and CD163 in M Φ , M1 and M2 macrophages after specific stimulation. *P<0.05, vs. M Φ -stimulated group; #P<0.05, vs. M Φ -stimulated group. (B) Protein levels of IL-1 β , TNF- α , HLA-DR, CCL17 and CD163 in M Φ -, M1- and M2-polarized macrophages. *P<0.05, vs. M Φ -stimulated group; #P<0.05, vs. M Φ -stimulated group. (C) CD86 and CD206 expression rates were measured. *P<0.05, vs. M Φ -stimulated group; #P<0.05, vs. M Φ -stimulated group. IL-1 β , interleukin- β ; TNF- α , tumor necrosis factor- α ; VEGF, vascular endothelial growth factor; HLA-DR, human leukocyte antigen-DR; CCL17, chemokine (C-C motif) ligand 17.

Cell counting kit (CCK)-8 assay. To determine the cell proliferation rate, a CCK-8 assay was performed. Originally, 2×10^4 A549, HeLa, HepG2 and MCF-7 cells were incubated in conditioned medium for 1 to 5 days. Each day, 10 μ l CCK-8 solution (Sigma-Aldrich; Merck KGaA) was added to each well and incubated at 37°C for 2 h in a humidified incubator. The absorbance value was measured at a wavelength of 450 nm on a Multiskan spectrum microplate reader (Thermo Fisher Scientific, Inc.), and the experiment was repeated three times.

Colony formation assay. Resuspended cells were plated in 6-well plates at a density of 1×10^3 cells/well, and incubated for 2 weeks. The cells were fixed with methanol containing 1% crystal violet for 30 min, and images were captured using a X71 (U-RFL-T) fluorescence microscope (Olympus Corporation; magnification, x40).

Tumor formation in soft agar. For each well, 1×10^4 A549, HeLa, HepG2 and MCF-7 cells were suspended in diluted 0.3% low-melt agar medium and added to pre-set 0.6% low-melt agar medium in 6-well plates. The plates were incubated for 2 weeks at 37°C and then stained with crystal violet (0.01% solution), washed with PBS, and imaged under a X71 (U-RFL-T) fluorescence microscope (Olympus Corporation) at x40 magnification.

Statistical analysis. SPSS 17.0 (SPSS, Inc.) software was used to conduct the statistical analyses, and all data are presented as the mean \pm SD. Differences between two groups were

compared using the Student's t-test, whilst the differences between ≥ 3 groups were assessed using one-way ANOVA followed by Tukey's post-hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Stimulation of M1 and M2 polarized macrophages. To obtain M1 and M2 polarized macrophages, THP-1 cells were first treated with PMA for 24 h to induce M Φ differentiation. Then, M Φ cells were further stimulated with IFN- γ /LPS or IL-4 for 48 h, and the cytokine and chemokine expression profiles of THP-1-derived M Φ , M1 and M2 macrophages were evaluated by RT-qPCR (Fig. 1A). The data showed that classically activated (M1-polarized) macrophages exhibited an upregulation of several proinflammatory mediators, including IL-1 β and TNF- α . By contrast, CCL17 and CD163 were significantly upregulated in M2-polarized macrophages. The changes in the expression of these cytokines and chemokines in specific macrophage subsets were then confirmed by western blotting, and the protein levels of these mediators showed similar trends (Fig. 1B). The correct macrophage polarization was also confirmed by detecting cell surface markers of M1 and M2 polarization. As shown in Fig. 1C, M1-polarized macrophages exhibited a significantly higher level of CD86 expression, which is reported to be an M1 macrophage-specific surface marker (25,26). However, M2-polarized macrophages exhibited a significantly higher level of CD206 compared with unpolarized M1 macrophages (25,26).

