



Original Article

C-X3-C motif chemokine ligand 1/receptor 1 regulates the M1 polarization and chemotaxis of macrophages after hypoxia/reoxygenation injury

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Received 30 January 2021

Available online 7 June 2021

Abstract

Background: Macrophages play an important role in renal ischemia reperfusion injury, but the functional changes of macrophages under hypoxia/reoxygenation and the related mechanism are unclear and need to be further clarified.

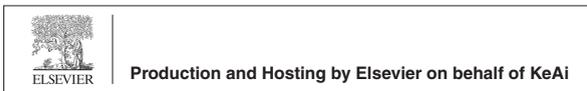
Methods: The effects of hypoxia/reoxygenation on functional characteristics of RAW264.7 macrophages were analyzed through the protein expression detection of pro-inflammatory factors TNF- α and CD80, anti-inflammatory factors ARG-1 and CD206. The functional implications of C-X3-C motif chemokine receptor 1 (CX3CR1) down-regulation in hypoxic macrophages were explored using small interfering RNA technology. Significance was assessed by the parametric *t*-test or nonparametric Mann–Whitney test for two group comparisons, and a one-way ANOVA or the Kruskal–Wallis test for multiple group comparisons.

Results: Hypoxia/reoxygenation significantly increased the protein expression of M1-related pro-inflammatory factors TNF- α , CD80 and chemokine C-X3-C motif chemokine ligand 1 (CX3CL1)/CX3CR1 and inhibited the protein expression of M2-related anti-inflammatory factors ARG-1 and CD206 in a time-dependent manner in RAW264.7 cells. However, the silencing of CX3CR1 in RAW264.7 cells using specific CX3CR1-siRNA, significantly attenuated the increase in protein expression of TNF- α ($P < 0.05$) and CD80 ($P < 0.01$) and the inhibition of ARG-1 ($P < 0.01$) and CD206 ($P < 0.01$) induced by hypoxia/reoxygenation. In addition, we also found that hypoxia/reoxygenation could significantly enhance the migration (2.2-fold, $P < 0.01$) and adhesion capacity (1.5-fold, $P < 0.01$) of RAW264.7 macrophages compared with the control group, and CX3CR1-siRNA had an inhibitory role (40% and 20% reduction, respectively). For elucidating the mechanism, we showed that the phosphorylation levels of ERK ($P < 0.01$) and the p65 subunit of NF- κ B ($P < 0.01$) of the RAW264.7 cells in the hypoxic/reoxygenation group were significantly increased, which could be attenuated by down-regulation of CX3CR1 expression ($P < 0.01$, both). ERK inhibitors also significantly blocked

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Peer review under responsibility of Chinese Medical Association.



the effects of hypoxic/reoxygenation on the protein expression of M1-related pro-inflammatory factors TNF- α , CD80 and M2-related anti-inflammatory factors ARG-1 and CD206. Moreover, we found that conditioned medium from polarized M1 macrophages induced by hypoxia/reoxygenation, notably increased the degree of apoptosis of hypoxia/reoxygenation-induced TCMK-1 cells, and promoted the protein expression of pro-apoptotic proteins bax ($P < 0.01$) and cleaved-caspase 3 ($P < 0.01$) and inhibited the expression of anti-apoptotic protein bcl-2 ($P < 0.01$), but silencing CX3CR1 in macrophages had a protective role. Finally, we also found that the secretion of soluble CX3CL1 in RAW264.7 macrophages under hypoxia/reoxygenation was significantly increased.

Conclusions: The findings suggest that hypoxia/reoxygenation could promote M1 polarization, cell migration, and adhesion of macrophages, and that polarized macrophages induce further apoptosis of hypoxic renal tubular epithelial cells by regulating of CX3CL1/CX3CR1 signaling pathway.

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Keywords: Macrophages; Hypoxia/Reoxygenation; C-X3-C motif chemokine ligand 1/receptor 1; Phenotypic polarization

Introduction

Macrophages are the major infiltrating inflammatory cells at sites of inflammation, tissue injury, and tumor growth.^{1,2} They play important roles in innate and acquired immune responses and in the process of tissue damage and repair by cytophagy, release of inflammatory cytokines and antigen-presenting.³ Moreover, macrophages are highly variable cells and can display two polarized phenotypes that are dependent on different organizational environments, the classically activated pro-inflammatory M1 and the alternatively activated anti-inflammatory M2.^{2,4}

Hypoxia, defined as an imbalance between oxygen supply and demand, is a common response that occurs in many pathological processes, such as atherosclerosis, ischemia, and tumors.^{5,6} Many recent studies have indicated that different hypoxic environments could induce different functional transformations in macrophages. The hypoxic areas of murine atherosclerotic lesions displayed marked M1 macrophage infiltration by the regulation of hypoxia-inducible factor-1 α (HIF-1 α). The HIF-1 α deficiency in macrophages could decrease the differentiation to proinflammatory phenotype and reduce atherosclerotic lesions of aorta in *Ldlr*^{-/-} mice.⁷ Low frequency intermittent hypoxia also has been shown to impair the adipogenesis of subcutaneous adipose tissue in lean mice by promoting macrophage M1 polarization.⁸ However, the hypoxic environment in idiopathic pulmonary fibrosis could mediate macrophage M2 polarization by HIF-1 α upregulating ADORA2B protein expression in macrophages and contribute to pulmonary fibrosis.⁹ In addition, the tumor hypoxia is also an important reason for inducing macrophage M2 polarization and tumor growth and metastasis.⁶

Ischemia-reperfusion injury (IRI) is a typical hypoxic disease and a pathological process that aggravates injury after reoxygenation. Renal IRI is the main cause of acute renal injury (AKI).¹⁰ It has been demonstrated that macrophages play a major role in ischemia-mediated renal injury.¹¹ Traditionally, macrophages are thought to originate from peripheral circulating monocytes, but it has recently been reported that there are tissue-resident macrophages found in almost all tissues including the kidney.¹ Furthermore, kidney containing complex vascular system has high blood flow and blood retention, in which there are a number of circulating mononuclear/macrophages.¹² These macrophages also undergo hypoxia/reoxygenation processes while the renal IRI occurs. However, the effects of hypoxia/reoxygenation on the functional characteristics of these macrophages and the related mechanisms remain unclear.

