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Interaction between bone marrow-derived dendritic cells and miR-21 of tubular renal epithelial cells under hypoxia

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Abstract. – OBJECTIVE: To investigate the intracellular response and role of microRNA 21 in the regulation of dendritic cell maturation and function.

MATERIALS AND METHODS: Bone marrow-derived DCs (BMDCs) isolated from male C57BL/6J mice and primary renal tubular epithelial cells were used as primary cells to perform this study. Flow cytometry was used to determine BMDCs and analyze the apoptosis effect. Transmission electron microscopy was used for the identification of the diameter of exosomes. Reverse transcription-Polymerase Chain Reaction (RT-PCR) and Western blotting were used to detect the effect after cells were transfected with oligo. ELISA was used to determine the tumor necrosis factor-α (TNF-α), interleukin-12 (IL-12), and IL-1beta in DC supernatants.

RESULTS: We found that the upregulation of microRNA 21 in dendritic cells induced by physical hypoxia contributed to decreased expressions of CD80 (cluster of differentiation 80), CD86 (cluster of differentiation 86), and MHCII (major histocompatibility complex class II molecules) of dendritic cells and suppressed secretion of inflammatory cytokines and chemokine receptor type 7. Co-culture with tubular epithelial cells or hypoxia-pretreated tubular epithelial cell-derived conditional medium promoted bone marrow-derived dendritic cell maturation. Exosomes purified from the supernatant of cultured marrow-derived dendritic cells showed upregulated microRNA 21 under hypoxia, whereas anti-microRNA 21 treated tubular epithelial cells promoted co-cultured marrow-derived dendritic cell maturation.

CONCLUSIONS: Both oxygen concentration and tubular epithelial cells participate in regulating dendritic cell maturation, directly or indirectly through the microRNA 21 signal pathway.

Key Words:
Dendritic cells, Hypoxia, Ischemic kidney injury, MiR-21, Tubular epithelial cells.

Introduction

Acute kidney injury (AKI), a common clinical complication, has a reported prevalence rate ranging from 3.49% to 23.2%[1-3]. Despite the rapid development of blood purification techniques, the mortality of critically ill AKI patients remains unchanged, with one reason being the lack of effective interventions.

Ischemic injury is one of the major pathological mechanisms of AKI[1]. Ischemia directly leads to hypoxia and renal tubular epithelial cell (TEC) injury, whereas the inflammatory immune response secondary to renal TEC injury leads to a variety of events triggering ischemic AKI[1]. Danger-associated molecular patterns (DAMPs) released by injured TECs trigger the downstream signaling pathways, resulting in the activation of various immune cells such as macrophages, dendritic cells, and neutrophils[4]. An adaptive immune response can be further triggered by an overwhelming immuno-inflammatory imbalance[5].

Dendritic cells (DCs) are the strongest antigen-presenting cells and relate to the innate and adaptive immune responses[6]. They develop from myeloid progenitors and undergo a maturation process via migration to the secondary lymph nodes. This is stimulated by inflammatory signals, such as DAMPs, with upregulation of costimulatory and migration molecules[7] (e.g., when DCs in the peripheral tissue come into contact with microbial components, such as lipopolysaccharides (LPS), they are activated and migrate to the draining lymph nodes)[7,8]. DCs can be licensed to T helper (Th)1-type or Th2-type immune response depending on the type and dose of the stimulation[9-11]. Kidney intrinsic DCs originate from the

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bone marrow and spleen. Under physiological conditions, most intrinsic DCs are located in the renal interstitial cells[12].

DCs travel via lymphatic vessels to transport antigens and present them to T cells in the lymph nodes. They directionally move toward lymphatic vessels by C-C chemokine receptor type 7 (CCR7)[13]. MicroRNAs (miRNAs), small noncoding RNAs that modulate gene expression, bind to the 3′-untranslated region of target messenger RNAs[14]. Reportedly, a bundle of miRNAs regulates DC functions[15-17]. A previous study[18] showed that CCR7 was the target gene of miR-21. In our precedent work, we showed that miR-21 was upregulated under hypoxia in HK2 cells and supernatant of HK2 cells[19]. Thus, we suggested that the phenotype and function of kidney intrinsic DCs can be regulated by miR-21 via a paracrine pathway[20]. Therefore, in this study, we aimed to investigate the intracellular response and the role of miR-21 in the regulation of DC-mediated inflammatory responses secondary to hypoxia.

Materials and Methods

Culture of Bone Marrow-Derived DCs (BMDCs)

This study was approved by the Animal Ethics Committee of Fudan University Animal Center. BMDCs were isolated from male C57BL/6J mice, as previously reported[21], and cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and supplemented with 10 ng/mL recombinant mouse granulocyte-macrophage colony-stimulating factor and 10 ng/mL IL-4 (PeproTech Inc., Rocky Hill, NJ, USA). After 48 h, non-adherent cells were removed from the dish, and the remaining cluster was cultured; the medium was changed every 48 h. On day 6, the cells were collected for treatment with hypoxia-pretreated TEC-derived conditional medium (HCM) or normal medium. The phenotypes of BMDCs were determined based on the protein levels of CD11c, CD80 (cluster of differentiation 80), CD86 (cluster of differentiation 86), MHCII (major histocompatibility complex class II molecules), and CCR7 (eBioscience, Frankfurt, Germany).

Primary Murine Renal TEC Culture

Primary renal TECs were bought from AllCells (Allcells, LLC, Berkeley, CA, USA) and cultured in a specific medium (AllCells, Berkeley, CA, USA). After reaching 80% confluency, the TECs were solely cultured or co-cultured with BMDCs.

Hypoxia (1% O_2, 5% CO_2, and 94% N_2) was set up in an oxygen control incubator (Thermo Fisher Scientific Inc, Waltham, MA, USA), and the indicated cells were cultured under hypoxia for 6-48 h.