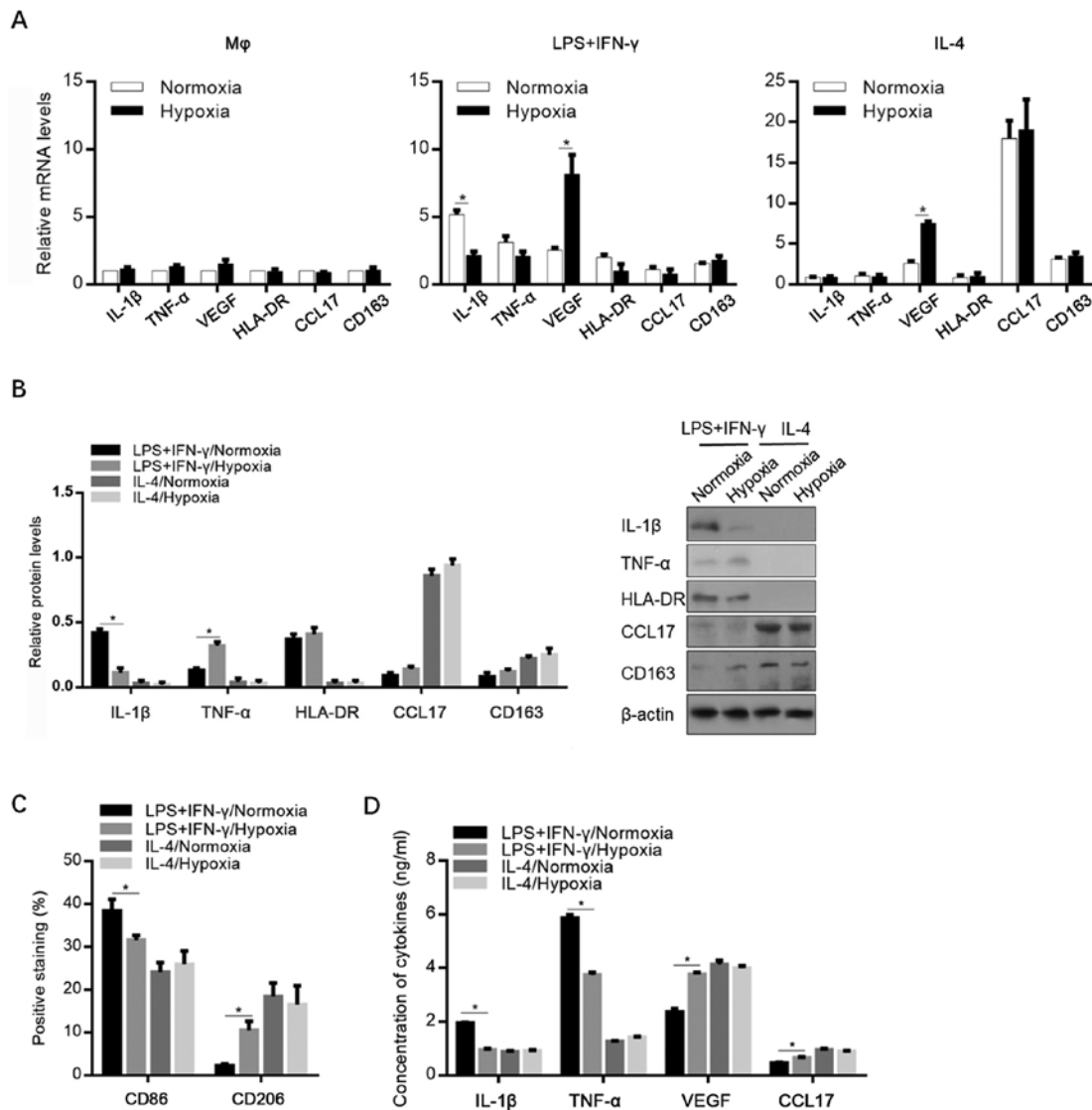


Figure 2. Effects of hypoxia on the expression levels of cytokines and chemokines. (A) mRNA levels of IL-1 β , TNF- α , VEGF, HLA-DR, CCL17 and CD163 in M Φ , M1 (LPS+IFN- γ) and M2 (IL-4) macrophages after specific stimulation under normoxic or hypoxic conditions. * P <0.05, vs. Normoxic group. (B) Protein levels of IL-1 β , TNF- α , HLA-DR, CCL17 and CD163 in M Φ -, M1- and M2-polarized macrophages under normoxic or hypoxic conditions. * P <0.05, vs. LPS+IFN- γ /Normoxic group. (C) CD86 and CD206 expression levels. * P <0.05, vs. LPS+IFN- γ /Normoxic group. (D) Secretion of IL-1 β , TNF- α , VEGF, and CCL17. * P <0.05, vs. LPS+IFN- γ /Normoxic group. IL-1 β , interleukin- β ; TNF- α , tumor necrosis factor- α ; VEGF, vascular endothelial growth factor; CCL17, chemokine (C-C motif) ligand 17; LPS, lipopolysaccharide; IFN- γ , interferon- γ .