Chemokine C-X3-C motif chemokine ligand 1/receptor 1 (CX3CL1/CX3CR1) signaling pathway has been shown to promote the progression of renal IRI by directly inducing monocyte/macrophage recruitment.¹³ However, researchers recently found that CX3CL1/CX3CR1 was also an important phenotypic regulator of macrophage during various pathological processes. The CX3CR1 knockdown in bleomycin-induced pulmonary fibrosis model mice did not reduce the number of pulmonary infiltrations of any type of leukocytes, but showed macrophage phenotype toward M1, and alleviated the progression of pulmonary fibrosis.¹⁴ Compared with wild-type normolipidemic recipients, CX3CR1^{-/-} recipients had less regression signature in transplanted atherosclerotic plaques that resulted from the decrease of macrophages of M2 polarization.¹⁵ Whereas, CX3CR1 deficiency in acute schistosomiasis mice has been shown to enhance M2 polarization of

macrophages by activating STAT6/PPAR- γ signaling, and to protect from hepatic granuloma formation.¹⁶

Therefore, we hypothesized that hypoxia/reoxygenation could affect the polarization phenotype of macrophages and that CX3CL1/CX3CR1 signaling is a key regulator. Here, we found that hypoxia/reoxygenation, by activating CX3CL1/CX3CR1/ERK/NF- κ B signaling, induced M1 polarization of RAW264.7 macrophages, which was shown through up-regulated proinflammatory cytokine production, increased cell adhesion and migration, and the role of promoting apoptosis of murine renal tubular epithelial cells.

Methods

Cell culture and hypoxic exposure

The murine macrophage cell line RAW264.7 and the renal tubular epithelial cell line TCMK-1 were purchased from ATCC (Manassas, VA). They were cultured in Dulbecco's Modified Eagle Medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY), and incubated at 37 °C in a humidified atmosphere of 5% CO₂. For the hypoxic exposure study, the cells were maintained in an anaerobic system (2% O₂, 5% CO₂, and 93% N₂), in which the hypoxic conditions were established for 6 h (RAW264.7) and 24 h (TCMK-1). Then, the cells were cultured under normoxic conditions (20% O₂ and 5% CO₂) for a reoxygenation phase of 24 h.

Small interfering RNA (siRNA) transient transfection

Murine CX3CR1-siRNA and negative control siRNA were purchased from JST scientific (Wuhan, China). The sequence of CX3CR1 specific siRNA was: 5'-CCGCCAACUCCAUGAACAATT-3' (sense), 5'-UUGUUCAUGGAGUUGGCGGTT-3' (antisense). Macrophages were transiently transfected for 48 h with CX3CR1-siRNA or negative-control siRNA using Lipofectamine™ 2000 Transfection Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction.

Western blot analysis

Western blot analysis of protein expressions in RAW264.7 and TCMK-1 cell lines was performed as previously described.¹⁷ The primary antibodies used were as follows: anti-CX3CL1 (DF12376), anti-ARG-1 (DF6657), anti-ERK (AF0155), and

anti-phosphorylated-ERK (AF1015) were purchased from Affinity Biosciences, Cincinnati, OH. Anti-CX3CR1 (13885-1-AP), anti-TNF- α (60291-1-IG), anti-CD206 (60143-1-Ig) and anti-NF- κ B p65 (10745-1-AP) were purchased from Proteintech Group Inc. (Rosemont, IL). The other antibodies used were as follows: anti-CD80 (bs-2211R, Bioss, Beijing, China), and anti-phosphorylated-NF- κ B p65 (#3033, Cell Signaling Technology, Danvers, MA). GAPDH (AB-P-R001, Xianzhi Biotechnology, Hangzhou, China) was used for normalization.

Quantitative real-time PCR

Total RNA was isolated using TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions, and reverse transcription was performed using the HiScript Reverse Transcriptase Reagent Kit (Vazyme, Nanjing, China). Quantitative real-time PCR was performed using SYBR Green Master Mix (Vazyme, Nanjing, China) on the QuantStudio 6 Flex Real-Time PCR system (Applied Biosystems, Foster City, CA).

Primer sequences were as follows: murine CX3CR1: 5'-GCTACTTCCGCATCATCCA-3' (forward), 5'-TCCCAGGTATCTTCTGAACCTTTT-3' (reverse) and murine GAPDH: 5'-ATGGGTGTGAACCACGAGA-3' (forward), 5'-CAGGGATGATGTTCTGGGCA-3' (reverse). The mRNA quantity was normalized to GAPDH and the relative mRNA expression was calculated using the $2^{-\Delta\Delta C_t}$ method.

Flow cytometry

Cells were collected, washed with PBS, and resuspended in PBS containing 0.5% fetal bovine serum. Fluorescence-conjugated antibodies against CD80 (104713) and CD206 (141705), both purchased from BioLegend (San Diego, CA, cc USA) were added and incubated for 30 min in the dark on ice. Cell fluorescence was detected using a BD Biosciences flow cytometer (Franklin Lakes, NJ, USA). The percentage of apoptotic cells was detected using an annexin V-FITC apoptosis detection kit purchased from KeyGEN Biotechnology Co., Ltd. (Nanjing, China).

Cell adhesion assay

The cells in each group were digested and produced single-cell suspensions which were then seeded onto 96-well plates prepared with Matrigel (#356234, BD

Biosciences, Bedford, MA) in advance and incubated under normal culture conditions. After 1 h, the non-adherent cells were removed by washing twice with PBS. Then, cells were incubated with 10 μ l CCK-8 (#C0037, Beyotime Biotechnology Co. Ltd., Shanghai, China) under normal culture conditions for 4 h. The absorbance was measured at 450nm.

Cell migration assay

Cell migration assays were performed using transwell chambers (24-well insert, #353097, BD Biosciences, Bedford, MA). Macrophages in each group were digested and resuspended in serum-free medium, and then plated in the upper chamber. Medium containing 10% FBS as a chemoattractant was plated in the lower chamber. Then, the cells were cultured under normoxic conditions for 48 h. Non-migrating macrophages on the upper surface were removed using a cotton swab. Macrophages on the lower surface were fixed with 70% cold ethanol solution for 1 h, and stained with 0.5% crystal violet for 20 min. Stained cells were counted in five randomly selected fields (at 200 \times magnification) using a light microscope.

Enzyme-linked immunosorbent assay (ELISA)

CX3CL1 levels in cell culture supernatants were analyzed using ELISA kits (Elabscience, Wuhan, China) according to the manufacturer's instruction.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software version 6 (GraphPad Software Inc., San Diego, CA). When comparing two groups, we used the parametric *t*-test or nonparametric Mann–Whitney test, and for multiple group comparisons, a one-way ANOVA or the Kruskal–Wallis test was performed. Statistical significance was obtained when $P < 0.05$ or $P < 0.01$.

