

MiR-451 ameliorates red blood cell storage damage and macrophage polarization-mediated transfusion immunity by regulating the AMPK/mTOR signalling pathway.

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Keywords: microRNA miR-451; ATF2; AMP-activated protein kinases/mTOR pathway; erythrocytes; blood preservation macrophages.

Abstract. Red blood cells (RBCs) undergo a series of structural and functional changes during storage, and miR-451 is crucial for maintaining homeostasis of RBCs. Inflammatory factors and miR-451 expression in whole blood at different storage times were detected by ELISA and qRT-PCR. THP-1 cells were induced into M0 macrophages. Subsequently, miR-451 mimics, miR-451 inhibitor, and activating transcription factor 2 (ATF2) were transfected into the cells, followed by the application of compound C. Macrophages were then polarized to the M1 phenotype. Macrophage markers were discovered through flow cytometry and Western blot. The adenosine monophosphate-activated protein kinase (AMPK)/mammalian target of rapamycin (mTOR) pathway protein levels were detected using Western blot. Finally, a mouse model of traumatic hemorrhagic shock was constructed, and blood transfusion and tail vein injection of agomir-451 were performed. The levels of M1 macrophage markers and inflammatory factors were detected by flow cytometry and ELISA, respectively. When the human whole blood storage time was 21 d and 35 d, the expression of miR-451 decreased, and the proinflammatory factor contents increased. When miR-451 was overexpressed, proinflammatory cytokines and M1 macrophage markers' expression in THP-1 cells were reduced, p-AMPK level was increased, and p-mTOR level was decreased. After overexpression of ATF2 or compound C, proinflammatory factors and M1 macrophage markers in THP-1 cells increased, and p-AMPK and p-mTOR expression reversed. Overexpression of miR-451 also inhibited macrophage M1 polarization and inflammation in shock mice. miR-451 inhibits ATF2-regulated AMPK/mTOR pathway, inhibits macrophage M1 polarization and inflammation, and improves RBC storage damage.

MiR-451 mejora el daño de almacenamiento de glóbulos rojos y la inmunidad de transfusión mediada por la polarización de macrófagos mediante la regulación de la señalización AMPK/mTOR.

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Palabras clave: micro ARN miR-451; ATF2; proteínas quinasa activadas por AMP/mTOR; eritrocitos; conservación de la sangre; macrófagos.

Resumen. Los glóbulos rojos (RBC) sufren una serie de cambios estructurales y funcionales durante el almacenamiento, y miR-451a es crucial para la homeostasis de los glóbulos rojos. MiR-451 puede mantener la homeostasis de los glóbulos rojos. Los factores inflamatorios y la expresión de miR-451 en sangre completa a diferentes tiempos de almacenamiento fueron detectados por ELISA y qRT-PCR. Las células THP-1 fueron inducida a macrófagos M0, luego el miR-451 imitador y el miR-451inhibidor, junto al factor de activación de transcripción 2 (ATF2) se transfectaron a las células, seguido de la aplicación del compuesto C. Los macrófagos son entonces polarizados a tipo M1. Los marcadores de macrófagos fueron evaluados mediante citometría de flujo y Western blot. Los niveles de proteínas de la vía de la proteína quinasa-activada por monofosfato de adenosina (AMPK)/blanco de la rapamicina en mamíferos (mTOR) fueron detectados usando Western blot. Finalmente, se construyó un modelo de shock hemorrágico traumático en ratón , y se realizó transfusión sanguínea e inyección en vena de la cola de agomir-451. Los marcadores de macrófagos tipo M1 y los niveles de factores inflamatorios fueron examinados por citometría de flujo y ELISA, respectivamente. Cuando el tiempo de almacenamiento de sangre total humana fue de 21 y 35 días, la expresión de miR-451 disminuyó y el contenido de factor pro-inflamatorio aumentó. Cuando mir-451 se superexpresa, la expresión de citocinas proinflamatorias y marcadores de macrófagos de tipo M1 en las células THP-1 disminuye, los niveles de p-AMPK aumentan y los niveles de p-mTOR disminuyen. La superexpresión de ATF2 o la aplicación del compuesto C aumenta el factor proinflamatorio y los marcadores de macrófagos de tipo M1 en las células THP-1, y la expresión de p-AMPK y p-mTOR se revierte. La sobreexpresión de miR-451 también inhibe la la polarización M1 y la inflamación en macrófagos de ratones en shock. El miR-451 inhibe el ATF2 para regular la vía AMPK/mTOR, inhibe la polarización e inflamación por los macrófagos M1 y mejora el daño de almacenamiento de RBC.