Effects of hypoxia on the microenvironment of M1- and M2-polarized macrophages. To identify the effect of hypoxic exposure on the expression profiles of specific cytokines and chemokines, M Φ -, M1- and M2-polarized macrophages were induced under normoxic and hypoxic conditions. As shown in Fig. 2A, hypoxia significantly decreased IL-1 β expression in M1 macrophages and increased the VEGF mRNA levels in both M1 and M2 macrophages. The changes in the protein levels of these cytokines were confirmed via western blot analysis (Fig. 2B). Interestingly, the detection of M1- and M2-specific cell surface markers revealed that macrophage differentiation towards M2 polarization was promoted, without affecting M2 polarization status by incubating with IL-4/hypoxia conditioned medium (Fig. 2C). The release of IL-1 β , TNF- α , VEGF (including the predominant isoforms VEGF₁₈₉, VEGF₁₆₅, VEGF₁₄₅ and VEGF₁₂₁) and CCL17 in the cell supernatants was then investigated. Consistent with the aforementioned results,

hypoxia decreased the secretion of proinflammatory mediators in M1-polarized macrophages (Fig. 2D).

Hypoxia-modified microenvironments promote malignant behavior in A549 cells. To detect the effect of hypoxia-modified microenvironments on A549 lung cancer cells, as well as MCF-7, HepG2 and HeLa cells, conditioned-medium was used to culture each cell line. Considering the less pronounced effect of hypoxia on M2-polarized macrophages, the conditioned medium of M1-polarized macrophages was employed for further analyses. The malignant behaviors of A549, HeLa, HepG2 and MCF-7 cells (including proliferation, colony formation and soft-agar tumor formation) were analyzed. The results showed that all of these malignant behaviors were decreased by normoxia-conditioned medium, compared with M Φ -conditioned medium (P <0.05). Moreover, hypoxia-conditioned medium exerted no detectable effects on

