

Macrophages induce the expression of lncRNA ATB via the secretion of TGF- β to relieve ischemia-reperfusion injury in cardiomyocytes

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Abstract. Cardiac ischemia-reperfusion can cause severe damage to cardiomyocytes. Previous studies have revealed that TGF- β can alleviate ischemia-reperfusion injury in cardiomyocytes by inducing the expression of long non-coding RNA (lncRNA) activated by TGF- β (ATB). However, M2 macrophages can secrete a large amount of TGF- β . However, whether M2 macrophages alleviate the ischemia-reperfusion-induced injury of cardiomyocytes by secreting TGF- β is unclear. In the present study, macrophages and cardiomyocytes were cultured under oxygen-glucose deprivation/reoxygenation (OGD/R) conditions to simulate ischemia-reperfusion injury. M2-type macrophage markers (IL-10, Arginase-1 and IL-13) were validated using reverse transcription-quantitative PCR and western blotting. Subsequently, the culture medium of M2-type macrophages was collected for the treatment of cardiomyocytes, which were cultured under OGD/R conditions. The levels of inflammatory factors and oxidase enzymes were detected with ELISA. The apoptotic rates of cardiomyocytes were detected by flow cytometry. The expression of cell apoptosis-related proteins and the phosphorylation levels of NF- κ B were analyzed by western blotting. The expression levels of specific inflammatory cytokines and the levels of malondialdehyde and lactate dehydrogenase were suppressed in cardiomyocytes following treatment with culture medium derived from M2-type

macrophages, which were cultured under OGD/R conditions. Furthermore, OGD/R-induced apoptosis of cardiomyocytes was also relieved following treatment of the cells with macrophage medium. It was found that M2-type macrophages could secrete TGF- β and that the culture medium of M2-type macrophages could activate the expression of lncRNA ATB in cardiomyocytes. TGF- β secreted by M2 macrophages relieved the inflammatory response, oxidative stress and apoptosis of cardiomyocytes by inducing the expression of lncRNA ATB.

Introduction

Internal heart ischemia is the main cause of death in most patients with heart-associated diseases (1). The restoration of the blood flow inside the heart is a therapeutic strategy used to relieve myocardial infarction caused by ischemia (2). However, certain studies suggested that ischemia-reperfusion could induce additional damage to cardiomyocytes (2,3). In addition, it was shown that ischemia-reperfusion can lead to functional damage to the heart and aggravate the structure of the heart muscle following a period of time (4). These injuries eventually lead to the injury of heart function and the development of severe arrhythmias (5-8). These studies have indicated that the ischemia-reperfusion process induces severe injury in the myocardium. Furthermore, TGF- β plays a crucial role in ventricular remodeling, cell apoptosis and the inflammatory response (9). A study revealed that TGF- β can improve myocardial function and inhibit hypoxia-induced apoptosis of cardiomyocytes by alleviating endoplasmic reticulum stress (10).

Macrophages are a type of monocyte phagocytic cells that are present in the body. Macrophages are composed of cells (blood monocytes and tissue macrophages) which originate from the bone marrow (11). Monocytes in the blood are eventually transferred to different tissues and transformed into macrophages. Furthermore, macrophages also play a crucial role in the occurrence, development and reduction of inflammation (12). Macrophages mainly exert roles in antigen presentation, immune regulation and phagocytosis by inducing the production of various cytokines and growth

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factors, which in turn affects the occurrence and development of the inflammatory response (13,14). In addition to these functions, macrophages are activated and polarized into different cell types (M1 and M2) within the tissue microenvironment (15). Macrophages are polarized to the M1-type in the early stages of the inflammatory response to secrete inflammatory cytokines and associated molecules, such as TNF- α , IL-1, IL-6, reactive oxygen species (ROS) and inducible nitric oxide synthase (iNOS). During the late stages of inflammation, macrophages are polarized into M2 macrophages and exert anti-inflammatory, repair and fibrotic effects through the release of cytokines, such as IL-10, IL-12, TGF- β and Arginase-1 (16,17). A previous study revealed that long non-coding RNA (lncRNA) activated by TGF- β (ATB) is a signaling mediator of and can be activated by TGF- β (18). TGF- β suppresses the MyD88/NF- κ B pathway by activating the expression of lncRNA ATB to relieve the inflammatory response of joint chondrocytes (19). However, whether macrophage cell (M2-type)-secreted TGF- β can relieve the ischemia-reperfusion injury of myocardial cells by enhancing the expression of lncRNA ATB remains unclear.

Therefore, in the present study, the polarization of macrophages was performed to generate M2-type macrophages. Subsequently, the medium of M2-type macrophages was used to treat cardiomyocytes, which were cultured under oxygen-glucose deprivation/reoxygenation (OGD/R) conditions. The degree of inflammatory injury, oxidative stress and apoptosis of these cardiomyocytes was detected. The effects of the M2-type macrophage secreted-TGF- β were evaluated on ischemia-reperfusion injury of cardiomyocytes based on the results of these assays.

Materials and methods

Cell culture and treatment. Cardiomyocytes (H9c2) and macrophage cells (RAW 264.7) were obtained from American Type Culture Collection. Cells were cultured in RPMI-1640 medium (Hyclone; Cytiva) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin. The cells were cultured in a 37°C humidified atmosphere with 5% CO₂ and glucose-free balanced salt solution in the presence of 95% N₂ for 6 h to mimic hypoxia and hypoglycemic conditions. Subsequently, these cells were cultured in RPMI-1640 medium in 95% air and 5% CO₂ at 37°C for 12, 24 and 48 h. This process was performed to simulate ischemia-reperfusion *in vitro* (20). LY364947 (Selleck Chemicals) is a receptor kinase I inhibitor that was added into the medium to inhibit TGF- β function. lncRNA ATB overexpression plasmid (Oe-ATB) and its negative control (Oe-NC) were synthesized by Shanghai GeneChem Co., Ltd. and transfected into H9c2 cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

ELISA. A TGF- β ELISA kit (Bendermed System Diagnostics; eBioscience; Thermo Fisher Scientific, Inc.; cat. no. BMS249/4) was used to determine the secretion of TGF- β from macrophage cells according to the manufacturer's instructions.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from cells with TRIzol® reagent (Invitrogen;

Thermo Fisher Scientific, Inc.). Subsequently, a cDNA reverse transcription kit (Invitrogen; Thermo Fisher Scientific, Inc.; cat. no. 4368814) was used to reverse transcribe mRNA into cDNA according to the manufacturer's instructions. SYBR Green (Thermo Fisher Scientific, Inc.) was used for the detection of the expression of the target genes on an ViiA™ 7 Real-Time PCR System (Applied Biosystems™; Thermo Fisher Scientific, Inc.). PCR conditions used were as follows: 95°C denaturation step for 30 sec and 55°C annealing for 1 min and 72°C for 2 min, carried out for 26 cycles. The relative expression of the target genes was analyzed with the 2^{- $\Delta\Delta$ C_q} method (21). Primer sequences are listed in Table I. GAPDH was used as a reference gene for normalization of quantitative PCR data.