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INTRODUCTION

Red blood cell (RBC) transfusion is a standard treatment that saves lives. The unique cell structure of RBCs enables them

to have good deformability and the ability to transport O₂. This structure also makes RBCs highly susceptible to environmental influences and has a short lifespan, making them prone to harmful lesions *in vitro*. At

present, the primary storage method of RBC is to suspend them in different components of RBC preservation solution¹. However, RBC storage is accompanied by a series of biochemical and morphological changes, which eventually lead to the decline of RBC quality and function, which is called RBC storage damage². There may be a potential negative relationship between RBC storage time and blood transfusion outcome, which will reduce the effect of clinical blood transfusion treatment and may even lead to the occurrence of serious adverse events of blood transfusion³. Therefore, RBC storage damage has become a key area of blood transfusion safety. Prolonging RBC storage time and effectiveness, and obtaining higher quality RBCs to meet clinical blood use needs, is of great significance.

Studies have found that infusion of red blood cells with extended storage time can cause persistent lung inflammation and injury⁵. The extracellular vesicles produced by stored red blood cells can induce the production of tumor necrosis factor- α (TNF- α), interleukin (IL) -6 and IL-8, resulting in a strong inflammatory response in the recipient. The vesicles also contain a large amount of coagulation substances, which alter the recipient's coagulation function, potentially leading to adverse outcomes from red blood cell transfusion. In addition, the storage of red blood cells will produce a large amount of free iron ions, which will activate macrophages to release a large number of inflammatory substances after infusion into the human body, potentially leading to a systemic inflammatory response in the recipient⁷. It can be inferred that inhibition of inflammation is significant in improving red blood cell storage damage.

MicroRNA (miRNA) plays a role in regulating gene expression throughout biological evolution⁸. In 2010, for the first time, researchers reported the miRNA differential map of stored RBC, which provided a new idea for a comprehensive understanding of RBC storage damage, and proposed that

there may be miRNA regulatory pathways in stored RBC⁹. Later, researchers disclosed the protective impact of miR-196a on stored RBC decay¹⁰, and the up-regulation of miR-203 expression can prolong the storage time of RBC by inhibiting apoptosis¹¹. However, so far, little is known about the exact mechanism of abnormal expression of miRNAs in RBC storage damage. miR-451 is the only miRNA found so far that does not depend on the Dicer enzyme for maturation. miR-451 is the most expressed miRNA in mature RBC, and its abnormal expression is also associated with a variety of blood diseases¹². In stored RBC, miR-451 is also abnormally up-regulated¹³. In conclusion, miR-451 may have an improvement effect on RBC storage damage.

miR-451 can regulate cell function through adenosine monophosphate-activated protein kinase (AMPK) / mammalian target of rapamycin (mTOR) pathway¹⁴. It is known that adenosine triphosphate (ATP) consumption and Ca²⁺ accumulation occur in stored RBCs. AMPK can activate and regulate downstream target phosphorylation through the above process¹⁵. The survival rate of RBC transfusion is negatively related to deformability-related phosphorylation¹⁶. mTOR is a highly conserved serine/threonine protein kinase. mTOR, as a key signaling pathway for cell metabolism regulation, can regulate biological macromolecules as cells' cornerstone¹⁷. In addition to the plasma membrane and cytoplasm, mature RBCs have no other organelles, cannot carry out aerobic oxidation of sugar, and can only use glycolysis for energy supply¹⁸. In addition to the plasma membrane and cytoplasm, mature RBCs have no other organelles, cannot carry out aerobic oxidation of sugar, and can only use glycolysis for energy supply. Inhibition of the mTOR signalling pathway can downregulate G-6-PD to regulate changes in cell biological function²⁰. In recent years, studies have found that exposure to drugs such as mTOR inhibitors can affect RBC number and redox metabolism, affect the

quality of RBC storage and the efficacy after blood transfusion²¹, suggesting that miR-451 may affect RBC metabolism by regulating AMPK/mTOR signalling and participate in RBC storage damage.