Results

Hypoxia/reoxygenation induces changes in the expression of TNF- α , CD80, ARG-1, CD206, and CX3CL1/CX3CR1 in macrophages

We first studied the effect of hypoxia/reoxygenation on the protein expression of pro-inflammatory factors TNF- α and CD80, anti-inflammatory factors ARG-1 and CD206, and chemokine CX3CL1/CX3CR1 at

different time of hypoxia. The results showed that hypoxia/reoxygenation significantly induced the protein expression of TNF- α in a time-dependent manner in RAW264.7 cells, reaching a 1.70-fold ($P < 0.01$) increase at 6 h hypoxia and a 1.90-fold ($P < 0.01$) increase at 8 h hypoxia compared with the normoxic control (Fig. 1A, D). Hypoxia/reoxygenation significantly inhibited the protein expression of ARG-1 in RAW264.7 cells at hypoxic exposure for 4 h (0.73-fold, $P < 0.01$), 6 h (0.48-fold, $P < 0.01$) and 8 h (0.40-fold, $P < 0.01$) compared with the control group (Fig. 1A, D). In addition, we also found that hypoxia/reoxygenation significantly upregulated the protein expression of pro-inflammatory factor CD80, and downregulated the anti-inflammatory factor CD206 in a time-dependent manner (Supplementary Fig. S1). CX3CL1 and CX3CR1 expressions in the hypoxia/reoxygenation group were significantly increased under hypoxic stimulus of 4 h (1.25-fold and 1.39-fold, respectively, both $P < 0.01$), 6 h (1.90-fold and 1.62-fold, respectively, both $P < 0.01$) and 8 h (1.96-fold and 1.71-fold, respectively, both $P < 0.01$) in a time-dependent manner compared with the normoxic group (Fig. 1B, E). Furthermore, we also found that the addition of CX3CL1 recombinant protein promoted a progressive change in the protein expression of TNF- α and ARG-1 induced by hypoxia/reoxygenation in a concentration-dependent manner (Fig. 1C, F).

CX3CR1-siRNA attenuates the M1 polarization of macrophages induced by hypoxia/reoxygenation

We analyzed the effect of down-regulated expression of CX3CR1 through siRNA technology on the polarization phenotype of macrophages under hypoxia/reoxygenation conditions. The silencing of CX3CR1 in RAW264.7 cells using specific CX3CR1-siRNA under hypoxic conditions was determined by RT-PCR and Western blotting. We found that specific siRNA-CX3CR1 used in our study significantly down-regulated the mRNA and protein expressions under normal and hypoxic exposure by approximately 60% (Supplementary Fig. S2). The siRNA material did not have an effect on the protein expression of TNF- α , ARG-1, CD80 and CD206, the phosphorylation of the p65 subunit of NF- κ B, and the capacity of migration and adhesion of RAW265.7 cells (Supplementary Fig. S3). As shown in Fig. 2A, B, hypoxia/reoxygenation significantly increased the protein expression of pro-inflammatory TNF- α , and inhibited the protein expression of anti-inflammatory ARG-1. The down-regulation of CX3CR1 expression significantly

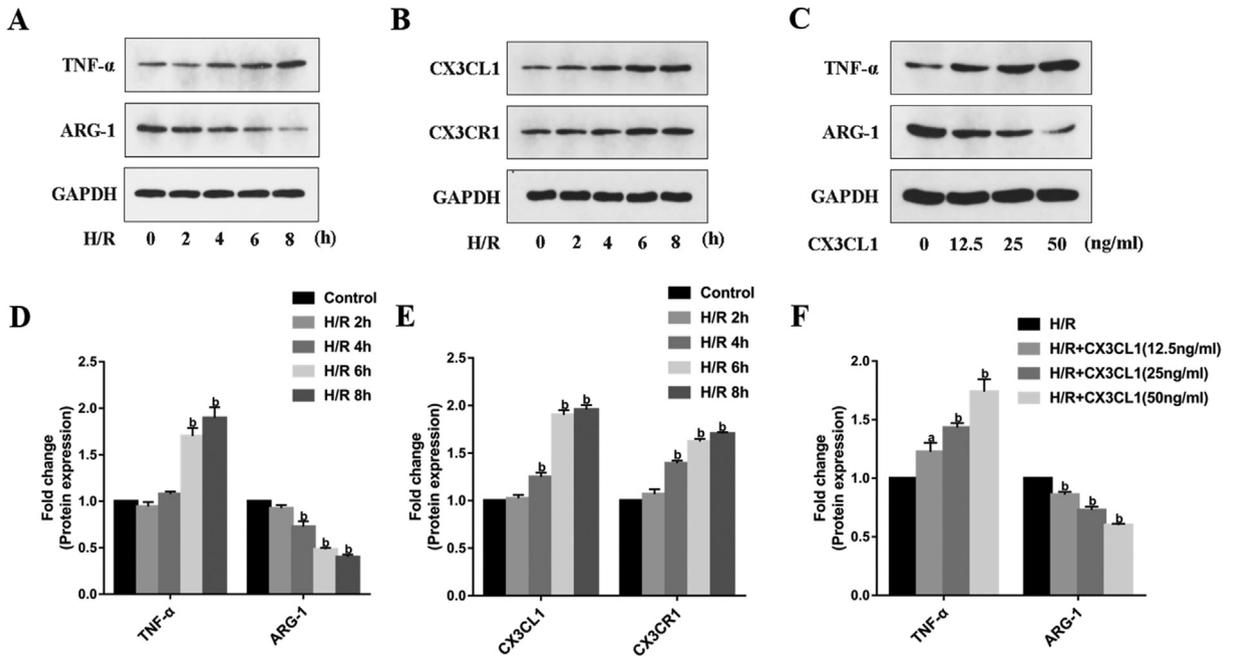


Fig. 1. Hypoxia/reoxygenation increased the protein expressions of TNF- α , CX3CL1, and CX3CR1, and inhibited the protein expression of ARG-1 in a time-dependent manner in RAW264.7 cells. (A and B) The proteins of RAW264.7 macrophages were tested for TNF- α , ARG-1, CX3CL1, and CX3CR1 at different hypoxia times. (C) The protein expression of TNF- α and ARG-1 in RAW264.7 macrophages under hypoxia/reoxygenation combined with different concentrations of CX3CL1 recombinant protein. (D, E, and F) The quantitative analysis of A, B and C, respectively. ^a $P < 0.05$, ^b $P < 0.01$ vs control or H/R. H/R: hypoxia/reoxygenation, hypoxia at 2, 4, 6 or 8 h and then reoxygenates for 24 h.