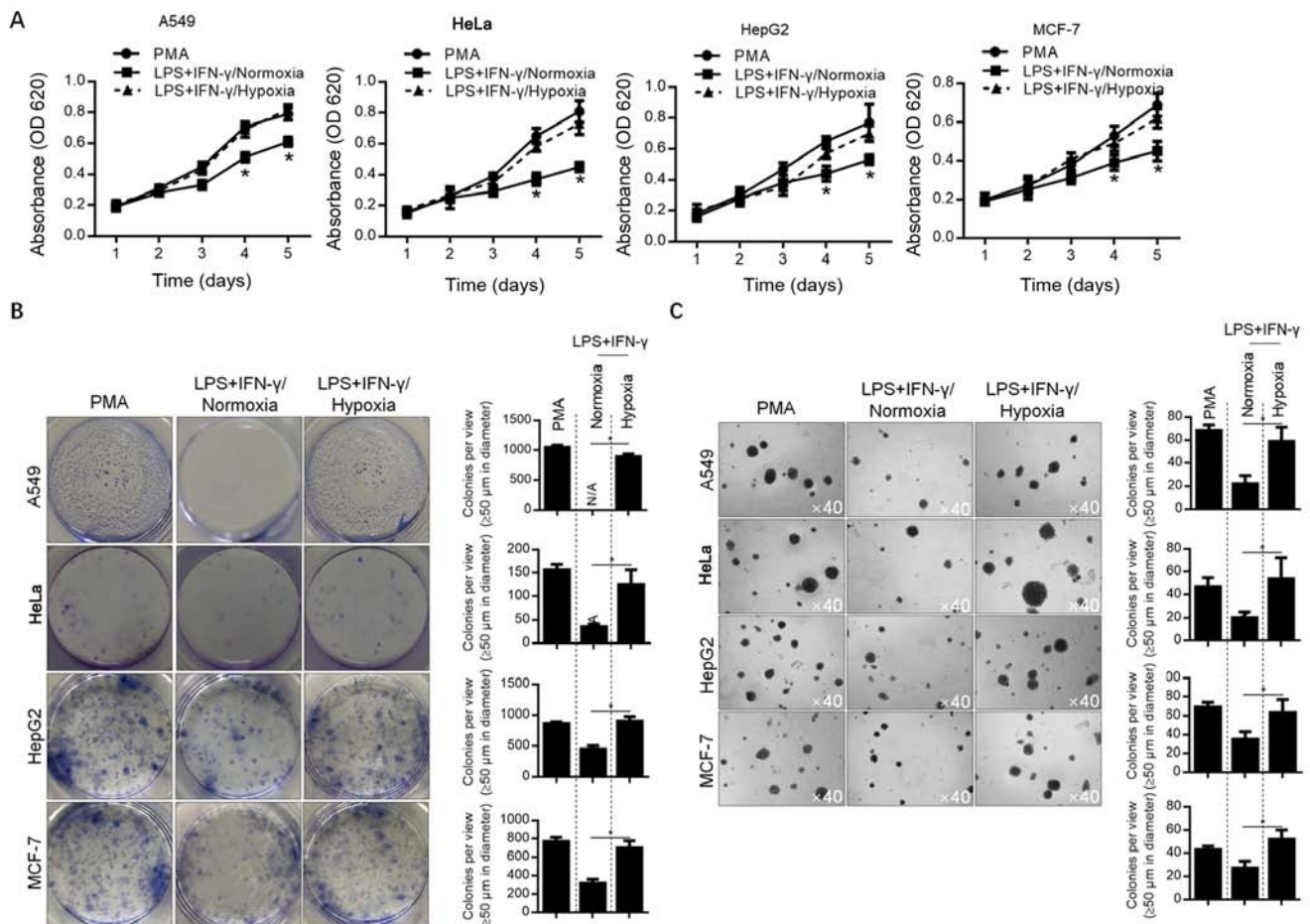


Figure 3. Effects of hypoxia-modified M1-polarized conditioned medium on malignant behaviors of cancer cells. (A) Proliferation of A549, HeLa, HepG2 and MCF-7 cells was analyzed by performing CCK-8 assay. The effects of hypoxia conditioned medium on (B) Colony formation and (C) Soft agar tumor formation. * $P < 0.05$ vs. LPS+IFN- γ /Normoxia. PMA, phorbol 12-myristate 13-acetate; CCK-8, cell counting kit-8; LPS, lipopolysaccharide; IFN- γ , interferon- γ .

these malignant behaviors (Fig. 3A-C). This indicates that the promotion of cancer cell malignant behavior is inhibited by the M1-modified microenvironment, which can subsequently be reversed by hypoxia.

Hypoxia-modified polarization of macrophages is independent of the expression of HIF-1 α , and partially dependent on the activation of p38. As a key transcriptional regulator, HIF-1 α serves a critical role in the adaptation of tumors to hypoxia via the regulation of multiple cytokines, including VEGF (27,28). Following hypoxia or CoCl₂ treatment, HIF-1 α expression in macrophages was determined; as expected, HIF-1 α was upregulated in both M1- and M2-polarized macrophages (Fig. 4A and B). The Addition of CoCl₂ to culture medium is commonly used to accumulate HIF-1 α , and to activate its transcriptional activity (29). However, the CoCl₂-conditioned medium of M1 macrophages failed to modify the release of cytokines and chemokines affected by the hypoxia-conditioned medium of M1 polarization (Fig. 4C and D). The detection of cell surface markers also indicated that CoCl₂ treatment failed to exert the same effect with hypoxia, indicating that the effect of hypoxic exposure is independent of the presence of HIF-1 α (Fig. 4E).

Considering that p38 is a central mediator in the production of pro-inflammatory cytokines (30), and that it is activated by

hypoxia in several cell types (31), p38 and phosphorylated p38 were detected after hypoxia, with or without the p38 signaling inhibitor SB203580. As shown in Fig. 4F, hypoxia significantly promoted the phosphorylation of p38. This was eradicated by the addition of 1 μM SB203580. Notably, it was observed that the addition of 1 μM SB203580 significantly decreased the level of HIF-1 α expression after hypoxia. This indicates that SB203580 may decrease HIF-1 α -activated p38 via different pathways. Importantly, pro-inflammatory cytokines decreased by hypoxia were recovered by the addition of SB203580, indicating that hypoxia-activated p38 signaling is, at least in part, responsible for the production of pro-inflammatory cytokines (Fig. 4G).

Discussion

The findings of the present study demonstrate that hypoxia can modify the polarization of macrophages, regulate the inflammatory microenvironment, and consequently regulate the malignant behaviors of A549 lung cancer cells. It was also indicated that the effects of hypoxia on the inflammatory microenvironment are regulated via the p38-signaling pathway.

In hypoxic conditions, macrophages are able to responsively accumulate HIF-1 α and HIF-2 α (32), and