In summary, we propose a hypothesis that miR-451 regulates the AMPK/mTOR pathway and is vital for RBC storage damage and transfusion-related complications. In this study, the miR-451 level in long-term stored blood was detected, and then its expression was interfered with. The macrophage polarization and inflammatory response indexes were measured, and the role of the AMPK/mTOR pathway was explored. The goal of this study is to prove that miR-451 can regulate the AMPK/mTOR signalling pathway to improve red blood cell storage damage and provide a theoretical basis for improving RBC storage damage and blood transfusion complications.

METHODS

Sample source

Whole blood was obtained from 20 healthy donors. All donors have read and signed the informed consent. The Medical Ethics Committee of Jiaying First Hospital has approved this study. Four hundred mL of whole blood was collected from each donor, and processed and stored according to the operation specifications of the blood station. Whole blood was collected in citrate-phosphate-glucose solution (CPD; 3 mg/mL citric acid, 26.30 mg/mL sodium citrate, 2.22 mg/mL sodium dihydrogen phosphate, and 25.51 mg/mL glucose), and a portion of the blood was taken out for immediate detection. The expression levels of inflammatory factors and miR-451 in the whole blood were detected by enzyme-linked immunosorbent assay (ELISA) and quantitative real-time polymerase chain reaction (qRT-PCR). The remaining blood was sub-packed in a 1.7 mL blood bag catheter and stored at 4 °C for up to 35 days.

Human monocytic leukemia THP-1 cells were purchased from Punosai Life Technology Co., Ltd. (CL-0233, Wuhan, China). THP-1 cells were cultured in RPMI 1640 medium (L220KJ, BasalMedia) containing 10% fetal bovine serum and 1% penicillin-streptomycin (S110JV, BasalMedia, Shanghai, China) at 37 °C and 5% CO₂. THP-1 cells in logarithmic growth phase were taken for subsequent experiments.

Macrophage differentiation

THP-1 cells were stimulated with 100 ng/mL PMA (IP1010, Solarbio) for 48 h to induce M0 macrophages. When the cells were transformed from suspension to a fusiform or irregular adherent state, THP-1 cells were induced to differentiate into macrophages²². M0 macrophages were then treated with 100 ng/mL LPS (lipopolysaccharides, LPS, IL2020, Solarbio) and 20 ng/mL IFN- γ (interferon- γ , IFN- γ , P00028, Solarbio) for 24 h to induce the polarization of M0 macrophages to M1 macrophages²³.

Macrophage treatment and transfection

M0 macrophages in logarithmic growth phase were inoculated in 6-well cell culture plates. The cells were randomly divided into the following groups: PMA group, M1 macrophage (LPS+IFN- γ) group, miR-451 mimics (mimics) group, miR-451 inhibitor (inhibitor) group, activating transcription factor 2 (ATF2) group, LPS+IFN- γ +miR-451 mimics (LPS+IFN- γ +mimics) group, LPS+IFN- γ +ATF2 group, and the corresponding negative control group. The cells in the PMA group were routinely cultured without any operation; miR-451 mimics, miR-451 inhibitor, ATF2 and corresponding negative controls were transfected into M0 macrophages according to the instructions of Lipofectamine 3000 (L3000001, Invitrogen, Austin, TX, USA) kit. Then, the transfected or untransfected M0 macrophages were polarized to M1 macrophages by LPS and IFN- γ .