Western blotting. RIPA buffer (Beyotime Institute of Biotechnology) was used to extract total proteins from H9c2 cells. Subsequently, protein concentration was determined with the BCA method (Beyotime Institute of Biotechnology). A total of 20 μ g protein was loaded per lane. Proteins were separated with 10% SDS-PAGE (Beyotime Institute of Biotechnology) and transferred to PVDF membranes (EMD Millipore). The membranes were blocked with 5% skimmed milk powder solution at room temperature for 1 h and incubated with the following primary antibodies: TGF- β (cat. no. 3711S; Cell Signaling Technology, Inc.), phosphorylated (p)-I κ B α (cat. no. 2859; Cell Signaling Technology, Inc.), I κ B α (cat. no. 4814; Cell Signaling Technology, Inc.), p-NF- κ B (cat. no. 3033S; Cell Signaling Technology, Inc.), NF- κ B (cat. no. 8242S; Cell Signaling Technology, Inc.), NADPH oxidase (Nox)-2 (cat. no. ab80508; Abcam), Nox-4 (cat. no. ab109225; Abcam), Bcl-2 (cat. no. ab32124; Abcam), Bax (cat. no. ab32503; Abcam), cleaved caspase-3 (cat. no. ab49822; Abcam), caspase-3 (cat. no. ab44976; Abcam), IL-1 β (cat. no. ab216995; Abcam), IL-6 (cat. no. ab6672; Abcam), IL-10 (cat. no. ab133575; Abcam), Arg-1 (cat. no. ab269541; Abcam), IL-13 (cat. no. ab260044; Abcam) and GAPDH (cat. no. ab8245; Abcam). The following day, membranes were washed three times with cold PBS-0.1% Tween-20 and incubated with HRP-conjugated secondary antibodies (1:10,000; cat. no. ab205718; Abcam) for 2 h at room temperature. Protein bands were finally developed using enhanced chemiluminescence reagent (EMD Millipore). Densitometry was quantified with NIH ImageJ 1.50 software (National Institutes of Health).

Apoptosis assay. Single-cell suspensions were prepared using trypsin (Beyotime Institute of Biotechnology) and washed three times with cold PBS to remove residual serum. Subsequently, these cells were incubated with Annexin V and PI in the dark and washed with PBS three times again. The apoptotic rates of these cells were detected by MACSQuant X flow cytometer (Miltenyi Biotec, Inc.). Flow cytometry data were analyzed with FlowJo V10 (FlowJo LLC).

Detection of malondialdehyde (MDA), superoxide dismutase (SOD) and lactate dehydrogenase (LDH). The production of MDA (cat. no. S0131S; Beyotime Institute of Biotechnology), SOD (cat. no. S0086; Beyotime Institute of Biotechnology) and LDH (cat. no. C0016; Beyotime Institute of Biotechnology) in cardiomyocytes was measured with commercial kits according to the manufacturer's instructions.

Table I. Primer sequences.

Gene	Primer sequences
Mouse TNF- α	F: 5'-CTCCAGGCGGTGCCTATG-3' R: 5'-GGCCATAGAAGTATGATGAGAGG-3'
Mouse iNOS	F: 5'-TTTGCTTCCATGCTAATGCGAAAG-3' R: 5'-GCTCTGTTGAGGTCTAAAGGCTCCG-3'
Mouse IL-1 β	F: 5'-GCACACCCACCCTGCA-3' R: 5'-ACCGCTTTTCCATCTTCTTCTT-3'
Mouse IL-6	F: 5'-TCCAGAAACCGCTATGAAGTTC-3' R: 5'-CACCAGCATCAGTCCCAAGA-3'
Mouse Arg-1	F: 5'-TCCACACGTCCAGAACAGTC-3' R: 5'-CCTTGAAACAGAGACAGGC-3'
Mouse IL-10	F: 5'-CAACATACTGCTAACCGACTCCT-3' R: 5'-TGAGGGTCTTCAGCTTCTCAC-3'
Mouse IL-13	F: 5'-GCCAGCCCACAGTTCTACAGC-3' R: 5'-GAGATGTTGCTCAGCTCCTCA-3'
Mouse GAPDH	F: 5'-AGGTCGGTGTGAACGGATTTG-3' R: 5'-TGTAGACCATGTAGTTGAGGTCA-3'
Rat TNF- α	F: 5'-CCAACAAGGAGGAGAAGTTC-3' R: 5'-CTCTGCTTGGTGGTTGCTAC-3'
Rat IL-1 β	F: 5'-GGAACCCGTGTCTTCTTAAAG-3' R: 5'-CTGACTTGGCAGAGGACAAAG-3'
Rat IL-6	F: 5'-TTGCCTTCTTGGGACTG-3' R: 5'-CTGGCTTTGTCTTTCTTGTTA-3'
Rat HMGB1	F: 5'-TGCTGCATATCGAGCTAAAGG-3' R: 5'-CCATACTGTACCAGGCAAGGT-3'
Rat lncRNA ATB	F: 5'-ACAAGCTGTGCAGTCTCAGG-3' R: 5'-CTAGGCCCAAAGACAATGGA-3'
Rat GAPDH	F: 5'-ACCACAGTCCATGCCATCAC-3' R: 5'-TCCACCACCCTGTTGCTGTA-3'

iNOS, inducible nitric oxide synthase; lncRNA ATB, long non-coding RNA activated by TGF- β ; HMGB1, high mobility group protein B1; Arg-1, Arginase-1.

Statistical analysis. Data in the present study were analyzed with GraphPad Prism 6.0 (GraphPad Software, Inc.). The data are presented as the mean \pm SD. Student's t-test or one-way ANOVA followed by Tukey-Kramer tests were performed to compare the data in two or multiple groups, respectively. All experiments were independently repeated three times. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Polarization of macrophages under OGD/R conditions. To determine the polarization of macrophages, RAW 246.7 cells were cultured under hypoxic conditions and subsequently placed under normal oxygen conditions for different time periods (12, 24 and 48 h). The levels of the M1-type macrophage markers (TNF- α , iNOS, IL-1 β and IL-6) were measured with RT-qPCR. The results indicated that the expression levels of

TNF- α , iNOS, IL-1 β and IL-6 gradually increased following reoxygenation (12 and 24 h) (Fig. 1A). However, the expression levels of these markers were significantly downregulated following 48 h of reoxygenation. The expression levels of the M2-type macrophage markers (IL-10, IL-13 and Arg-1) were determined with RT-qPCR and western blotting. The expression levels of IL-10, IL-13 and Arg-1 were increased following reoxygenation of macrophages (Fig. 1B and C). The maximum increase of the expression of these markers occurred at 48 h following reoxygenation. Therefore, the data suggested that the macrophages exhibited the characteristics of M2-type macrophages following 48 h of reoxygenation. These macrophages were selected in subsequent experiments as the main research cell type. TGF- β is a crucial cytokine secreted by M2-type macrophages (22). Furthermore, a study revealed that higher levels of TGF- β could relieve myocardial injury, which was induced following ischemia reperfusion (10). Therefore, the protein levels of TGF- β in these macrophages were measured. The results indicated that TGF- β levels were significantly enhanced following 48 h of reoxygenation compared with controls (Fig. 1D). In addition, TGF- β levels in the cell medium of the macrophage culture were also significantly elevated compared with controls (Fig. 1E).