attenuated the increase in TNF- α expression and inhibition of ARG-1 induced by hypoxia/reoxygenation. Flow cytometry analyses were also performed to assess the expression of M1 marker CD80 and M2 marker CD206 in RAW264.7 cells of different groups. The

results showed that CD80 expression was higher and CD206 expression was lower in macrophages in the hypoxia/reoxygenation group than in the normoxia and control-transfected groups. Knock-down of CX3CR1 expression significantly decreased the expression of

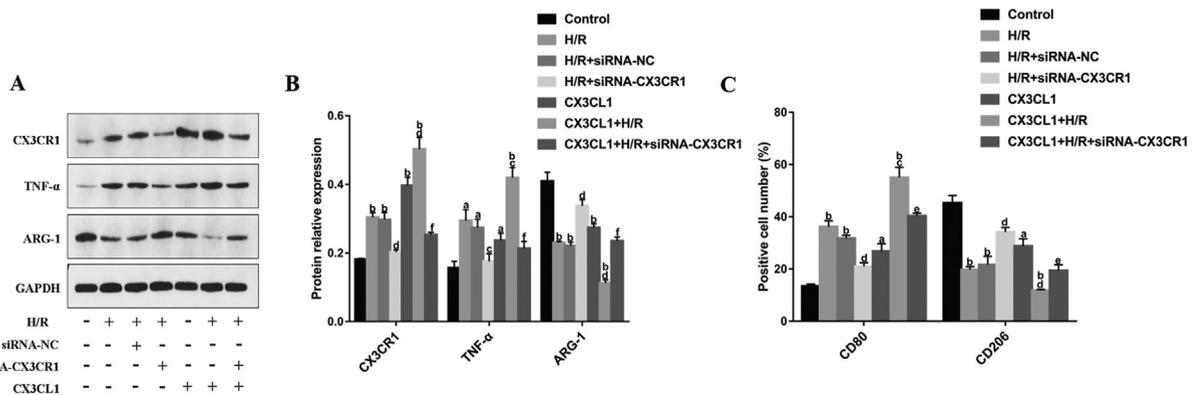


Fig. 2. Down-regulation of CX3CR1 with siRNA attenuated the M1 polarization of macrophages induced by hypoxia/reoxygenation. (A and B) Representative Western blots and summarized data showing the protein levels of CX3CR1, TNF- α , and ARG-1 in different groups of RAW264.7 macrophages. (C) The expression analysis of CD80 and CD206 by flow cytometry in different groups of RAW264.7 macrophages. ^a $P < 0.05$, ^b $P < 0.01$ vs Control; ^c $P < 0.05$, ^d $P < 0.01$ vs H/R; ^e $P < 0.05$, ^f $P < 0.01$ vs CX3CL1 + H/R. H/R: hypoxia/reoxygenation. Cells were exposed to hypoxia condition for 6 h, then reoxygenation for 24 h. The concentration of CX3CL1 recombinant protein used was 50 ng/mL.

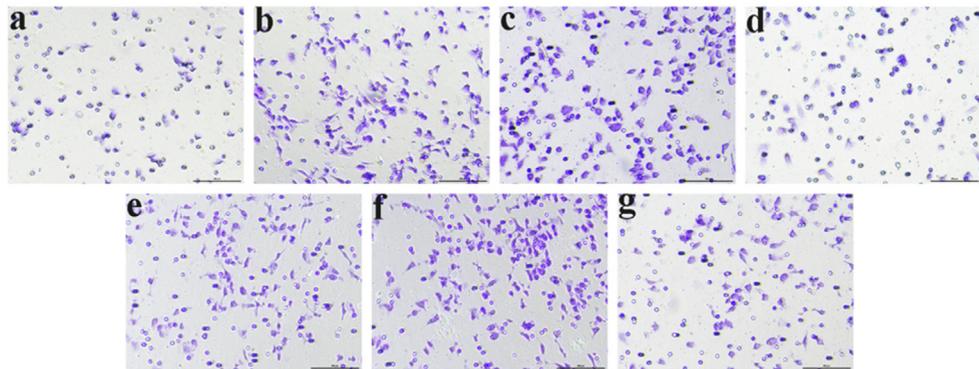
CD80 and increased the expression of CD206 in hypoxia/reoxygenation-treated RAW264.7 cells (Fig. 2C). In this study, we also demonstrated that CX3CL1 recombinant protein could potentiate hypoxia-induced M1 polarization of macrophages and down-regulation of CX3CR1 had an inhibiting role.

CX3CR1-siRNA inhibits cell adhesion and migration capacity of macrophages in hypoxia/reoxygenation

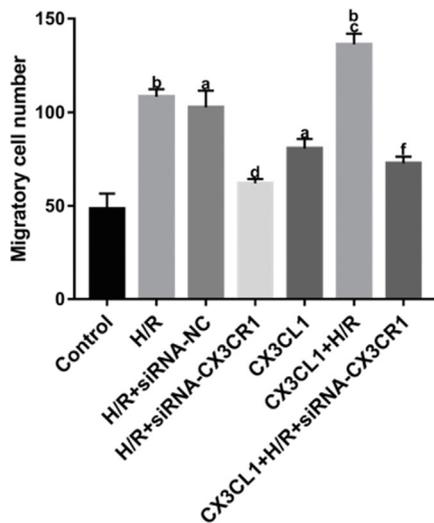
As shown in Fig. 3, hypoxia preconditioning for 6 h induced a 2.2-fold ($P < 0.01$) increase in the cell

migration capacity and an approximately 1.5-fold ($P < 0.01$) increase in the cell adhesive capacity of RAW264.7 cells, compared with the normoxic control cells. CX3CR1-siRNA not only significantly weakened the cell migration of RAW264.7 under hypoxia/reoxygenation conditions (60% of the cells transfected with negative control siRNA, $P < 0.01$), but also inhibited hypoxic preconditioning-induced cell adhesion (80% of the cells transfected with negative control siRNA, $P < 0.05$). CX3CL1 recombinant protein had a significant potentiation effect on the increase in adhesion and migration abilities of RAW264.7 cells after