BMDC Analysis by Flow Cytometry

In this work, we used LPS to stimulate mature DCs (mDCs), and no-LPS-stimulated DCs were defined as immature DCs (imDCs). BMDCs were analyzed by flow cytometry for surface marker expression using antibodies against CD80, CD86, MHCII, and CCR7 (eBioscience, Frankfurt, Germany).

Exosome Purification

BMDCs were cultured in RPMI-1640 medium without serum. Further, the medium was collected to purify exosomes after 24 h exposure to hypoxia. Supernatant fractions collected from the cell cultures were subjected to exosome extraction. Briefly, the supernatant was centrifuged at 300 rpm for 15 min to remove cells, followed by filtration through 0.22 μm filter to remove cell debris. Exosomes in the medium were precipitated with ExoQuick TC (System Biosciences, Palo Alto, CA, USA), according to the manufacturer's instructions, and exosomes pellet were resuspended in 100 μL phosphate-buffered saline (PBS) and stored at -80°C until further use.

Transmission Electron Microscopy

For transmission electron microscopy (TEM) morphology analysis, 5 μL exosome pellets were placed on Formvar/carbon-coated 200-mesh copper electron microscopy grids. The grids were incubated for 10 min at room temperature, followed by standard uranyl acetate staining. Then, the grids were washed with three changes of PBS and allowed to semi-dry at room temperature before observation under TEM (Hitachi H7500, Tokyo, Japan). The obtained micrographs were used to quantify the diameter of the exosomes.

Transfection of Anti-MiR Oligo

The synthesized anti-miR-21 and anti-scramble (Exiqon, Vedbaek, Denmark) were transfected into TECs with Lipofectamine (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Briefly, TECs (10^6 per well) were plated onto 24-well plates and allowed to grow for 12 h. Anti-miR-21 (50 nM) or anti-scramble with

1 μL Lipofectamine were added to the cells. After transfection, the cells were incubated at 37°C for 24-72 h; next, the cells were cultured under hypoxia or co-cultured with BMDCs. The cells were harvested for analysis at the indicated time points as described before; proteins and RNA were assessed using Western blot assay and Real-Time reverse-transcriptase-Polymerase Chain Reaction (RT-PCR) assay, respectively.

Western Blot Analysis

The cells or exosomes were lysed, and the total cell protein was extracted. The protein concentration was estimated by a bicinchoninic acid assay reagent (Beyotime, Shanghai, China). Then, the proteins were resolved on a 10% sodium dodecyl sulfate bis-tris gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membrane was blocked with 5% non-fat milk in TBST buffer, followed by incubation with rabbit anti-CD63 (1:1000) (Abcam, Cambridge, MA, USA), PDCD4 (1:1000) (Cell Signaling Technology [CST], Danvers, MA, USA), CCR7 (1:1000) (CST, Danvers, MA, USA), pp65 (1:1000, CST, Danvers, MA, USA), and p65 (1:1000, CST, Danvers, MA, USA) overnight at 4°C, washed with TBST, and incubated with HRP-linked goat anti-rabbit IgG (1:10000) (CST, Danvers, MA, USA). Further, the protein bands were visualized using an automatic imager (General Electric, Fairfield, CT, USA).

RNA Extraction and MiRNA Real-Time RT-PCR

The cells were washed with ice-cold phosphate buffer, and total RNA was extracted using the TRIzol agent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Complementary deoxyribose nucleic acid (cDNA) was synthesized using one-step prime script miRNA cDNA Synthesis kit (PrimeScript RT Reagent Kit) (TaKaRa, Otsu, Shiga, Japan). miRNA-specific primers were designed according to the manufacturer's instructions and synthesized at Applied Biosystems (Foster City, CA, USA). U6 small nuclear RNA was used as endogenous control for miRNA. The level of miR-21 expression was quantified using Real-Time RT-PCR with TaqMan chemistry (Applied Biosystems, Foster City, CA, USA) and monitored by a 7500 RT-PCR system (Life Technologies, Grand Island, NY, USA). The samples were analyzed in duplicate. The level of miRNA expression was calculated according to the $2^{-\Delta\Delta Ct}$ method. The relative gene level is expressed as ratio to control. Primer sequences used in this study were as follows: PCAT6, F: 5'-CAGAATTGCCCAGGCTTTTA-3', R: 5'-AATCCATGAGGCAAGGTGAC-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'.

Apoptosis Analysis by Flow Cytometry

The cells were harvested by ice-cold PBS from the culture dishes. The apoptosis was detected using Annexin V-FITC/PI staining kit (Vazyme, Nanjing, China), according to the manufacturer's instructions. Before starting flow cytometry, the cells were again rinsed twice with phosphate buffer. Flow cytometry was conducted using FACSCalibur flow cytometer (Thermo Scientific, Waltham, MA, USA).

Enzyme-Linked Immunosorbent Assay

The concentration of tumor necrosis factor-α (TNF-α), interleukin-12 (IL-12), and IL-1beta in DC supernatants was measured using specific kits purchased from eBioscience, in accordance with the manufacturer's instructions.

Statistical Analysis

The Student's t-test was used while comparing the difference between the two groups. Data are presented as mean ± standard error of the mean. Statistical analyses were conducted using Statistical Product and Service Solutions (SPSS) Version 16.0 (Chicago, IL, USA). One-way analysis of variance with Tukey's multiple comparison tests was used to analyze multiple group comparisons. p-value <0.05 was considered statistically significant.