Inflammatory damage of cardiomyocytes is relieved by M2-type macrophage culture medium. H9c2 cells were cultured under OGD/R conditions. Subsequently, the medium of the macrophages cultured under normal or OGD/R conditions (48 h reoxygenation) was used to treat H9c2 cells. RT-qPCR was performed to detect the levels of the inflammatory factors [TNF- α , IL-1 β , IL-6 and high mobility group protein B1 (HMGB1)] of H9c2 cells which were cultured with the medium of the macrophages. The results indicated that the levels of these inflammatory factors were significantly increased when the H9c2 cells were cultured under OGD/R conditions (Fig. 2A). However, the mRNA expression levels of TNF- α , IL-1 β , IL-6 and HMGB1 were significantly inhibited following treatment with the culture medium of macrophages that were previously incubated under OGD/R conditions. Western blotting was performed to detect the levels of inflammation-associated proteins (p-NF- κ B and p-I κ B α) in H9c2 cells. Compared with the Control group, the levels of p-NF- κ B and p-I κ B α were significantly increased in OGD/R group, whereas the levels of p-NF- κ B and p-I κ B α in OGD/R+CM of macrophage group were significantly inhibited following treatment of the cells with the culture medium of the M2-type macrophages (Fig. 2B).

M2-type macrophage culture medium alleviates oxidative stress injury in cardiomyocytes. Oxidative stress injury is another type of damage which is induced following ischemia and reperfusion (23,24). The production of oxidative stress-associated enzymes was examined in the present study. The results indicated that the culture medium of the macrophages, which were incubated under OGD/R conditions, could reduce the increase SOD levels induced by OGD/R (Fig. 3B). OGD/R-induced MDA and LDH levels were also reduced in cells incubated with the culture medium of the macrophages grown under OGD/R conditions (Fig. 3A

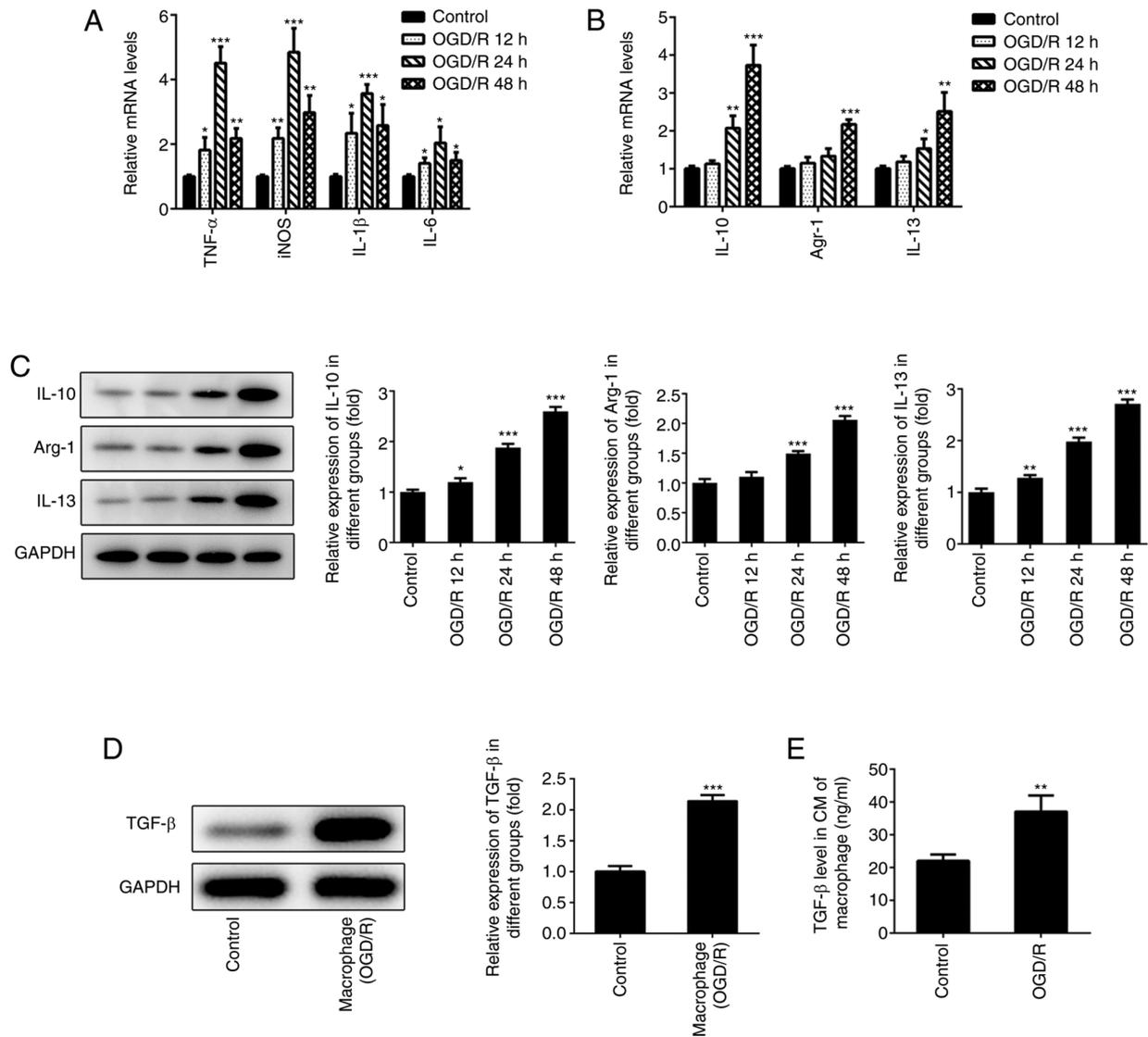


Figure 1. Polarization of macrophages under OGD/R conditions. (A) The expression of TNF- α , IL-1 β , IL-6, iNOS in macrophages were detected using RT-qPCR. The expression of M2-type macrophages (IL-10, Arg-1 and IL-13) were detected using (B) RT-qPCR and (C) western blotting. (D) The expression of TGF- β in macrophages was detected using western blotting after 48 h of reoxygenation. (E) The expression of TGF- β in the cell medium of macrophages was determined using ELISA after 48 h of reoxygenation. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. control. OGD/R, oxygen-glucose deprivation/reoxygenation; OGD/R, H9c2 cardiomyocytes under OGD/R conditions; macrophage (OGD/R), macrophages under OGD/R conditions; iNOS, inducible nitric oxide synthase; Arg-1, Arginase-1; RT-qPCR, reverse transcription-quantitative PCR.

and C). Furthermore, Nox-2 and Nox-4 has been shown to promote ROS production and aggravate oxidative damage in numerous types of cells (25). Therefore, the expression levels of Nox-2 and Nox-4 were detected in H9c2 cells using western blot analysis. The results indicated that the levels of Nox-2 and Nox-4 were increased when H9c2 cells were cultured under OGD/R conditions (Fig. 3D). However, the expression levels of Nox-2 and Nox-4 were suppressed following treatment of the cells with medium derived from M2-type macrophages.