A



B



C

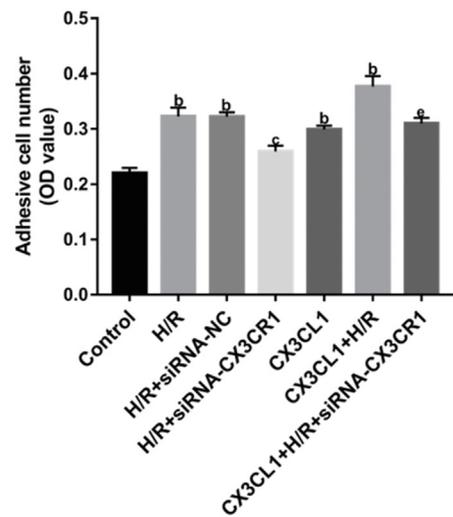


Fig. 3. CX3CR1-siRNA inhibited cell adhesion and migration capacity of macrophages in hypoxia/reoxygenation. (A and B) Representative photomicrographs and quantitative analysis of migratory RAW264.7 macrophages in different groups. The grouping situation included control (a), H/R (b), H/R + siRNA-NC (c), H/R + siRNA-CX3CR1 (d), CX3CL1 (e), CX3CL1 + H/R (f) and CX3CL1 + H/R + siRNA-CX3CR1 (g). (C) The adhesive capacity analysis of RAW264.7 macrophages of different groups is shown. ^a $P < 0.05$, ^b $P < 0.01$ vs Control; ^c $P < 0.05$, ^d $P < 0.01$ vs H/R; ^e $P < 0.05$, ^f $P < 0.01$ vs CX3CL1 + H/R. H/R: hypoxia/reoxygenation. NC: negative control. Cells were exposed to hypoxia condition for 6 h, then reoxygenation for 24 h. The concentration of CX3CL1 recombinant protein used was 50 ng/mL.

hypoxia/reoxygenation treatment, which could be attenuated by the knock-down of CX3CR1 expression.

The M1 phenotype polarization of macrophages induced by hypoxia/reoxygenation through regulation of the CX3CL1/CX3CR1/ERK/NF- κ B signaling pathway

To further elucidate the mechanism of hypoxia/reoxygenation-induced macrophage polarization, we assessed whether hypoxia/reoxygenation promotes the activation of ERK/NF- κ B signaling through the chemokine CX3CL1/CX3CR1 by Western blotting. Compared with the normoxic group, the phosphorylation of ERK and the p65 subunit of NF- κ B of the RAW264.7 cells in the hypoxia/reoxygenation group were significantly increased. Down-regulation of CX3CR1 expression in RAW264.7 cells markedly attenuated this increase (Fig. 4A, B). Moreover, we found that the ERK protein inhibitors PD98059 and U0126 significantly blocked the protein expression alteration of pro-inflammatory factor TNF- α and anti-inflammatory factor ARG-1 induced by hypoxia/reoxygenation with or without CX3CL1 recombinant protein treatment (Fig. 5A–D). Furthermore, the protein expression changes of pro-inflammatory CD80 and anti-inflammatory CD206 under hypoxia/reoxygenation were also significantly alleviated by the ERK protein inhibitors PD98059 and U0126 (Supplementary Fig. S4).

Conditioned medium from polarized M1 macrophages induced by hypoxia/reoxygenation aggravates apoptosis level in renal tubular epithelial cells

We then analyzed the effect of conditioned medium of hypoxia/reoxygenation-treated RAW264.7 cells on the apoptosis of mouse renal tubular epithelial TCMK-1 cells. Hypoxia/reoxygenation and the conditioned medium from polarized M1 macrophages induced by hypoxia/reoxygenation both significantly promoted apoptosis in TCMK-1 cells ($8.73\% \pm 1.12\%$ and $9.95\% \pm 0.95\%$, respectively) compared with the control group ($2.60\% \pm 0.58\%$). Moreover, the supernatant of RAW264.7 cells stimulated by hypoxia/reoxygenation could worsen the degree of apoptosis of hypoxia/reoxygenation-induced TCMK-1 cells with a 58% increase (Fig. 6A). The expression of pro-apoptotic proteins bax and cleaved-caspase 3 was remarkably increased, and the expression of anti-apoptotic protein bcl-2 was significantly decreased in TCMK-1 cells under hypoxia/reoxygenation conditions or treated with the conditioned medium from polarized M1 macrophages compared with normoxic control cells (Fig. 6B, C). In contrast, the conditioned medium from hypoxia/reoxygenation-treated RAW264.7 cells with knock-down of CX3CR1 expression had no significant deteriorating effect on the apoptosis of TCMK-1 cells under normoxic hypoxia/reoxygenation conditions (Fig. 6).

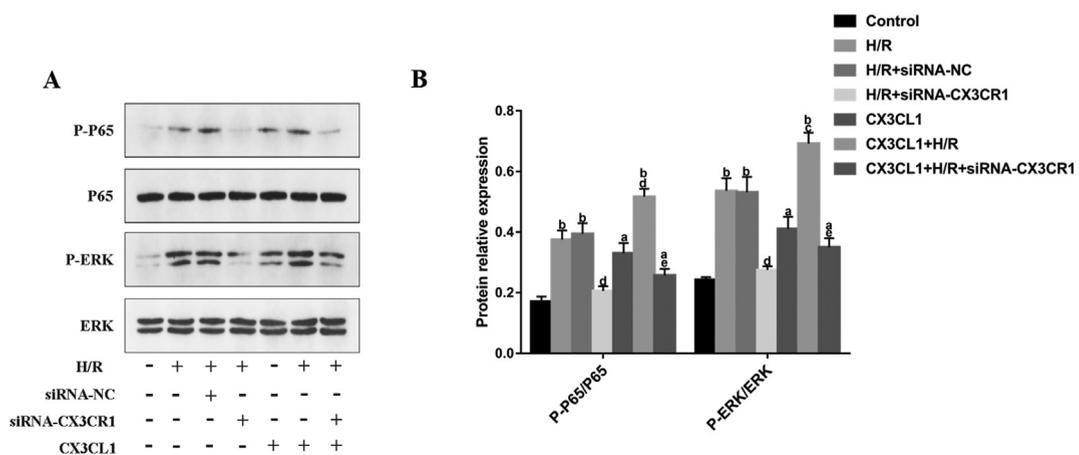


Fig. 4. Hypoxia/reoxygenation increased the phosphorylation levels of ERK and the p65 subunit of NF- κ B of the RAW264.7 cells and the CX3CR1 siRNA had an inhibitory role. (A and B) Representative Western blots and summarized data showing phosphorylation levels of ERK and the p65 subunit of NF- κ B in different groups of RAW264.7 macrophages. ^a $P < 0.05$, ^b $P < 0.01$ vs Control; ^c $P < 0.05$, ^d $P < 0.01$ vs H/R; ^e $P < 0.01$ vs CX3CL1 + H/R. H/R: hypoxia/reoxygenation. NC: negative control. Cells were exposed to hypoxia condition for 6 h, then reoxygenation for 24 h. The concentration of CX3CL1 recombinant protein used was 50 ng/mL.