Results

In Vitro Culture of Immature DCs (imDCs) and Mature DCs (mDCs)

CD11c+MHCII+ double-positive cells have been identified as BMDCs that differ from bone marrow-derived macrophages[22] (Figure 1A). Based on whether the toll-like receptor 4 of the BMDCs had been stimulated by LPS, our research aim included *in vitro* culture of imDCs and mDCs[23]. Flow cytometry revealed elevated expressions of MHCII (mDCs *vs.* imDCs, 1493 ± 44 *vs.* 1259 ± 59, $p<0.05$), CD80 (mDCs *vs.* imDCs, 624 ± 11 *vs.* 386 ± 31, $p<0.01$), and CD86 (mDCs *vs.* imDCs, 427 ± 11 *vs.* 311 ± 10, $p<0.01$) in mDCs (Figure 1B).

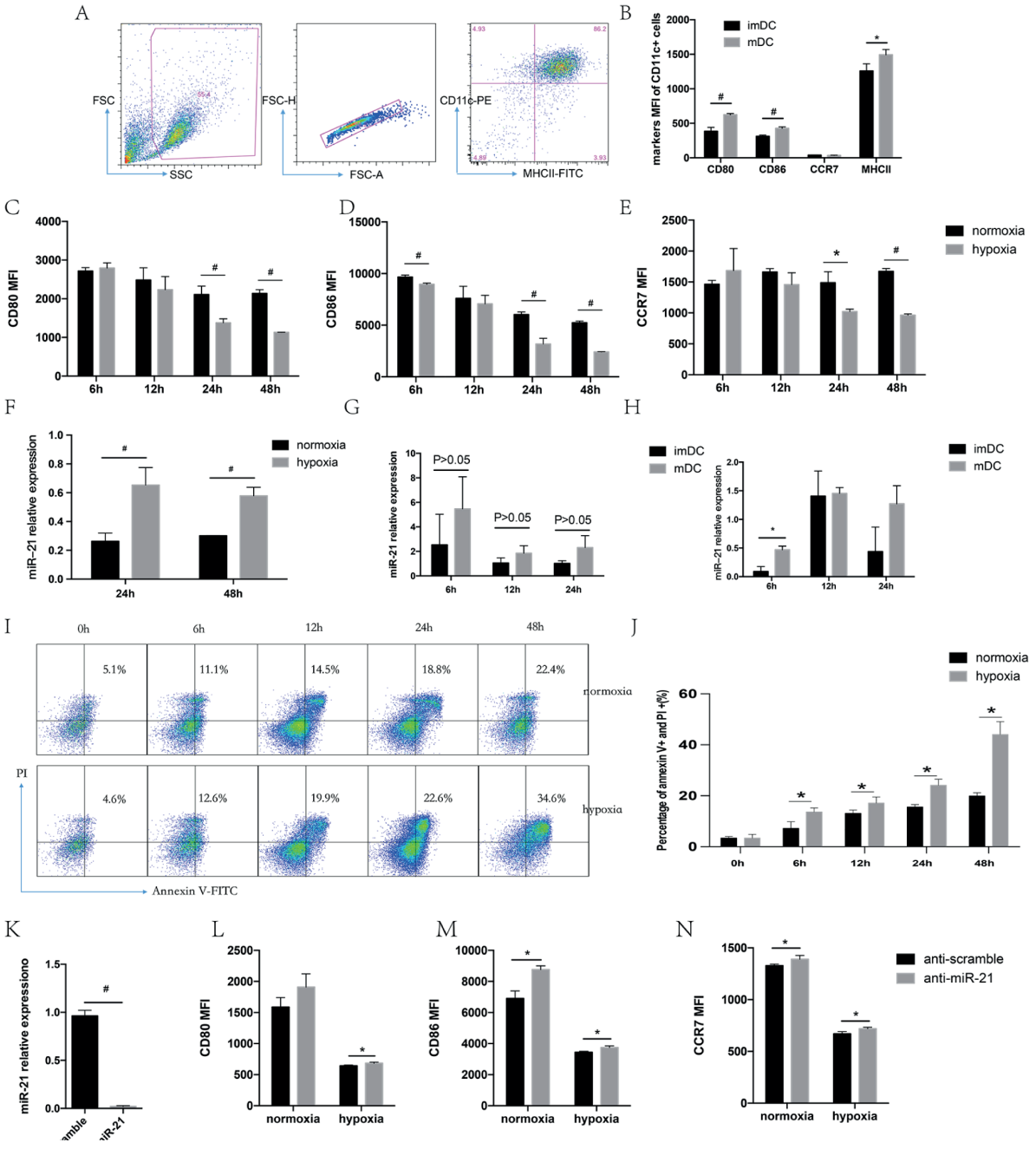

Figure 1. Hypoxia had a suppressive effect on the maturation of BMDCs. ***A,*** Induction rate of BMDCs gated from CD11c+MHCII+ double-positive cells. ***B,*** LPS stimulation elevated antigen-presenting marker expression of BMDC. ***C-E***, Hypoxia exposure suppressed maturation marker CD80, CD86, and CCR7 expressions of BMDCs at indicated time points, tested by flow cytometry. *$p< 0.05$, $^{\#}p< 0.01$, *vs.* normoxia group. N = 4. ***F,*** Exposure to hypoxia for 24–48 h upregulated the miR-21 levels in BMDCs. ***G,*** 24-h LPS stimulation suppressed miR-21 levels in BMDCs. ***H*** and ***I***, Time-dependent apoptosis rate of BMDCs at 1% O_2 concentration. ***J,*** Successful transfection of anti-miR-21 to TECs. ***K-M,*** Transfection of anti-miR-21 to BMDC elevated the levels of CD80, CD86, and CCR7 MFI of BMDCs under normoxia or hypoxia.

Hypoxia Inhibited BMDC Maturation

ImDCs were exposed to normoxia at 21% O_2 (N-imDCs) or hypoxia at 1% O_2 (H-imDCs). In H-imDCs, expressions of CD80 (H-imDCs 24 h *vs.* N-imDCs 24 h, 1377 ± 60 *vs.* 2107 ± 128, $p<0.05$) (Figure 1C), CD86 (H-imDCs 24 h *vs.* N-imDCs 24 h, 3182 ± 314 *vs.* 6033 ± 140, $p<0.05$) (Figure 1D), and CCR7 (H-imDCs 24 h *vs.* N-im-

DCs 24 h, 1022 ± 23 *vs.* 1489 ± 102, $p<0.05$) (Figure 1E) were significantly lower than those in N-imDCs.