Macrophage markers were detected by flow cytometry (FCM)

Cells were collected, resuspended into a single cell suspension, stained with polarization markers (M0 macrophages: 5 μ L anti-human CD68-PE (333807, Biolegend); M1 macrophages: 5 μ L anti-human CD86-FITC (374203, Biolegend)), incubated in a refrigerator at 4 °C for 30 min, and fixed with 500 μ L paraformaldehyde for flow cytometry (BD FACSCalibur™, BD Biosciences, San Jose, CA, USA). The Cell Quest software was used for data analysis.

CCK-8 assay

After the polarization of M1 macrophages was completed, the medium was replaced with a medium containing 10% CCK-8 reagent and cultured for two hours. The OD_{450 nm} was detected by a microplate reader (MultiskanGo1510, Thermo, Massachusetts, USA), and the cell viability was calculated according to the formula.

Bioinformatics analysis

The potential target genes of miR-451 were screened by miRWalk (<http://129.206.7.150/>), TargetScan (https://www.targetscan.org/vert_80/), mirRDB (<https://mirdb.org/>), and ENCORI/starBase (<https://rnasysu.com/encori/>) databases. The binding site of miR-451 to ATF2 was predicted by the TargetScan database.

Dual-luciferase assay

WT-ATF2 or MUT-ATF2 targeted binding site fragments were ligated to the pmirGLO vector to construct a dual luciferase vector, and then transfected with miR-451 mimics, NC or mimics into M0 macrophages using transfection reagents. After 48 h, the cells were digested with trypsin and transferred to a 96-well microplate, and then the luciferase activity of each group was measured using the Promega dual Glo® luciferase assay system.

qRT-PCR

Total RNA was extracted from the whole blood of 20 healthy donors using TransZol Up (ET111-01-V2, TRANS, Beijing, China). Using the TransScript® Green One-Step qRT-PCR SuperMix kit (AQ211-01, TRANS), RNA was reversely transcribed into cDNA and subjected to qRT-PCR amplification. The data were quantified by the 2^{- $\Delta\Delta$ Ct} method. GAPDH and U6 were used as internal references.

Primer sequences: miR-451: F: TCCGATTGAGTCATTACCAT; R: GTGCAGGGTCCGAGGT; ATF2: F: AATTGAGGAGCCTTCTGTTGTAG; R: CATCACTGGTAGTAGACTCTGGG; GAPDH: F: 5'-TGGCCTTCCGTGTTCTTAC-3'; R: 5'-GAGTTGCTGTTGAAGTCGCA-3'; U6: F: 5'-CAGGTCTCCAAGACGACATAGA-3'; R: 5'-CGCCTTTTCGATTCATGTACTGC-3'.

Western blot (WB)

Ten μ M Compound C, an AMPK inhibitor, was applied at the same time as mimics transfection, followed by induction of M1 macrophage polarization. Then, the cells of each group were collected, and the total protein was extracted by adding the lysate. The protein concentrations were detected using the BCA kit (PC0020, Solarbio), and the protein was denatured at high temperature. The appropriate concentration of the gel was prepared, and electrophoresis was performed after loading the sample. The gel was then immersed in the transfer solution and transferred to the PVDF membrane at a low temperature. At the end of the incubation, the protein band was immersed in a rapid blocking solution for 30 min. CD86 (ab239075, abcam), p-mTOR (ab109268, abcam), iNOS (ab283655, abcam), TNF- α (ab215188, abcam), ATF2 (ab239361, abcam), p-AMPK (ab92701, abcam), AMPK (ab271188, abcam), mTOR (ab134903, abcam) and GAPDH (ab128915, abcam) primary antibodies were incubated overnight at 4 °C. The corresponding secondary antibody was re-incubated, followed by PBST wash-

ing. The gel was then exposed to luminescent liquid (G2161, Servicebio) and imaged using the gel imaging system (SCG-W1000, Servicebio). Image J software was used to analyze the protein gray value. Finally, the results were saved and analyzed.