M2-type macrophage culture medium reduces the induction of cardiomyocyte apoptosis. Ischemia-reperfusion injury induces cell apoptosis in different tissues (26-28). Therefore, the apoptotic rates of H9c2 cells were determined using flow cytometry. The results indicated that the apoptotic rates of H9c2 cells were promoted when these cells were cultured

under OGD/R conditions (Fig. 4A and B). However, the induction of H9c2 cell apoptosis was reduced following treatment of the cells with medium derived from macrophages cultured under OGD/R conditions. Subsequently, the expression levels of apoptosis-associated proteins were determined using western blotting. The expression levels of Bcl-2 were suppressed, whereas the levels of pro-apoptotic proteins (Bax and cleaved caspase 3) were increased in H9c2 cells cultured under OGD/R conditions. However, the levels of Bcl-2 were increased and the expression levels of the pro-apoptotic proteins (Bax and cleaved caspase 3) were inhibited in H9c2 cells following treatment with medium derived from M2-type macrophages (Fig. 4C).

TGF- β -mediated induction of lncRNA ATB is required for the protective effects of M2-type macrophage medium on cardiomyocytes. It was reported that TGF- β can increase the

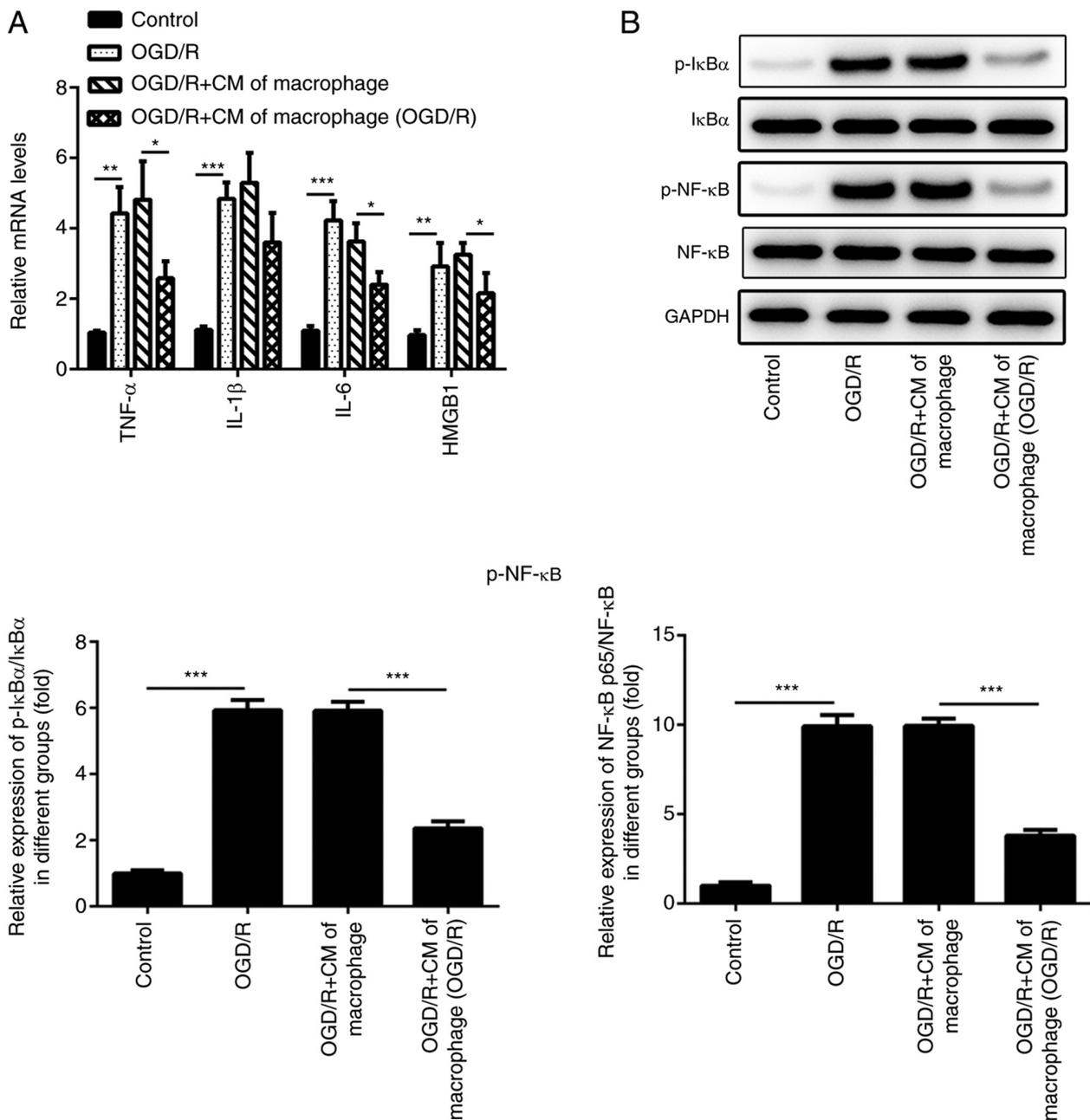


Figure 2. Inflammatory damage of cardiomyocytes is relieved by M2-type macrophage culture medium. (A) The levels of inflammatory factors (TNF- α , IL-1 β , IL-6 and HMGB1) in H9c2 cells were determined by reverse transcription-quantitative PCR. (B) The levels of p-I κ B α and NF- κ B p65 in H9c2 cells were detected by western blotting. *P<0.05, **P<0.01 and ***P<0.001. OGD/R, oxygen-glucose deprivation/reoxygenation; CM of macrophage, normal macrophage culture medium; CM of macrophage (OGD/R), macrophage culture medium under OGD/R conditions; HMGB1, high mobility group protein B1; p, phosphorylated.

expression levels of lncRNA ATB, which relieves the inflammatory response of chondrocytes (19). The present study demonstrated that a large amount of TGF- β was secreted by M2-type macrophages (Fig. 1D). Therefore, the expression of lncRNA ATB was determined in H9c2 cells following treatment with LY364947. The levels of lncRNA ATB in H9c2 cells exposed to OGD/R were decreased, while this effect was reversed following addition of M2-type macrophage medium (Fig. 5A). The effects of M2-type macrophage medium were reversed following treatment with LY364947, an inhibitor of TGF- β receptor kinase I (29). To investigate the effects of lncRNA ATB in H9c2 cells, Oe-ATB was transfected into

H9c2 cells (Fig. 5B). This cell group demonstrated elevated expression levels of lncRNA ATB. In addition, the anti-inflammatory effects of M2-type macrophage medium were inhibited following LY364947 treatment. However, Oe-ATB partly abrogated the role of LY364947 (Fig. 5C and D). Similarly, the protective roles of M2-type macrophage medium against oxidative stress and apoptosis were impaired by LY364947 (Fig. 5E and F), whereas Oe-ATB could reduce the harmful effects triggered by LY364947 (Fig. 5G), indicating that TGF- β secreted by M2-type macrophage medium could protect H9c2 cells from oxidative stress and apoptosis via activation of lncRNA ATB.