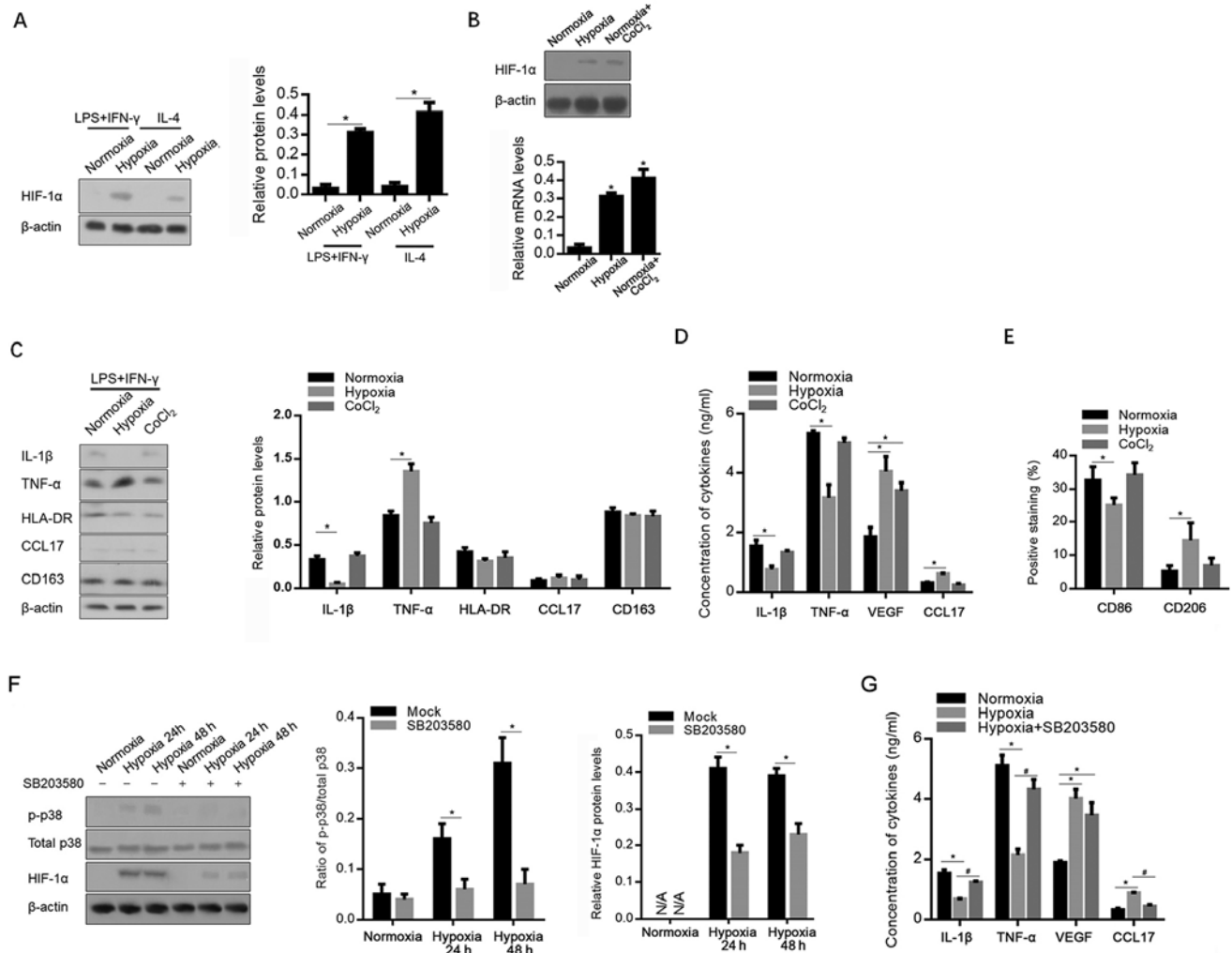


Figure 4. Hypoxia affects the inflammatory microenvironment in a p38-dependent and HIF-1 α -independent manner. HIF-1 α protein expression level was measured following (A) hypoxia and (B) CoCl₂ treatment. (C) mRNA levels of IL-1 β , TNF- α , VEGF, HLA-DR, CCL17 and CD163 were measured after hypoxic exposure or CoCl₂ treatment. * P <0.05, vs. Normoxia-exposed group. (D) Release of IL-1 β , TNF- α , VEGF and CCL17 in supernatant was measured by ELISA. (E) CD86 and CD206 expression was also assessed. * P <0.05, vs. Normoxia-exposed group. (F) p38 and phosphorylated p38 levels were measured under hypoxic conditions with or without 1 μ M SB203580. (G) IL-1 β , TNF- α , VEGF and CCL17 in the supernatant was measured by ELISA under hypoxic conditions with or without 1 μ M SB203580. * P <0.05, vs. Normoxia-exposed group; # P <0.05, vs. Hypoxia-exposed group. HIF, hypoxia inducible factor; 1 β , interleukin- β ; TNF- α , tumor necrosis factor- α ; VEGF, vascular endothelial growth factor; HLA-DR, human leukocyte antigen-DR; CCL17, chemokine (C-C motif) ligand 17; p-, phosphorylated; LPS, lipopolysaccharide; IFN- γ , interferon- γ .