Animal experiment

Sixty-four SPF 6-week-old male C57BL/6 mice, weighing (20 ± 2) g, were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The mice were fed in a (22 ± 2) °C, 50%-60% relative humidity, light/dark 12h/12h environment. During the feeding period, the mice were fed freely, and the experimental process was started after one week of adaptive feeding. All experiments were conducted following guidelines approved by animal protection organizations and the Jiaying First Hospital committee.

After anesthesia with 1% pentobarbital sodium, the mice were subjected to cardiac puncture and sterile blood collection. Thirty-two mL of whole blood from 40 mice was mixed with 5.2 mL of CPDA-1 anticoagulant (14% CPDA-1 was eventually used for storage), and the whole blood of the mice was stored for 14 days. All procedures in the blood collection process were performed under strict aseptic conditions. Whole blood was mixed in a 50 mL sterile tube (Corning, New York, USA) and stored in the dark at 4 °C.

The remaining 24 mice were used to establish a mouse model of traumatic hemorrhagic shock²⁴, and the stored blood samples were injected into the shocked mice through the tail vein. Shock-mice were randomly divided into four groups with six mice in each group: group 1 without exchange transfusion + equal amount of normal saline (Control), group 2 exchange transfusion + equal amount of normal saline (Model), group 3 exchange transfusion + agomir-NC (agomir-NC) and group 4 exchange transfusion + agomir-451 (agomir-451). After the exchange transfusion was completed, the mice were immediately injected with five

nmol agomir-NC and agomir-451 via the tail vein. The levels of macrophage polarization markers and inflammatory cytokines were evaluated after 72 h.

After 72 hours of treatment, the mice were sacrificed, and the lymph node tissues of the mice in each group were fully ground and digested with collagenase (C8160, Solarbio) at 37 °C for 0.5 h. The DMEM medium containing 20% fetal bovine serum was added to the mixture to terminate the digestion entirely. After being blown, it was passed through a 70 μ m sieve and centrifuged to prepare a single cell suspension. 100 μ L suspension was added with CD86 antibody, incubated at 4 °C for 30 min in the dark, washed three times with PBS solution, and each tube was resuspended with 200 μ L PBS solution and detected by flow cytometry.

ELISA

The blood sample was transferred to a sterile centrifuge tube, and the supernatant was collected after centrifugation. IL-10 (ab34843, abcam), TNF- α (ab183218, abcam), IL-6 (ab11449, abcam; sEKM-0007, Solarbio), IL-1 β (ab2105, abcam; SEKM-0002, Solarbio), IL-12 (ab9992, abcam), inducible nitric oxide synthase (iNOS, ab205529, abcam), IL-18 (SEKM-0019, Solarbio), CCL22 (SEKM-0110, Solarbio) and transforming growth factor- β (TGF- β , SEKM-0035, Solarbio) levels were measured by ELISA. A total of 90 μ L of sample serum and standard were added to the reaction well, and 10 μ L of horseradish peroxidase-labelled antibody was added. After mixing, the reaction well was sealed with a sealing membrane and incubated for two hours in the dark. Washed with detergent three times and dried on absorbent paper. 100 μ L chromogenic agent TMB solution was added, the reaction hole was sealed with a sealing film, and the reaction was incubated for 20 min. Fifty μ L of stop solution was added, the OD value was immediately measured after mixing, and the corresponding concentration of the sample was determined according to the standard curve.

Statistical analysis

The Prism software (Graphpad 9.0) was used for statistical analysis, and the data with normal distribution were expressed as mean \pm standard deviation. One-way ANOVA analysis was used for comparison among multiple groups. The Pearson correlation was performed for fold-change in the level of miR-451 and expression level of inflammatory factors (IL-1 β , IL-6, IL-12, and TNF- α). $p < 0.05$ was considered statistically significant.