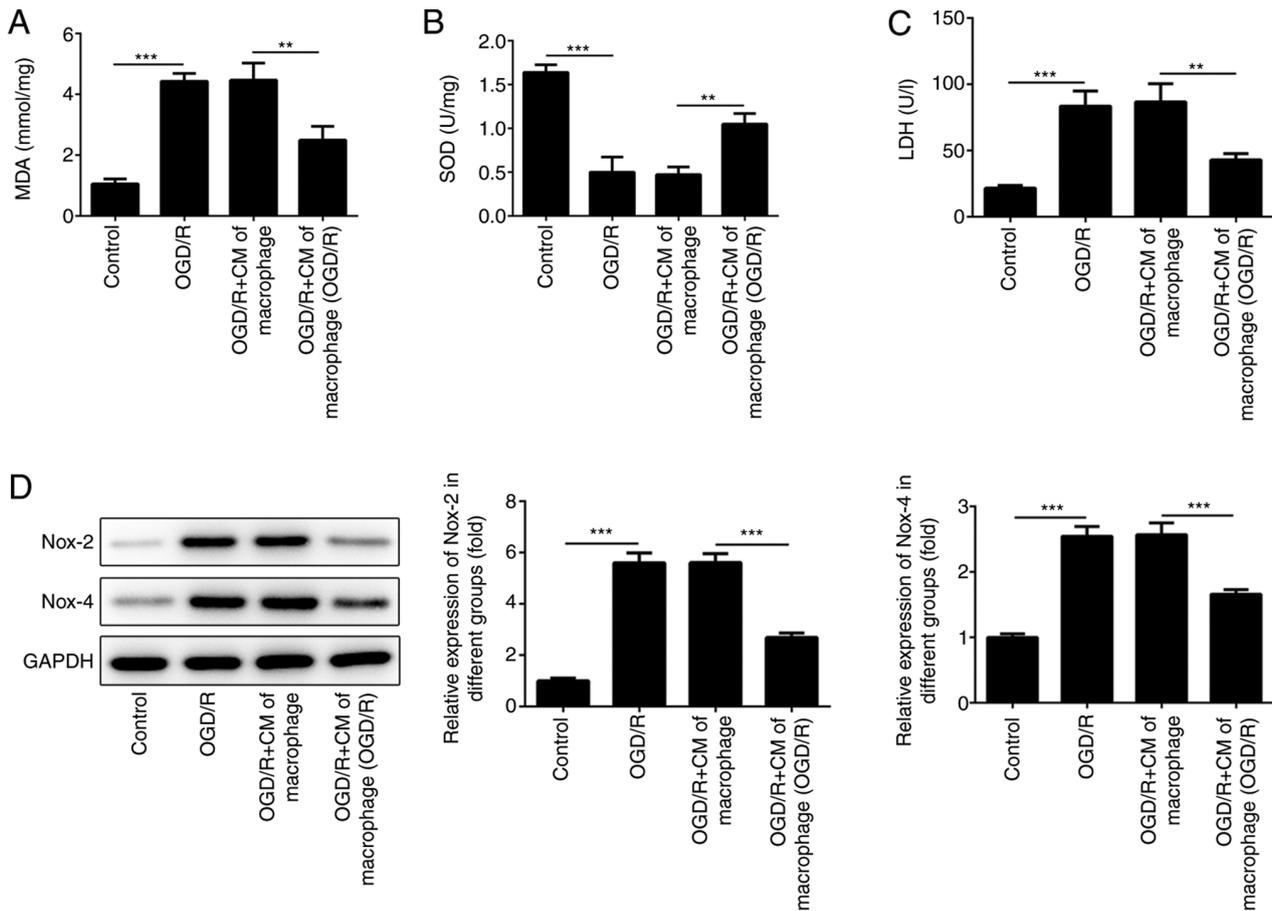


Figure 3. M2-type macrophage culture medium alleviates oxidative stress injury in cardiomyocytes. The levels of (A) MDA, (B) SOD and (C) LDH in H9c2 cells were determined using commercial kits. (D) The expression of Nox-2 and Nox-4 in H9c2 cells was detected using western blotting. ** $P < 0.01$ and *** $P < 0.001$. OGD/R, oxygen-glucose deprivation/reoxygenation; CM of macrophage, normal macrophage culture medium; CM of macrophage (OGD/R), macrophage culture medium under OGD/R conditions; MDA, malondialdehyde; SOD, superoxide dismutase; LDH, lactate dehydrogenase; Nox, NADPH oxidase.

Discussion

Myocardial ischemia and reperfusion cause severe damage to the heart tissue and in the absence of timely and effective therapy, can lead to coronary heart disease and heart failure (30,31). The production of excess ROS induced by ischemia-reperfusion, the overload of calcium ions in cardiomyocytes, myocardial contractile dysfunction and the induction of cardiomyocyte apoptosis are considered the main mechanisms of myocardial damage caused by ischemia-reperfusion (32-34). Furthermore, TGF- β plays a crucial role in ventricular remodeling, inflammatory injury and apoptosis of cardiomyocytes (35). Previous studies have revealed that TGF- β can manipulate myocardial fibrosis by regulating the expression of Smad2/Smad3 and Wnt/ β -catenin pathway-associated proteins (36,37). A study suggested that higher levels of TGF- β could alleviate the apoptosis of cardiomyocytes, which was induced by ischemia-reperfusion, leading to the protection of the normal function of cardiomyocytes (10). In addition, previous studies revealed that M2-type macrophages could secrete TGF- β (38,39). Macrophages are a type of autoimmune cells in the body, which can alleviate the inflammatory damage caused in various tissues of the body by secreting diverse cytokines. In addition, macrophages are polarized and transformed into M1- and M2-type macrophages (40).

Furthermore, M1- and M2-type macrophages secrete different cytokines and protect the tissues from the induction of the inflammatory response (15). However, whether TGF- β secreted by M2 macrophages can relieve cardiomyocyte injury induced by ischemia-reperfusion is unclear. In the present study, macrophages were cultured under OGD/R conditions. However, the expression levels of M1 and M2 macrophage markers differed according to the different time periods (12, 24 and 48 h) of reoxygenation. The expression levels of M2 macrophage markers (IL-10, Arg-1 and IL-13) were the highest following 48 h of reoxygenation. The levels of TGF- β were also higher in these macrophages following 48 h of reoxygenation. Therefore, these macrophages were considered M2 macrophages and were used for subsequent experiments.

lncRNAs are non-coding RNAs consisting of >200 nucleotides. lncRNAs participate in the regulation of several physiological activities in different types of cells (41,42). A previous study revealed that lncRNA ATB is the signal transmitting medium of TGF- β (17). lncRNA ATB activated by TGF- β relieves the inflammatory injury of chondrocytes by suppressing the expression levels of NF- κ B and MyD88 (19). According to these findings, the expression levels of lncRNA ATB in cardiomyocytes were increased following treatment of macrophage culture medium, which were cultured under OGD/R conditions. These results indicated that TGF- β