stimulation by different mediators can induce different isotypes of HIF. IL-4-stimulated polarization of macrophages towards a wound-healing phenotype is associated with increased levels of HIF-2 α , which is barely detected in classically activated macrophages (33). HIF-1 α and -2 α are detectable in hypoxic tumor microenvironments; this indicates that in hypoxia-modified inflammatory microenvironments, macrophages can alter their phenotype, resulting in a mixed macrophage population and allowing simultaneous accumulation of HIF-1 α and -2 α (34,35). Populations of macrophages with mixed phenotypes tightly regulate the inflammatory microenvironment by producing both pro- and anti-inflammatory cytokines and chemokines. Thus, determining how hypoxia affects the polarization of macrophages is important for further understanding the effects of hypoxia on the inflammatory microenvironment (36). There has been much research into the effect of hypoxia on macrophage polarization. Despite this, whether hypoxia or HIF contribute to macrophage

polarization remains unknown (36). In the present study, M1- and M2-polarized macrophages were stimulated under hypoxia, and it was found that hypoxia affected the ratio of CD86 and CD206 expression in M1-polarized macrophages without affecting expression in M2-polarized macrophages. This may indicate that hypoxia modifies the polarization of macrophages towards the M2 phenotype.

To understand the effects of hypoxia on the inflammatory microenvironment, macrophages were exposed to hypoxia during stimulation. The data revealed that hypoxia significantly decreased the release of pro-inflammatory cytokines, and increased the expression of VEGF, which is reported to regulate macrophage functions, including tumor promotion (37,38). This indicates that hypoxia may promote an inflammatory microenvironment. In M2-polarized macrophages, hypoxia increased the VEGF mRNA level without altering that of VEGF protein, indicating that hypoxia-induced VEGF is not secreted. Considering that hypoxia-induced

HIF-1 α expression may contribute to the promotion of an inflammatory microenvironment, CoCl₂ was employed to induce HIF-1 α expression. However, only a minor effect was observed following CoCl₂ treatment, indicating that hypoxia may modify the microenvironment independently of HIF-1 α signaling. As a limitation of the present study, the results were not verified *in vivo*. In further investigations, it would be worth evaluating the potential modification of hypoxia on the tumor microenvironment in a mouse model (39).

The link between p38-mitogen-activated protein kinase (MAPK) activation and hypoxia, and the resulting regulation of physiological processes, is well established (40). It is reported that under hypoxic conditions, the p38 MAPK signaling complex is also strongly associated with inflammation-mediated apoptotic cell death in a variety of cell types (41). Also reported is that the inhibition of p38 reduces the release of pro-inflammatory cytokines under hypoxic conditions (42). Thus, in the present study, the levels of p38 and phosphorylated p38 (p-p38) were determined after hypoxic exposure. SB203580, a p38 MAPK signaling inhibitor, was employed. According to the results, p-p38 was significantly increased without altering the p38 protein level, and was completely inhibited in the presence of SB203580. Notably, the hypoxia-induced regulation of cytokines was significantly reversed by treatment with SB203580, demonstrating that p-p38 is critical for hypoxia-regulated cytokine release. Furthermore, SB203580 treatment was found to significantly decrease HIF-1 α expression, which indicates an interesting point for further investigation. No significant effect on malignant behavior was detected from the conditioned medium of SB203580-treated cells, which included proliferation, colony and tumor formation. However, the effect of SB203580 on cytokine levels indicates its potential effects on these malignant behaviors, which should be explored in further investigations.