After exposure to hypoxia for 24-48 h, H-imDCs expressed a two-fold level of miR-21 expression compared with N-imDCs (Figure 1F). Further, mDCs expressed lower level of miR-21 expression than imDCs (Figure 1G). Furthermore, we measured the apoptosis rate of imDCs using flow cytometry. Compared with N-imDCs, H-imDCs showed a significantly increased apoptosis rate, which was aggravated by a longer exposure to hypoxia (6-h H-imDCs *vs.* N-imDCs, 13.6% ± 0.9% *vs.* 7.2% ± 1.5%, $p<0.05$; 12-h H-imDCs *vs.* N-imDCs, 17.2% ± 1.4% *vs.* 13.1% ± 0.7%, $p<0.05$; 24-h H-imDCs *vs.* N-imDCs, 24.1% ± 1.4% *vs.* 15.5% ± 0.6%, $p<0.05$; and 48-h H-imDCs *vs.* N-imDCs, 44.0% ± 2.9% *vs.* 19.9% ± 0.7%, $p<0.05$) (Figure 1H, 1I).

MiR-21 Negatively Regulated DC Maturation

The knockdown of miR-21 (Figure 1J) augmented the expression of maturation molecules such as CD80 (hypoxia anti-miR-21 *vs.* anti-scramble, 683 ± 10 *vs.* 642 ± 8, $p<0.05$), CD86 (normoxia anti-miR-21 *vs.* anti-scramble, 8759 ± 143 *vs.* 6902 ± 279, $p<0.01$; hypoxia anti-miR-21 *vs.* anti-scramble, 3746 ± 61 *vs.* 3445 ± 33, $p<0.05$), and CCR7 (normoxia anti-miR-21 *vs.* anti-scramble, 1391 ± 21 *vs.* 1328 ± 8, $p<0.05$; hypoxia anti-miR-21 *vs.* anti-scramble, 719 ± 8 *vs.* 671 ± 11, $p<0.05$) (Figure 1K-N). Compared with imDCs under normoxia, the level of miR-21 expressed by the imDCs under hypoxia increased by more than two-fold (Figure 1F). These results suggest that the lower antigen presentation ability of H-imDCs could be attributed to the negative regulatory role of miR-21 in DC maturation.

Supernatant of TEC Cultured Under Hypoxia Promoted DC Maturation

The link of kidney intrinsic and inflammatory cells may function via miR-21 signaling. To test this hypothesis, we first pretreated TECs under 1% O_2 for 24 h. Then, we collected the supernatant (also regarded as a pretreated TEC-derived HCM) to further incubate BMDCs under normoxia for 24 h. As opposed to the negative effects of direct hypoxia on the maturation markers expression of BMDCs (Figure 1C–1E), the BMDCs cultured with HCM exhibited an upregulated expression of CD80 and MHCII median fluorescence intensity levels compared with the control group (Figure 2A, B). Compared with a control group (normal TEC-derived conditional medium, NCM), HCM upregulated the pro-inflammatory cytokine secretion of BMDCs, which was verified by IL-12 overexpression (Figure 2C) and IL-4 downregulation in the supernatant of BMDCs (Figure 2D). Notably, we studied imDCs in this part, rather than the mature DCs.

Transwell Co-Culture System Mimics the Interaction Between DCs and TECs Under Hypoxia

To better study the relationship between TECs and DCs, we used a transwell co-culture system with BMDCs cultured in the upper room (approximate cell amount, 10^5/well), whereas TECs were cultured in the bottom room (10^6/well). First, we observed that in the co-cultured groups, co-culture with TEC resulted in a significant upregulation of CD80 and CD86 expressions under hypoxia (Figure 3A) or normoxia (Figure 3B). Second, after 48-h co-culturing, BMDCs secreted more pro-inflammatory cytokine IL-1β (BMDC + TEC *vs.* BMDC, 248.0 ± 26.41 ng/mL *vs.* 87.82 ± 4.89 ng/mL, $p<0.05$) (Figure 3C) and TNF-α (BMDC + TEC *vs.* BMDC, 2695 ± 48.22 ng/mL *vs.* 2403 ±20.43 ng/mL, $p<0.05$) (Figure 3D), but it had no significant effect on the secretion of IL-12 (Figure 3E). Third, hypoxia had significantly suppressed mDC maturation, such as of CD80 at each indicated time point (48-h hypoxia *vs.* normoxia, 11060 ± 108.7 *vs.* 21080 ± 316.5, $p<0.05$; 24-h hypoxia *vs.* normoxia, 6958 ± 101.3 *vs.* 10120 ± 82.30, $p<0.05$; 12-h hypoxia *vs.* normoxia, 5840 ± 420.7 *vs.* 6334 ± 732.7, $p<0.05$; and 6-h hypoxia *vs.* normoxia, 4680 ± 17.87 *vs.* 5515 ± 106.6, $p< 0.05$) (Figure 3F) and migration molecule CCR7 (48-h hypoxia *vs.* normoxia, 1506 ± 12.15 *vs.* 3359 ± 96.70, $p<0.05$; 24-h hypoxia *vs.* normoxia, 1155 ± 14.77 *vs.* 1702 ± 10.35, $p<0.05$; and 6-h hypoxia *vs.* normoxia, 960.5 ± 7.963 *vs.* 1211 ± 10.85, $p<0.05$) (Figure 3G). These results indicate that co-culture with TECs promoted BMDCs antigen-presenting function, migration, and pro-inflammatory activity under hypoxia.