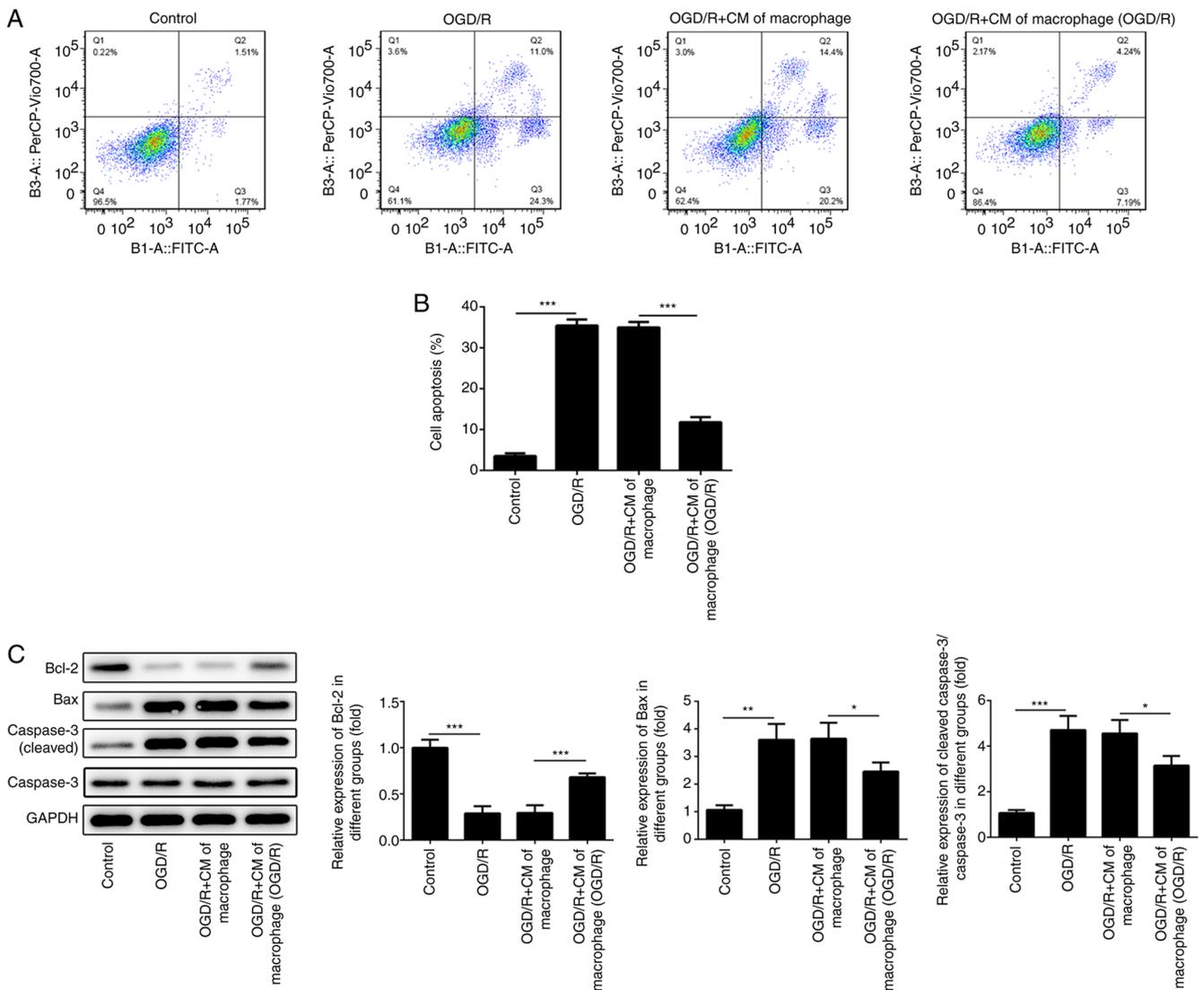


Figure 4. M2-type macrophage culture medium reduces the induction of cardiomyocyte apoptosis. (A and B) The apoptosis rates of H9c2 cells were measured using flow cytometry. (C) The expression of apoptosis-related proteins in H9c2 cells were determined using western blotting. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. OGD/R, oxygen-glucose deprivation/reoxygenation; CM of macrophage, normal macrophage culture medium; CM of macrophage (OGD/R), macrophage culture medium under OGD/R conditions.

secreted by M2 macrophages could increase the expression levels of lncRNA ATB in cardiomyocytes.

Given that inflammatory damage is the main symptom of ischemia and reperfusion injury (43,44), the levels of inflammatory factors in the cardiomyocytes cultured under OGD/R conditions were examined. The results indicated that the expression levels of inflammatory factors (TNF- α , IL-1 β , IL-6, HMGB1, p-I κ B α and NF- κ B p65) were inhibited in cardiomyocytes following treatment of cells with medium derived from M2 macrophages cultured under OGD/R conditions. Previous studies revealed that ischemia and reperfusion could also induce oxidative damage and apoptosis of cells (45,46). In the present study, the induction of oxidative stress and apoptosis in cardiomyocytes were relieved following treatment of the cells with culture medium derived from M2 macrophages, which were cultured under OGD/R conditions. The results of the present study indicated that TGF- β secreted by M2 macrophages alleviated OGD/R-induced inflammatory damage, oxidative stress, and

apoptosis by upregulating the expression levels of lncRNA ATB in cardiomyocytes. However, there are limitations in the present study. Firstly, TGF- β could induce myocardial fibrosis and remodeling, which adversely affects the heart according to previous literature (47,48). Hence, the effects of TGF- β on myocardial fibrosis while protecting cardiomyocytes from ischemia-reperfusion injury should be further investigated. Secondly, the specific role and mechanisms of anti-inflammatory factors secreted by macrophages in myocardial ischemia-reperfusion injury have not been fully elucidated, and the experimental contents of the study need to be repeated with soluble TGF- β alone. These will be investigated in future studies.

In conclusion, the present study demonstrated the effects of TGF- β secreted by M2 macrophages on OGD/R-induced injury of cardiomyocytes. The present study suggested that macrophages may be the source of TGF- β in the process of myocardial ischemic injury. TGF- β alleviated OGD/R-induced inflammatory damage, oxidative injury and apoptosis of