The data of the present study suggest that hypoxia during macrophage polarization towards the M1 or M2 phenotype significantly modifies the release of cytokines, and thus regulates the inflammatory microenvironment. Moreover, it was revealed that hypoxia-induced p38 phosphorylation, but not HIF-1 α , is necessary for regulating the inflammatory microenvironment, and that a hypoxia-modified inflammatory microenvironment may contribute to the promotion of malignant behaviors in A549 lung cancer cells. These results indicate a novel approach to targeting cancer cells by modifying the inflammatory microenvironment via the regulation of macrophage polarization.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XK, CC, YS, GX and DL designed the experiments. XK performed cell culture and data analysis. QC, JL and YT performed gene expression analysis and protein analysis. XH, WQ, AC and HW performed research on molecular mechanisms. GX and DL supervised the experiments, writing and revisions.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Liu Y and Cao X: The origin and function of tumor-associated macrophages. *Cell Mol Immunol* 12: 1-4, 2015.
2. Biswas SK, Sica A and Lewis CE: Plasticity of macrophage function during tumor progression: Regulation by distinct molecular mechanisms. *J Immunol* 180: 2011-2017, 2008.
3. Biswas SK and Mantovani A: Macrophage plasticity and interaction with lymphocyte subsets: Cancer as a paradigm. *Nat Immunol* 11: 889-896, 2010.
4. van Ravenswaay Claassen HH, Kluin PM and Fleuren GJ: Tumor infiltrating cells in human cancer. On the possible role of CD16+ macrophages in antitumor cytotoxicity. *Lab Invest* 67: 166-174, 1992.
5. Coussens LM and Werb Z: Inflammation and cancer. *Nature* 420: 860-867, 2002.
6. Franklin RA, Liao W, Sarkar A, Kim MV, Bivona MR, Liu K, Pamer EG and Li MO: The cellular and molecular origin of tumor-associated macrophages. *Science* 344: 921-925, 2014.
7. Schultze JL, Schmieder A and Goerdts S: Macrophage activation in human diseases. *Semin Immunol* 27: 249-256, 2015.
8. Sica A and Mantovani A: Macrophage plasticity and polarization: In vivo veritas. *J Clin Invest* 122: 787-795, 2012.
9. Martinez FO, Sica A, Mantovani A and Locati M: Macrophage activation and polarization. *Front Biosci* 13: 453-461, 2008.
10. Jeannin P, Paolini L, Adam C and Delneste Y: The roles of CSFs on the functional polarization of tumor-associated macrophages. *FEBS J* 285: 680-699, 2018.
11. Murdoch C, Muthana M and Lewis CE: Hypoxia regulates macrophage functions in inflammation. *J Immunol* 175: 6257-6263, 2005.
12. Lewis C and Murdoch C: Macrophage responses to hypoxia: Implications for tumor progression and anti-cancer therapies. *Am J Pathol* 167: 627-635, 2005.
13. Roda JM, Wang Y, Sumner LA, Phillips GS, Marsh CB and Eubank TD: Stabilization of HIF-2 α induces sVEGFR-1 production from tumor-associated macrophages and decreases tumor growth in a murine melanoma model. *J Immunol* 189: 3168-3177, 2012.
14. Burke B, Giannoudis A, Corke KP, Gill D, Wells M, Ziegler-Heitbrock L and Lewis CE: Hypoxia-induced gene expression in human macrophages: Implications for ischemic tissues and hypoxia-regulated gene therapy. *Am J Pathol* 163: 1233-1243, 2003.
15. Lewis JS, Lee JA, Underwood JC, Harris AL and Lewis CE: Macrophage responses to hypoxia: Relevance to disease mechanisms. *J Leukoc Biol* 66: 889-900, 1999.
16. Huber R, Meier B, Otsuka A, Fenini G, Satoh T, Gehrke S, Widmer D, Levesque MP, Mangana J, Kerl K, *et al*: Tumour hypoxia promotes melanoma growth and metastasis via High Mobility Group Box-1 and M2-like macrophages. *Sci Rep* 6: 29914, 2016.