Effect of Transwell Co-Culture on the PDCD4-CCR7 Signaling Pathway of BMDCs

Co-culture with TECs or LPS stimulation showed a slight but insignificant elevation of PDCD4 protein expression in BMDCs (Figure 4A, B). An efficient transfection of anti-miR-21 to BMDCs upregulated CCR7 and PDCD4 expres-

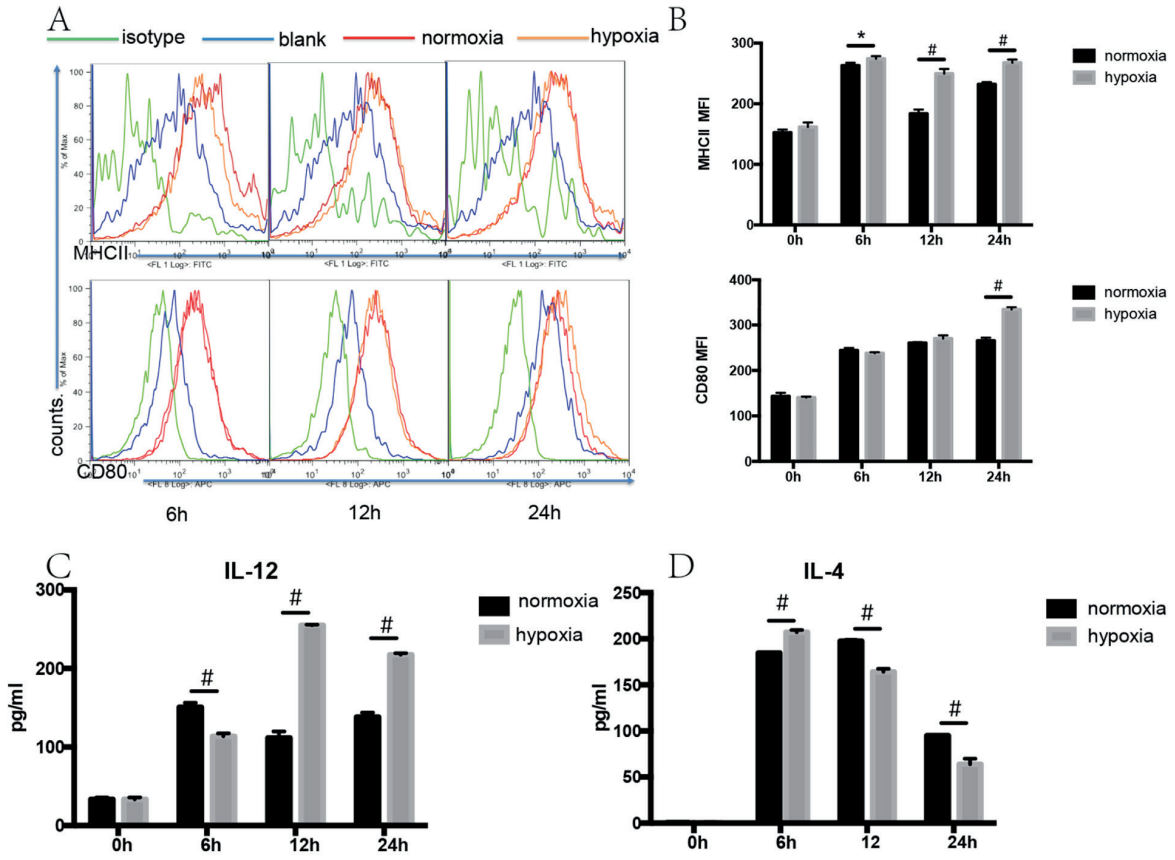

Figure 2. Hypoxia-pretreated TEC-derived conditional medium promoted the maturation marker expression and secretion of inflammatory cytokines by BMDCs. ***A,*** and ***B,*** Hypoxia-pretreated TEC-derived conditional medium (HCM) upregulated MHCII and CD80 expression of BMDCs (N = 4, *p<0.05, #p<0.01 *vs.* NCM-cultured BMDCs). ***C,*** HCM treatment promoted IL-12 secretion of BMDCs. D, HCM treatment suppressed the secretion of IL-4 of BMDCs supernatant ($^{*}p$<0.05, $^{\#}p$<0.01, *vs.* NCM group. N=4).

sions (Figure 4C, D). Co-culture with anti-miR-21 transfected TECs had no significant effect on PDCD4 expression of BMDCs (Figure 4E, F).

Exosomes May Act as the Potential Determinant Between the Interaction of TECs and BMDCs

We investigated the BMDC-derived exosome miR-21 under hypoxia. BMDC-derived exosomes (Figure 5A, B) showed upregulated miR-21 expression under 1% O_2 concentration (Figure 5C). Conversely, inhibiting miR-21 levels in TECs with anti-miR-21 promoted the median fluorescence intensity level of CD80 (anti-miR-21 *vs.* anti-scramble: 683.7 ± 10.48 *vs.* 642.3 ± 7.881, p< 0.05), CD86 (anti-miR-21 *vs.* anti-scramble: 3746 ± 61.28 *vs.* 3445 ± 33.39, p< 0.05), and migration molecule CCR7 (anti-miR-21 *vs.* anti-scramble: 719.0 ± 8.386 *vs.* 671.0 ± 11.27, p< 0.05) of the co-cultured mDCs under hypoxia (Figure 5D) or normoxia (Figure 5E). Overall, these data indicate that TECs can indirectly regulate the function of DCs.