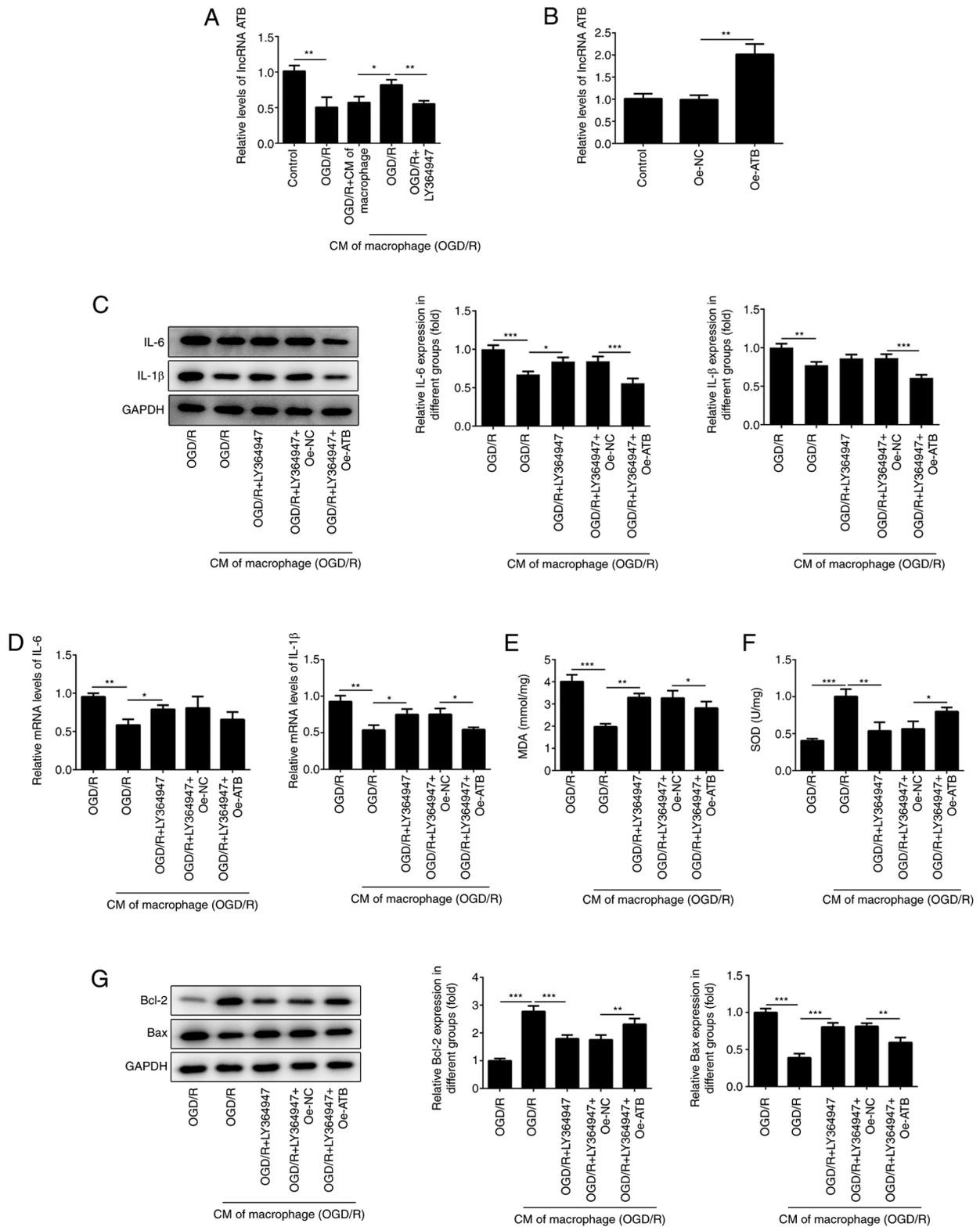


Figure 5. TGF- β -mediated induction of lncRNA ATB is required for the protective effects of M2-type macrophage medium on cardiomyocytes. (A) The expressions of lncRNA ATB in H9c2 cells was detected using RT-qPCR following treatment with LY364947 (5 μ g/ml). (B) The levels of lncRNA ATB in H9c2 cells were determined using RT-qPCR. The expression of IL-1 β , IL-6 in H9c2 cells was detected using (C) western blotting and (D) RT-qPCR. The levels of (E) MDA and (F) SOD in H9c2 cells were determined using commercial kits. (G) The expression of apoptosis-related proteins in H9c2 cells were determined using western blotting. * P <0.05, ** P <0.01 and *** P <0.001. OGD/R, oxygen-glucose deprivation/reoxygenation; CM of macrophage, normal macrophage culture medium; CM of macrophage (OGD/R), macrophage culture medium under OGD/R conditions; MDA, malondialdehyde; SOD, superoxide dismutase; lncRNA ATB, long non-coding RNA activated by TGF- β ; Oe, overexpression plasmid; NC, negative control.

cardiomyocytes via suppressing the NF- κ B signaling pathway by activating lncRNA ATB, which will provide a basis for

clinical treatment of myocardial ischemic injury targeting the inflammatory response.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

HL and WSX acquired the data. XWL and ZW analyzed the data. JY and TZ contributed to the design of the study. All authors read and approved the final manuscript.

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

- Zhou M, Bao Y, Li H, Pan Y, Shu L, Xia Z, Wu D, Lam KSL, Vanhoutte PM, Xu A, *et al*: Deficiency of adipocyte fatty-acid-binding protein alleviates myocardial ischaemia/reperfusion injury and diabetes-induced cardiac dysfunction. *Clin Sci (Lond)* 129: 547-559, 2015.
- Lejay A, Fang F, John R, Van JAD, Barr M, Thaveau F, Chakfe N, Geny B and Scholey JW: Ischemia reperfusion injury, ischemic conditioning and diabetes mellitus. *J Mol Cell Cardiol* 91: 11-22, 2016.
- Wang S, Wang C, Yan F, Wang T, He Y, Li H, Xia Z and Zhang Z: N-acetylcysteine attenuates diabetic myocardial ischemia reperfusion injury through inhibiting excessive autophagy. *Mediators Inflamm* 2017: 9257291, 2017.
- Xia KP, Cao HM and Shao CZ: Protective effect of notoginsenoside R1 in a rat model of myocardial ischemia reperfusion injury by regulation of Vitamin D3 upregulated protein 1/NF- κ B pathway. *Pharmazie* 70: 740-744, 2015.
- Dressing A, Graeter Z and Bardutzky J: Safe intravenous thrombolysis after traumatic cardiopulmonary resuscitation with Rib fractures: A case report. *Case Rep Neurol* 9: 156-160, 2017.
- Page PS, Sethi P, Freed JK, Boettcher BT and Hossein Almassi G: A rare complication of cardiopulmonary resuscitation after mitral valve replacement. *J Cardiothorac Vasc Anesth* 31: 770-772, 2017.
- Rosoff PM and Schneiderman LJ: Response to open peer commentaries on 'Irrational Exuberance: Cardiopulmonary resuscitation as Fetish'. *Am J Bioeth* 17: W1-W3, 2017.
- Sarma S, Bucuti H, Chitnis A, Klacman A and Dantu R: Real-time mobile device-assisted chest compression during cardiopulmonary resuscitation. *Am J Cardiol* 120: 196-200, 2017.
- Dobaczewski M, Chen W and Frangogiannis NG: Transforming growth factor (TGF)-beta signaling in cardiac remodeling. *J Mol Cell Cardiol* 51: 600-606, 2011.
- Wang Y, Zong L and Wang X: TGF- β improves myocardial function and prevents apoptosis induced by anoxia-reoxygenation, through the reduction of endoplasmic reticulum stress. *Can J Physiol Pharmacol* 94: 9-17, 2016.
- Fujiwara N and Kobayashi K: Macrophages in inflammation. *Curr Drug Targets Inflamm Allergy* 4: 281-286, 2005.
- Lu CH, Yeh DW, Lai CY, Liu YL, Huang LR, Lee AY, Jin SC and Chuang TH: USP17 mediates macrophage-promoted inflammation and stemness in lung cancer cells by regulating TRAF2/TRAF3 complex formation. *Oncogene* 37: 6327-6340, 2018.
- Cochain C and Zerneck A: Macrophages in vascular inflammation and atherosclerosis. *Pflugers Arch* 469: 485-499, 2017.
- Essandoh K, Li Y, Huo J and Fan GC: MiRNA-mediated macrophage polarization and its potential role in the regulation of inflammatory response. *Shock* 46: 122-131, 2016.
- Zhang M, Hutter G, Kahn SA, Azad TD, Gholamin S, Xu CY, Liu J, Achrol AS, Richard C, Sommerkamp P, *et al*: Anti-CD47 treatment stimulates phagocytosis of glioblastoma by M1 and M2 polarized macrophages and promotes M1 polarized macrophages in vivo. *PLoS One* 11: e0153550, 2016.
- Sun YY, Li XF, Meng XM, Huang C, Zhang L and Li J: Macrophage phenotype in liver injury and repair. *Scand J Immunol* 85: 166-174, 2017.
- Sarie Y, Takafumi S, Takeshi M, Yutaro M, Norio I, Kazuki F, Saiko MN, Shuhei N, Shuji K, Hiroyuki M, *et al*: Inhibition of local macrophage growth ameliorates focal inflammation and suppresses atherosclerosis. *Arterioscler Thromb Vasc Biol* 38: 994-1006, 2018.
- Yuan JH, Yang F, Wang F, Ma JZ, Guo YJ, Tao QF, Liu F, Pan W, Wang TT, Zhou CC, *et al*: A long noncoding RNA activated by TGF-beta promotes the invasion-metastasis cascade in hepatocellular carcinoma. *Cancer Cell* 25: 666-681, 2014.
- Ying H, Wang Y, Gao Z and Zhang Q: Long non-coding RNA activated by transforming growth factor beta alleviates lipopolysaccharide-induced inflammatory injury via regulating microRNA-223 in ATDC5 cells. *Int Immunopharmacol* 69: 313-320, 2019.
- Zhi W, Li K, Wang H, Lei M and Guo Y: Melatonin elicits protective effects on OGD/R-insulted H9c2 cells by activating PGC-1 α /Nrf2 signaling. *Int J Mol Med* 45: 1294-1304, 2020.
- 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.
- Italiani P and Boraschi D: From monocytes to M1/M2 macrophages: Phenotypical vs. functional differentiation. *Front Immunol* 5: 514, 2014.
- Higashida K, Kim SH, Jung SR, Asaka M, Holloszy JO and Han DH: Effects of resveratrol and SIRT1 on PGC-1 α activity and mitochondrial biogenesis: A reevaluation. *PLoS Biol* 11: e1001603, 2013.
- Chen Y, Li Y, Xu H, Li G, Ma Y and Pang YJ: Morin mitigates oxidative stress, apoptosis and inflammation in cerebral ischemic rats. *Afr J Tradit Complement Altern Med* 14: 348-355, 2017.
- Lou Z, Wang AP, Duan XM, Hu GH, Song GL, Zuo ML and Yang ZB: Upregulation of NOX2 and NOX4 mediated by TGF- β signaling pathway exacerbates cerebral ischemia/reperfusion oxidative stress injury. *Cell Physiol Biochem* 46: 2103-2113, 2018.
- Takeda T: A pathomorphological study on damage and repair process of tubuli after renal ischemia. *Nihon Jinzo Gakkai Shi* 38: 493-501, 1996 (In Japanese).
- Fliiss H and Gatteringer D: Apoptosis in ischemic and reperfused rat myocardium. *Circ Res* 79: 949-956, 1996.
- Du C, Hu R, Csernansky CA, Hsu CY and Choi DW: Very delayed infarction after mild focal cerebral ischemia: A role for apoptosis? *J Cereb Blood Flow Metab* 16: 195-201, 1996.
- Wang XL and Huang C: Difference of TGF- β /Smads signaling pathway in epithelial-mesenchymal transition of normal colonic epithelial cells induced by tumor-associated fibroblasts and colon cancer cells. *Mol Biol Rep* 46: 2749-2759, 2019.
- Alleman RJ, Tsang AM, Ryan TE, Patteson DJ, McClung JM, Spangenburg EE, Shaikh SR, Neuffer PD and Brown DA: Exercise-induced protection against reperfusion arrhythmia involves stabilization of mitochondrial energetics. *Am J Physiol Heart Circ Physiol* 310: H1360-H1370, 2016.