17. Didkowska J, Wojciechowska U, Manczuk M and Łobaszewski J: Lung cancer epidemiology: Contemporary and future challenges worldwide. *Ann Transl Med* 4: 150, 2016.
18. Dizon DS, Krilov L, Cohen E, Gangadhar T, Ganz PA, Hensing TA, Hunger S, Krishnamurthi SS, Lassman AB, Markham MJ, *et al*: Clinical cancer advances 2016: Annual report on progress against cancer from the American Society of Clinical Oncology. *J Clin Oncol* 34: 987-1011, 2016.
19. Ugel S, De Sanctis F, Mandruzzato S and Bronte V: Tumor-induced myeloid deviation: When myeloid-derived suppressor cells meet tumor-associated macrophages. *J Clin Invest* 125: 3365-3376, 2015.
20. Quatromoni JG and Eruslanov E: Tumor-associated macrophages: Function, phenotype, and link to prognosis in human lung cancer. *Am J Transl Res* 4: 376-389, 2012.
21. Meng X, Kong FM and Yu J: Implementation of hypoxia measurement into lung cancer therapy. *Lung Cancer* 75: 146-150, 2012.
22. Karetzi E, Ioannou MG, Kerenidi T, Minas M, Molyvdas PA, Gourgoulialis KI and Paraskeva E: Differential expression of hypoxia-inducible factor 1 α in non-small cell lung cancer and small cell lung cancer. *Clinics (Sao Paulo)* 67: 1373-1378, 2012.
23. Swinson DE, Jones JL, Cox G, Richardson D, Harris AL and O'Byrne KJ: Hypoxia-inducible factor-1 α in non small cell lung cancer: Relation to growth factor, protease and apoptosis pathways. *Int J Cancer* 111: 43-50, 2004.
24. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2 $^{-\Delta\Delta C_T}$ method. *Methods* 25: 402-408, 2001.
25. Chavez-Galan L, Ollerios ML, Vesin D and Garcia I: Much more than M1 and M2 macrophages, there are also CD169(+) and TCR(+) macrophages. *Front Immunol* 6: 263, 2015.
26. Roszer T: Understanding the mysterious M2 macrophage through activation markers and effector mechanisms. *Mediators Inflamm* 2015: 816460, 2015.
27. Wan J, Ma J, Mei J and Shan G: The effects of HIF-1 α on gene expression profiles of NCI-H446 human small cell lung cancer cells. *J Exp Clin Cancer Res* 28: 150, 2009.
28. Wan J, Chai H, Yu Z, Ge W, Kang N, Xia W and Che Y: HIF-1 α effects on angiogenic potential in human small cell lung carcinoma. *J Exp Clin Cancer Res* 30: 77, 2011.
29. Goldberg MA and Schneider TJ: Similarities between the oxygen-sensing mechanisms regulating the expression of vascular endothelial growth factor and erythropoietin. *J Biol Chem* 269: 4355-4359, 1994.
30. Bachstetter AD and Van Eldik LJ: The p38 MAP kinase family as regulators of proinflammatory cytokine production in degenerative diseases of the CNS. *Aging Dis* 1: 199-211, 2010.
31. Xu L, Pathak PS and Fukumura D: Hypoxia-induced activation of p38 mitogen-activated protein kinase and phosphatidylinositol 3'-kinase signaling pathways contributes to expression of interleukin 8 in human ovarian carcinoma cells. *Clin Cancer Res* 10: 701-707, 2004.
32. Takeda N, O'Dea EL, Doedens A, Kim JW, Weidemann A, Stockmann C, Asagiri M, Simon MC, Hoffmann A and Johnson RS: Differential activation and antagonistic function of HIF-1 α isoforms in macrophages are essential for NO homeostasis. *Genes Dev* 24: 491-501, 2010.
33. Imtiyaz HZ, Williams EP, Hickey MM, Patel SA, Durham AC, Yuan LJ, Hammond R, Gimotty PA, Keith B and Simon MC: Hypoxia-inducible factor 2 α regulates macrophage function in mouse models of acute and tumor inflammation. *J Clin Invest* 120: 2699-2714, 2010.
34. Mosser DM and Edwards JP: Exploring the full spectrum of macrophage activation. *Nat Rev Immunol* 8: 958-969, 2008.
35. Talks KL, Turley H, Gatter KC, Maxwell PH, Pugh CW, Ratcliffe PJ and Harris AL: The expression and distribution of the hypoxia-inducible factors HIF-1 α and HIF-2 α in normal human tissues, cancers, and tumor-associated macrophages. *Am J Pathol* 157: 411-421, 2000.
36. Brune B, Dehne N, Grossmann N, Jung M, Namgaladze D, Schmid T, von Knethen A and Weigert A: Redox control of inflammation in macrophages. *Antioxid Redox Signal* 19: 595-637, 2013.
37. Douglas NC, Zimmermann RC, Tan QK, Sullivan-Pyke CS, Sauer MV, Kitajewski JK and Shawber CJ: VEGFR-1 blockade disrupts peri-implantation decidual angiogenesis and macrophage recruitment. *Vasc Cell* 6: 16, 2014.
38. Li N, Qin J, Lan L, Zhang H, Liu F, Wu Z, Ni H and Wang Y: PTEN inhibits macrophage polarization from M1 to M2 through CCL2 and VEGF-A reduction and NHERF-1 synergism. *Cancer Biol Ther* 16: 297-306, 2015.
39. Almendros I, Gileles-Hillel A, Khalyfa A, Wang Y, Zhang SX, Carreras A, Farré R and Gozal D: Adipose tissue macrophage polarization by intermittent hypoxia in a mouse model of OSA: Effect of tumor microenvironment. *Cancer Lett* 361: 233-239, 2015.
40. Fu S, Bai R, Zhao Z, Zhang Z, Zhang G, Wang Y, Wang Y, Jiang D and Zhu D: Overexpression of hypoxia-inducible factor-1 α and vascular endothelial growth factor in sacral giant cell tumors and the correlation with tumor microvessel density. *Exp Ther Med* 8: 1453-1458, 2014.
41. Shologu N, Scully M, Laffey JG and O'Toole D: Human mesenchymal stem cell secretome from bone marrow or adipose-derived tissue sources for treatment of hypoxia-induced pulmonary epithelial injury. *Int J Mol Sci* 19: pii: E2996, 2018.
42. Sakiyama S, dePerrot M, Han B, Waddell TK, Keshavjee S and Liu M: Ischemia-reperfusion decreases protein tyrosine phosphorylation and p38 mitogen-activated protein kinase phosphorylation in rat lung transplants. *J Heart Lung Transplant* 22: 338-346, 2003.



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