Discussion

Pathogenesis of ischemia-reperfusion (IR) and the related molecular mechanisms that regulate inflammatory injury in local ischemic tissue have recently gained much attention[24]. Tissue hypoxia is regarded as an important factor in the pathophysiology of AKI and chronic kidney disease[25,26]. In recent years, inflammatory reactions have been shown to play key roles in the pathophysiology of organ damage following IR[27]. DAMPs are released by intrinsic kidney cells, such as TECs, endothelial and parenchymal cells, which are subsequently presented to antigen-presenting cells; this further triggers downstream adaptive immune cell activity and pro-inflammatory cytokine se-

cretion, which, in response, causes an exaggerated renal injury[28]. DCs are the strongest specific APC and have anti- and pro-inflammatory effects depending on their maturation state[29]. In this study, we developed an *in vitro* hypoxic model that mimicked an *in vivo* IR microenvironment. With our model, we validated the DC phenotypes and function variations when DCs were under hypoxia, either incubated alone or with TECs.

Because kidney DCs migrate from the spleen and bone marrow, BMDCs were cultured from the bone marrow cells induced by IL-4 and GM-CSF in this experiment. CD11c+MHCII+ double-positive cells were identified as BMDCs. LPS stimulation of the toll-like receptor 4 of BMDCs' completely activated BMDCs to mDCs. This effect was evidenced by the upregulation of CD80, CD86, and CCR7.

In this study, we first found that hypoxia suppressed imDC maturation and upregulated miR-21 expressions. mDCs showed lower-level miR-21 expressions compared with imDCs. These data suggest an opposite tendency for BMDC maturation and miR-21 levels. The transfection of anti-miR oligos further verified this hypothesis. Second, we used hypoxia-pretreated TEC-derived medium or employed a transwell co-culture system at 1% O_2 to study the interaction between DCs and TECs and to mimic the AKI microenvironment *in vitro*. We found that a co-culture of HCM and TECs contributed to the promotion of imDC maturation, which was evidenced by the upregulation of DC maturation markers and was accompanied by elevated pro-inflammatory cytokine secretion. Lastly, anti-miR treatment of co-cultured TECs further clarified the negative

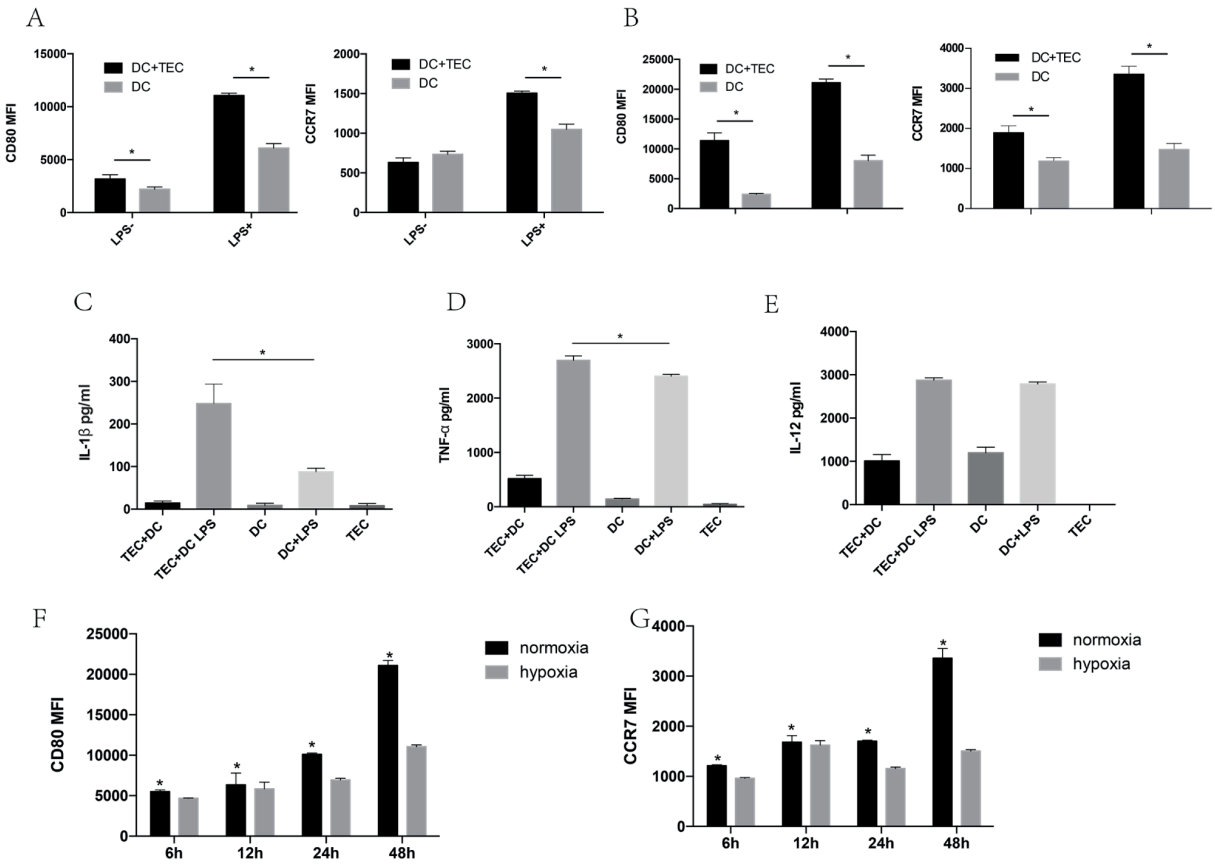

Figure 3. Transwell co-culture with TEC elevated the inflammatory activity of BMDCs under hypoxia. A, and B, Whether exposed to hypoxia ***(A)*** or normoxia ***(B)***, co-culture with TECs elevated the levels of CD80 MFI and CCR7 MFI of imDCs and mDCs. **p*<0.05, *vs.* sole DCs culture group. ***C-E***, 48-h transwell co-culture with TECs under hypoxia promoted inflammatory cytokine secretion of BMDCs compared with those in the sole DC culture. **p*<0.05, *vs.* sole DCs culture group. *F* and ***G***, Co-culture with TECs under hypoxia suppressed the expression of BMDC maturation markers compared with normoxic co-culture group. **p*<0.05, *vs.* normoxia group. N = 4.