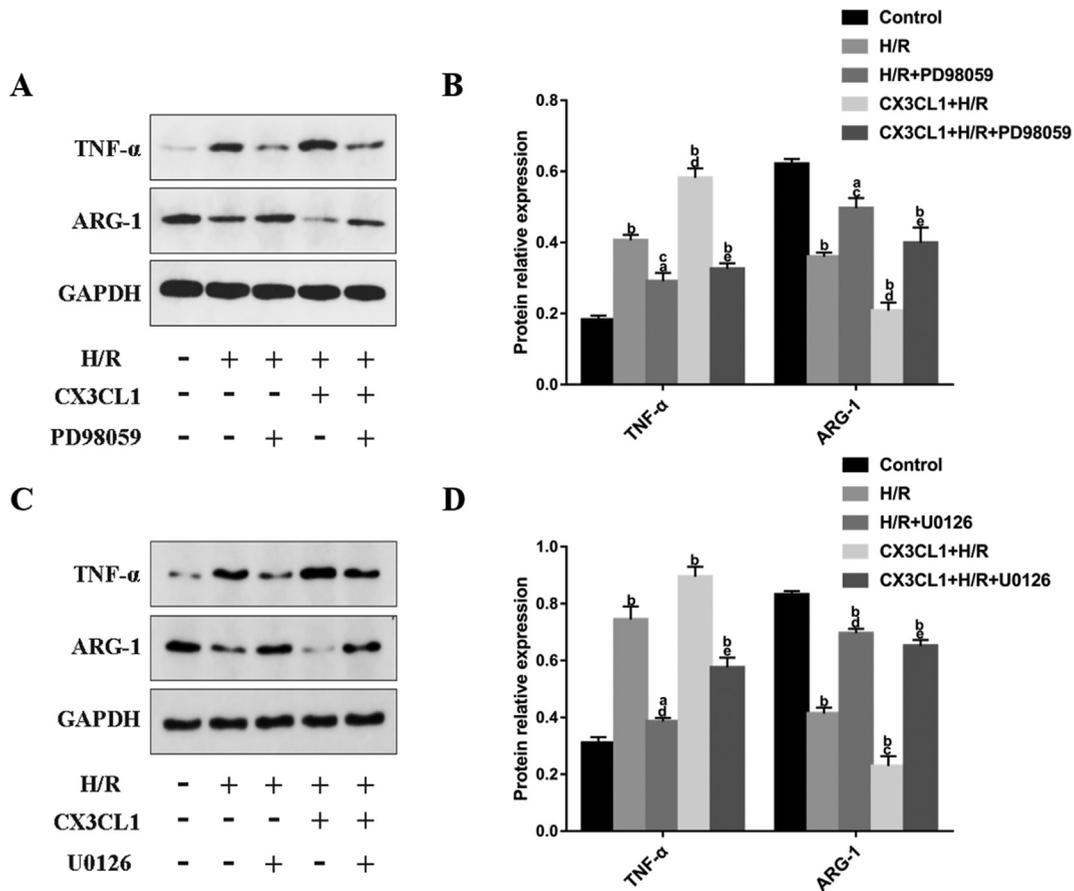


Fig. 5. ERK protein inhibitor reversed the protein expression alteration of TNF- α and ARG-1 induced by hypoxia/reoxygenation. (A and B, C and D) Representative Western blots and summarized data showing the protein expressions of pro-inflammatory TNF- α , and anti-inflammatory marker ARG-1 in different groups of RAW264.7 macrophages. ^a $P < 0.05$, ^b $P < 0.01$ vs Control; ^c $P < 0.05$, ^d $P < 0.01$ vs H/R; ^e $P < 0.01$ vs CX3CL1 + H/R. H/R: hypoxia/reoxygenation. Cells were exposed to hypoxia condition for 6 h, then reoxygenation for 24 h. The concentration of CX3CL1 recombinant protein and ERK protein inhibitor PD98059 and U0126 used was 50 ng/mL, 20 μ mol/L and 10 μ mol/L, respectively.

Soluble chemokine CX3CL1 levels in cellular supernatant of macrophages are upregulated by hypoxia/reoxygenation

Finally, the secretion of soluble CX3CL1 in macrophages after hypoxia/reoxygenation exposure was analyzed. Hypoxia/reoxygenation significantly increased the concentration of soluble CX3CL1 in the cellular supernatant of RAW264.7 macrophages, after hypoxic exposure for 4 h (1.51-fold, $P < 0.01$), 6 h (2.43-fold, $P < 0.01$) and 8 h (2.88-fold, $P < 0.01$) compared with the control group (Fig. 7A). CX3CR1-siRNA could significantly inhibit the increase in soluble CX3CL1 levels in the cellular supernatant of RAW264.7 macrophages after hypoxia/reoxygenation exposure (Fig. 7B). Moreover, we also found that ERK protein inhibitor PD98059 and U0126 could also markedly inhibit the

secretion of soluble CX3CL1 in RAW264.7 macrophages under hypoxia/reoxygenation (Fig. 7C).

Discussion

Macrophages have been reported to be involved in post-ischemic renal injury by producing various pro-inflammatory or anti-inflammatory cytokines, and promoting acquired immune responses as antigen-presenting cells.³ Whereas, we mainly studied bone marrow-derived macrophages and focused on the pathological changes in renal parenchymal cells in the past, and did not focus on the functional changes in original renal macrophages which also experienced ischemia and hypoxia during ischemic reperfusion injury. Our preliminary study found that pre-ischemic renal lavage that washed out the residual circulating leukocytes, could clearly reduce the