31. Arsenian M: Potential cardiovascular applications of glutamate, aspartate, and other amino acids. *Clin Cardiol* 21: 620-624, 1998.
32. Hausenloy DJ and Yellon DM: Myocardial ischemia-reperfusion injury: A neglected therapeutic target. *J Clin Invest* 123: 92-100, 2013.
33. Kalogeris T, Baines CP, Krenz M and Korthuis RJ: Cell biology of ischemia/reperfusion injury. *Int Rev Cell Mol Biol* 298: 229-317, 2012.
34. Prasad A, Stone GW, Holmes DR and Gersh B: Reperfusion injury, microvascular dysfunction, and cardioprotection: The 'dark side' of reperfusion. *Circulation* 120: 2105-2112, 2009.
35. Marunouchi T and Tanonaka K: Cell death in the cardiac myocyte. *Biol Pharm Bull* 38: 1094-1097, 2015.
36. Działo E, Tkacz K and Błyszczuk P: Crosstalk between the TGF- β and WNT signalling pathways during cardiac fibrogenesis. *Acta Biochim Pol* 65: 341-349, 2018.
37. Khalil H, Kanisicak O, Prasad V, Correll RN, Fu X, Schips T, Vagnozzi RJ, Liu R, Huynh T, Lee SJ, *et al*: Fibroblast-specific TGF- β -Smad2/3 signaling underlies cardiac fibrosis. *J Clin Invest* 127: 3770-3783, 2017.
38. Zhang F, Wang H, Wang X, Jiang G, Liu H, Zhang G, Wang H, Fang R, Bu X, Cai S and Du J: TGF- β induces M2-like macrophage polarization via SNAIL-mediated suppression of a pro-inflammatory phenotype. *Oncotarget* 7: 52294-52306, 2016.
39. Zhu L, Fu X, Chen X, Han X and Dong P: M2 macrophages induce EMT through the TGF- β /Smad2 signaling pathway. *Cell Biol Int* 41: 960-968, 2017.
40. Milich LM, Ryan CB and Lee JK: The origin, fate, and contribution of macrophages to spinal cord injury pathology. *Acta Neuropathol* 137: 785-797, 2019.
41. Fatica A and Bozzoni I: Long non-coding RNAs: New players in cell differentiation and development. *Nat Rev Genet* 15: 7-21, 2014.
42. Guttman M and Rinn JL: Modular regulatory principles of large non-coding RNAs. *Nature* 482: 339-346, 2012.
43. Chen Z, Ding T and Ma CG: Dexmedetomidine (DEX) protects against hepatic ischemia/reperfusion (I/R) injury by suppressing inflammation and oxidative stress in NLRC5 deficient mice. *Biochem Biophys Res Commun* 493: 1143-1150, 2017.
44. Sharma AK, Charles EJ, Zhao Y, Narahari AK, Baderdinni PK, Good ME, Lorenz UM, Kron IL, Bayliss DA, Ravichandran KS, *et al*: Pannexin-1 channels on endothelial cells mediate vascular inflammation during lung ischemia-reperfusion injury. *Am J Physiol Lung Cell Mol Physiol* 315: L301-L312, 2018.
45. Han SJ, Choi HS, Kim JI, Park JW and Park KM: IDH2 deficiency increases the liver susceptibility to ischemia-reperfusion injury via increased mitochondrial oxidative injury. *Redox Biol* 14: 142-153, 2018.
46. Liu Y, Shi B, Li Y and Zhang H: Protective effect of Luteolin against renal ischemia/reperfusion injury via modulation of pro-inflammatory cytokines, oxidative stress and apoptosis for possible benefit in kidney transplant. *Med Sci Monit* 23: 5720-5727, 2017.
47. Huo L, Shi W, Chong L, Wang J, Zhang K, Li Y: Asiatic acid inhibits left ventricular remodeling and improves cardiac function in a rat model of myocardial infarction. *Exp Ther Med* 11: 57-64, 2016.
48. Meng XM, Nikolic-Paterson DJ and Lan HY: TGF- β : The master regulator of fibrosis. *Nat Rev Nephrol* 12: 325-338, 2016.



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