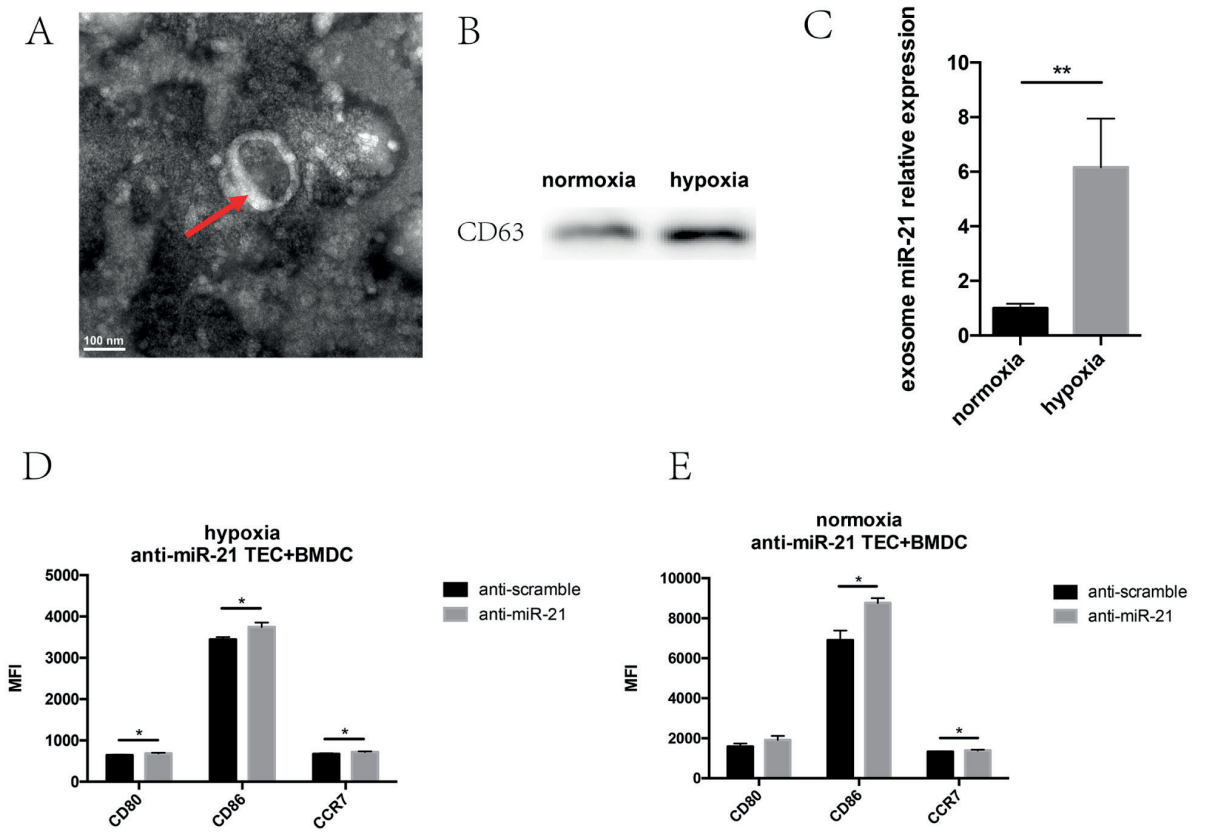

Figure 4. Protein expressions of PDCD4 in BMDCs under different conditions. ***A***, and ***B***, Western blot analysis revealed that co-culture with TECs under hypoxia or LPS treatment had no significant effect on PDCD4 expression of BMDCs. ***C***, and ***D***, Anti-miR-21 treatment upregulated the protein expression of CCR7 and PDCD4 in BMDCs (*p<0.05, *vs*. anti-scramble group. N=4). ***E*** and ***F***, Co-culture with anti-miR-21 transfected TECs had no significant effect on the PDCD4 expression of BMDCs.

role of miR-21 in the relationship between BMDCs and TECs. The PDCD4-CCR7 signaling pathway was negatively regulated by miR-21, which was subsequently attributed to the phenotypic profiles of BMDCs.

Several miRNAs have been reported as important regulators of IR immune responses, such as miR-146a, miR142, miR-155, and miR-150. Wu et al[30-32] reported that miR-181a overexpression downregulated CD40 and CD83 and suppressed the necrotic myocyte-induced upregulation of CD40, CD83, and CD864. MiR-21 has emerged as one of the most upregulated mRNAs after ischemia injury in some organs, such as the brain[33], the heart[34], and the liver[35]. In our *in vitro* study, we found that hypoxia-induced the upregulation of miR-21 in BMDCs, but suppressed the expression of CD80, CD86, and CCR7, when imDCs were solely cultured, which are consistent with the previous finding that miR-21 alleviated hypoxic injury[36]. In addition, we found that the inhibition of miR-21 in TECs or imDC promoted the maturation of BMDCs, with overexpression of CD80, CD86, and CCR7. Additionally, CCR7 was proven to be one of the miR-21 targets; the reciprocal suppression between miR-21 and CCR7 in our study further proved that hypoxia-induced upregulation of miR-21 and contributed to the immature state of BMDCs[37].

Hypoxia, a common condition related to the ischemic environment secondary to AKI, triggers renal intrinsic cell and immune cell apoptosis[38]. We investigated the role of miR-21 in BMDCs apoptosis, and our study showed that hypoxia solely increased the apoptosis rate of BMDCs under hypoxia (at 1% O_2) for 6 h. Additionally, the apoptosis rate kept increasing with the increase in exposure time. The apoptosis was also aggregated in miR-21$^{-/-}$ BMDCs under normoxia (data not shown). These data provided additional evidence

to support the renoprotective effect of miR-21 in a hypoxic microenvironment, which was present in the DC-mediated inflammatory response. However, the precise mechanism by which miR-21 modulates apoptosis in renal intrinsic and immune cells is yet to be elucidated. Thus, future studies should aim to identify relevant targets of miR-21, such as PDCD4 that participates in response to hypoxia and inflammation.