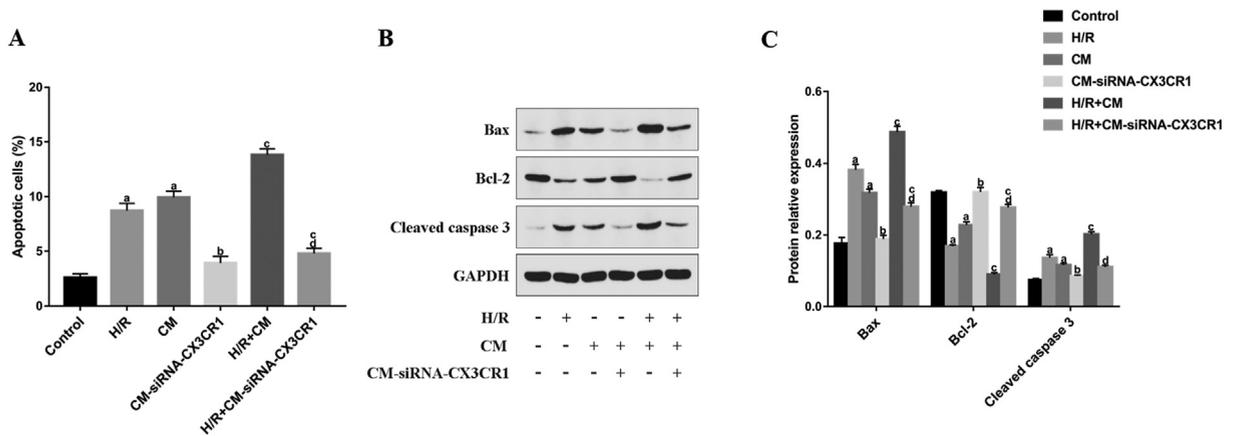


Fig. 6. The apoptosis degree of renal tubular epithelial TCMK-1 cells was increased when exposed to conditioned medium from polarized M1 macrophages induced by hypoxia/reoxygenation. (A) The ratio analysis of apoptosis of TCMK-1 cells by flow cytometry in different groups is shown. (B and C) Representative Western blots and summarized data showing the protein expressions of cell apoptosis-related proteins in different groups of TCMK-1. ^a $P < 0.01$ vs Control; ^b $P < 0.01$ vs CM; ^c $P < 0.01$ vs H/R; ^d $P < 0.01$ vs H/R + CM. H/R: hypoxia/reoxygenation, CM: conditioned medium of hypoxic macrophages, CM-CX3CR1-siRNA: conditioned medium of hypoxic macrophages transfected with CX3CR1-siRNA.

renal damage after IRI by attenuating inflammation.¹⁸ We speculated that the original macrophages in the kidney may be mainly derived from the recently discovered resident renal macrophages and the residual circulating macrophages in the renal vessels. When experiencing ischemia, these macrophages may be the first to undergo functional changes, infiltrate the interstitial space, and cause tissue damage. Therefore, the purpose of this study was to analyze the effect of hypoxia/reoxygenation on the functional characteristics of macrophages and the

relevant mechanism *in vitro*. This study is a continuation of our previous research, and more attention was paid to the functional changes in the residual circulating macrophages in the renal vessels during ischemia. We found that hypoxia/reoxygenation could promote the M1-type transformation of macrophages through the activation of the CX3CL1/CX3CR1/ERK/NF- κ B signaling pathway, in addition to enhancing the ability of migration and adhesion, and increasing the apoptosis level of hypoxic renal tubular epithelial cells.

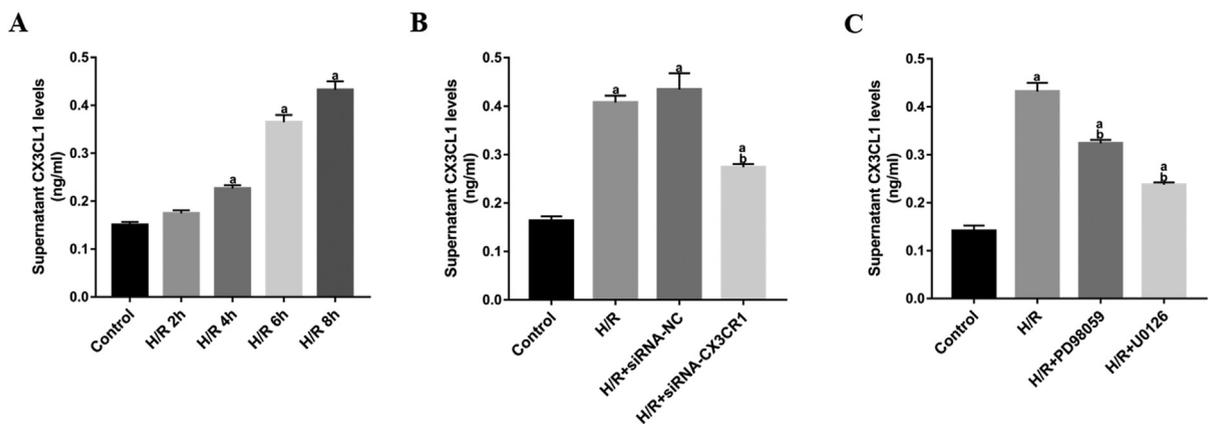


Fig. 7. Hypoxia/reoxygenation promoted the secretion of soluble CX3CL1 of RAW264.7 macrophages, which could be inhibited by CX3CR1-siRNA or ERK inhibitors. (A) Summarized data showing cell supernatant CX3CL1 levels of RAW264.7 macrophages after different hypoxia time. (B and C) Summarized data show the effects of CX3CR1-siRNA and ERK inhibitors on the cell supernatant CX3CL1 levels of RAW264.7 macrophages under hypoxia/reoxygenation. ^a $P < 0.01$ vs Control; ^b $P < 0.01$ vs H/R. H/R: hypoxia/reoxygenation. Cells were exposed to hypoxia condition for the indicated time or 6 h, then reoxygenation for 24 h. The concentration of ERK protein inhibitor PD98059 and U0126 used was 20 μ mol/L and 10 μ mol/L, respectively.

Although hypoxia is a common pathological factor that induces functional changes in macrophages, the direction of functional changes is complex. Our *in vitro* study found that hypoxia/reoxygenation can induce macrophages to transform into M1-type, which is similar to results of studies on adipose tissue¹⁹ and coronary atherosclerosis,⁷ while hypoxia promotes macrophages to transform into M2-type in pulmonary fibrosis¹⁴ and tumors.⁶ Why does hypoxia induce macrophages to exhibit different functional characteristics in different pathological environments? First, macrophages are heterogeneous, multifunctional and sensitive to microenvironmental changes. Second, the specific types of macrophages studied in different studies vary, such as circulating macrophages or tissue residential macrophages. Thirdly, different pathological environments, different adjacent cell types or tissue components may play a key role in the functional changes of macrophages through interaction, and even different degrees and durations of hypoxia have obvious effects on their functions. Four, under different pathological environments, hypoxia-inducible factor or CX3CL1/CX3CR1 signaling pathway can mediate gene regulatory networks in different directions, including positive or negative feedback regulation and immune metabolic rearrangement. In-depth research in these areas is of great significance for the precise intervention and treatment of our diseases.

CX3CL1 is the only member of the CX3C chemokine class and is mainly expressed in endothelial cells, epithelial cells, dendritic cells, smooth muscle cells, neurons, liver cells and fibroblasts. Due to its transmembrane macromolecular structure, CX3CL1 exists in two forms, membrane-bound and soluble forms.²⁰ CX3CR1 is a single specific receptor of the chemokine CX3CL1 with a binding ratio of 1:1. It is mainly expressed in monocytes, macrophages, mast cells, T cells and NK cells.^{20,21} Researchers first recognized that the role of CX3CL1/CX3CR1 in mediating the development of a variety of diseases by regulating inflammatory cell infiltration, including atherosclerosis,²¹ ischemia reperfusion injury,¹³ rheumatoid arthritis²² and asthma.²³ Similarly, most studies have shown that CX3CL1/CX3CR1 promotes chemotaxis of peripheral circulation macrophages and inflammatory response and induces pathological development of renal ischemia-reperfusion injury. Li et al²⁴ reported that mononuclear macrophage recruitment to ischemia reperfusion kidney was CX3CR1 dependent. Renal IRI in CX3CR1^{-/-} mice was significantly reduced with few F4/80^{low} macrophage infiltrates. Emal D et al¹³ found that depletion of gut microbiota decreased the migratory capacity toward CX3CL1 and CCL2 in bone marrow monocytes and

lowered the expression of F4/80, CX3CR1, and CCR2 in the F4/80 renal resident macrophage and bone marrow monocytes, and alleviated the renal ischemia-reperfusion injury. In recent years, researchers have gradually realized that chemokines CX3CL1/CX3CR1 not only promote inflammatory cell infiltration, but also mediate changes in the function and phenotypic status of immune cells. Studies have shown that the chemokine CX3CL1/CX3CR1 has significant and different regulatory effects on the proliferation, phagocytosis, and functional phenotype of macrophages in different pathological environments.²⁵ Our study focused for the first time on the functional changes of macrophages remaining in renal vessels, which experienced ischemia-reperfusion injury together with renal parenchymal cells and may be the first to cause ischemic renal damage. The study also found for the first time that hypoxia/reoxygenation could promote M1 transformation of macrophages by activating the CX3CL1/CX3CR1 signaling pathway. Silencing CX3CR1 using siRNA not only inhibited the functional transformation of macrophages induced by hypoxia/reoxygenation, but also inhibited their adhesion and migration abilities and reduced the apoptosis level of hypoxic renal tubular epithelial cells. These data suggest again that CX3CL1/CX3CR1 could be a therapeutic target for attenuating the inflammatory response in ischemia reperfusion kidney. However, there have also been studies showing that CX3CR1 promotes renal fibrosis during renal ischemia-reperfusion injury. Furuichi et al²⁶ found that gene knockout or neutralizing antibodies of CX3CR1 alleviated renal fibrosis after IRI, by inhibiting macrophage aggregation and reducing the expression of macrophage and platelet-derived growth factor B in the outer medulla. Stroo et al²⁷ reported that CX3CR1 expression is significantly upregulated during the reparative phase of renal ischemia-reperfusion injury. This indicates that chemokines CX3CL1/CX3CR1 may play different pathological roles in early and late renal ischemia-reperfusion injury. Our study mainly focused on the functional study of macrophages in the early stages of ischemic injury.

Researchers have begun to focus on the effects of acute kidney injury on distant organs, including the lung, heart, liver, brain and gut. Rossi et al²⁸ found that hemin-induced HO-1 attenuated the AKI-induced inflammatory response and injury in the lung by reducing systemic inflammation. Fox et al²⁹ reported that ischemic AKI altered the cardiac metabolome, characterized by ATP depletion, increased oxidative stress, and anaerobic forms of energy production, which were the most significant at 24 h after AKI. In our study,

hypoxia/reoxygenation enhanced the expression of proinflammatory factors and the ability of macrophages to migrate. We speculated that hypoxic macrophages secreted pro-inflammatory factors to be released into the circulatory system, or some of the hypoxic macrophages may migrate into distant organs after reperfusion, resulting in organ damage, which deserves further study.

In order to explain the specific mechanism by which CX3CL1/CX3CR1 signaling pathway mediates the change in cytokine expression in hypoxic macrophages, we then studied the effect of CX3CR1 silencing with CX3CR1-siRNA on the ERK/NF- κ B signaling pathway, which has been reported to be involved in the inflammatory response of renal IRI.³⁰ We found that hypoxia/reoxygenation promoted the phosphorylation of ERK and p65 subunit of NF- κ B in macrophages, which was inhibited by CX3CR1 silencing. In addition, ERK inhibitors prevented M1 macrophage transformation under hypoxia reoxygenation treatment. Increased macrophage-soluble CX3CL1 secretion induced by hypoxia/reoxygenation was also significantly inhibited by CX3CR1 silencing or ERK inhibitors. Therefore, these findings suggest that the activation of the CX3CL1/CX3CR1/ERK/NF- κ B signaling pathway is one of the mechanisms by which hypoxia/reoxygenation promotes M1 macrophage transformation.c.

Researchers have previously reported the effect of hypoxic renal tubular epithelial cells on macrophage function. Li et al³¹ reported that miRNA-23a-enriched exosomes released from hypoxic tubular epithelial cells in a HIF-1 α dependent manner could promote the reprogramming of macrophages into a pro-inflammatory phenotype. Masola et al¹¹ found that conditioned medium from hypoxic HK-2 renal epithelial tubular cells could regulate macrophage polarization to the M1 phenotype. However, the effect of hypoxic macrophages on renal tubular epithelial cells remains unclear. In this study, we found that conditioned medium from hypoxia/reoxygenation-treated macrophages promoted the apoptosis of hypoxic renal tubular epithelial cells through the regulation of apoptotic proteins. This effect was attenuated by CX3CR1 silencing in macrophages. We also speculated that intercellular interactions may be mediated by direct cytokine secretion or vesicle delivery.

In conclusion, our study demonstrated that hypoxia/reoxygenation could induce macrophage polarization to the M1 phenotype, promote the abilities of migration and adhesion of macrophages, and potentiate apoptosis in hypoxic renal tubular epithelial cells, in

which the chemokine CX3CL1/CX3CR1 signaling pathway plays an important role.

Funding

This work was supported by Scientific Research Starting Foundation of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (No. 2014KYQD01, 2018YJJA04).

Conflicts of interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cdtm.2021.05.001>.

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