Further, we investigated the source of miR-21 of BMDCs. Exosomes have been a hotspot of studies about cell-to-cell interaction. We primarily observed the upregulated miR-21 in BMDC-derived exosomes from the supernatant of hypoxia-cultured BMDCs. Conversely, inhibiting miR-21 levels in TECs promoted maturation of the co-cultured mDCs, which also indicated that TECs regulates the function of DCs in a paracrine way.

This work has certain relevant limitations. First, the effect of DCs on the differentiation of T cells was not investigated. Second, this was an *in vitro* study, and the findings need to be further validated for clinical application. Our team has recently published a paper reporting that delayed ischemic preconditioning protected against renal IR injury by inhibiting DC maturation in a mouse model[39]; however, many questions remain to be answered. Third, we were not able to yield conclusive evidence showing the downstream regulation of CCR7 by miR-21. Lastly, compared with previous investigations, our research showed some controversial results such as the effect of hypoxia on the CCR7 expression due to different cell models. Therefore, further research is warranted to clarify the specific mechanisms involved in the interaction between TECs and DCs.

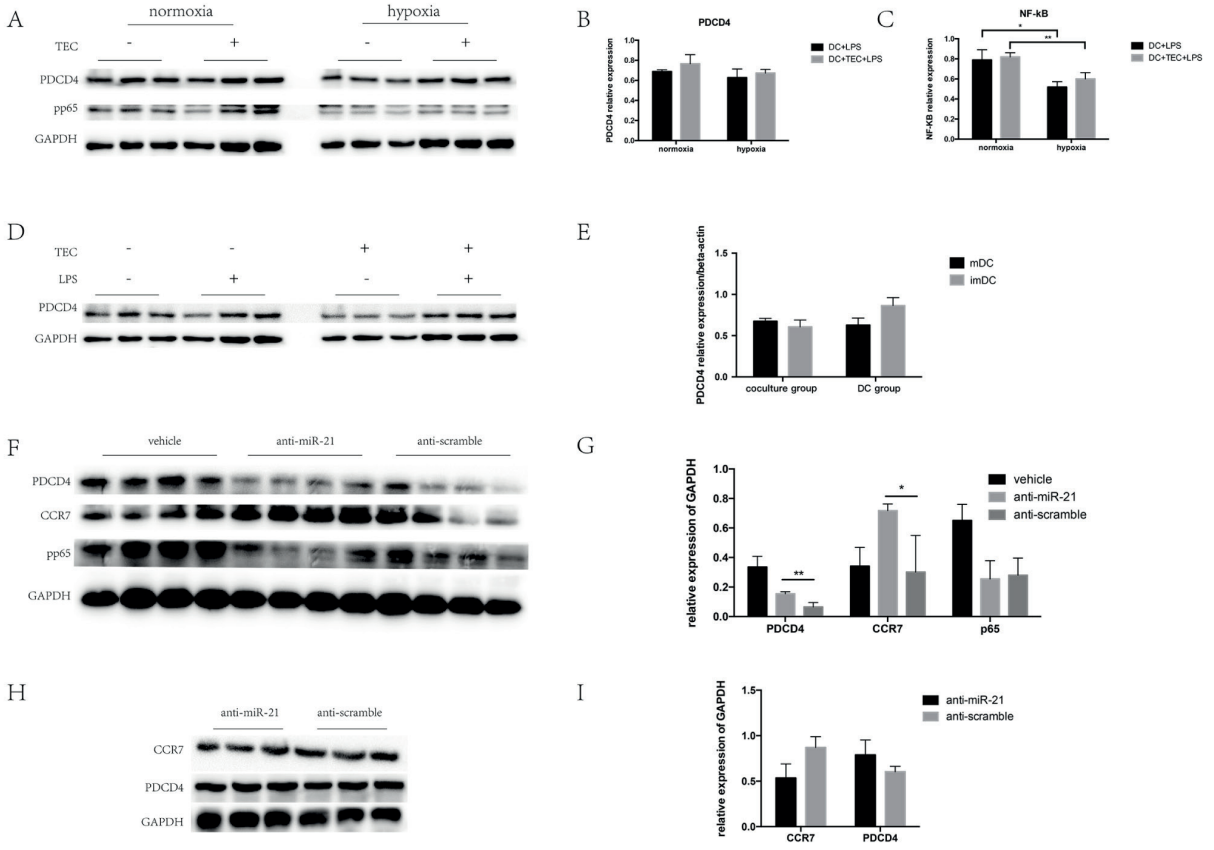

Figure 5. Anti-miR-21 treatment of TEC promoted maturation of co-cultured BMDCs. ***A,*** Electronic microscopy image of the BMDC supernatant-derived exosomes (red arrow), scale bars, 100 nm. ***B,*** Vesicle content of exosome marker CD63 confirmed by Western blot. ***C,*** MiR-21 levels in exosomes extracted from the supernatant of cultured mDCs. ***D*** and ***E,*** Whether exposed to normoxia or hypoxia, co-culture with transfected TEC elevated CD80, CD86, and CCR7 MFI of imDCs. *$p<0.05$, **$p<0.01$, N = 4.

Conclusions

We revealed the novel effect of miR-21 in DC inflammatory responses against hypoxia and TEC stimulation. Further studies and clinical trials that can provide valuable insight into the mechanisms involved in DC maturation and its clinical application are needed.

Conflict of Interest

The Authors declare that they have no conflict of interest.

Acknowledgments

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