RESULTS

The levels of inflammatory factors and the expression of miR-451 in whole blood storage

More and more studies have shown that RBCs will age or even undergo apoptosis after two weeks of storage due to biochemical and morphological changes. Therefore, we tested the stored whole blood samples on days 0, 21 and 35. We evaluated the fold-change in miR-451 levels and the expression

levels of inflammatory factors (IL-1 β , IL-6, IL-12, and TNF- α), which are typically released or overexpressed upon macrophage M1 type activation, using Pearson correlation analysis. This analysis revealed a correlation between them (Table 1). The results showed that inflammatory factors IL-1 β , IL-6, IL-12 and TNF- α contents were markedly increased in the whole blood samples stored for 21 d and 35 d, and the inflammation level progressively rose with the increase of storage time (Fig. 1A-D). Simultaneously, miR-451 expression was significantly decreased in whole blood samples stored for 21 d and 35 d (Fig. 1E). Notably, miR-451 had a protective effect on RBC storage damage. Compared with fresh blood samples, a large number of inflammatory factors accumulate in long-term stored blood samples. These inflammatory factors are related to M1 macrophages. Therefore, we speculate that miR-451 may be involved in improving RBC storage loss by regulating macrophage M1 polarization and mediating inflammatory response.

Table 1. Statistical analysis of correlations between levels of miR-451 and inflammatory cytokines.

		qIL-1 β	qIL-6	qIL-12	qTNF- α
miR-451	Pearson Correlation	-.444*	-.519*	-.450*	-.414
	Sig. (2-tailed)	0.049	0.019	0.046	0.069
n=20					

qIL-1 β = qIL-1 β Day 35/ qIL-1 β Day 0, qIL-6 = IL6 Day 35/IL6 Day 0, qIL-12 = IL-12 Day35/ IL-12 Day 0, qTNF- α = qTNF- α Day 35/ qTNF- α Day 0.

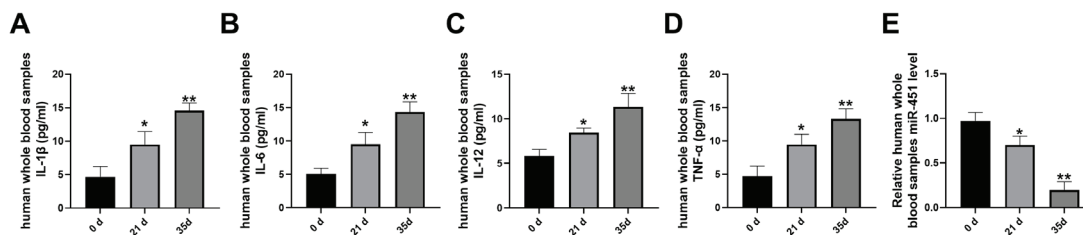


Fig. 1. Inflammatory factor contents and miR-451 expression in whole blood storage.

A-D: IL-1 β , IL-6, IL-12 and TNF- α levels in human whole blood samples stored for 0 d, 21 d and 35 d were detected by ELISA, which were significantly increased at 21 d and 35 d.

E: miR-451 level in human whole blood samples stored for 0 d, 21 d and 35 d was detected by qRT-PCR. It can be seen that it was significantly reduced at 21 d and 35 d.

n=20, * $p < 0.05$, ** $p < 0.01$ vs 0 d group vs 0 d group, one-way ANOVA was used for statistical analysis.

miR-451 attenuates macrophage M1 polarization and inhibits the release of inflammatory factors

To clarify the specific function of miR-451 on improving RBC storage damage, this study used PMA to induce THP-1 cells into M0 macrophages, and detected the expression of its marker CD68 by FCM. The results showed that PMA could significantly increase CD68-positive cells (Fig.2A-B), suggesting that M0 macrophages were successfully induced. Then,

M0 macrophages were induced to polarize into M1 macrophages by LPS and IFN- γ , and the marker CD86-positive cells were significantly increased by FCM (Fig.2C-D), indicating that M1 macrophages were successfully polarized. miR-451 expression was obviously lessened in M1 macrophages (Fig.2E), suggesting that miR-451 may regulate macrophage polarization. Therefore, we next transfected miR-451 mimics into M0 macrophages; miR-451 expression was notably increased (Fig.2F),

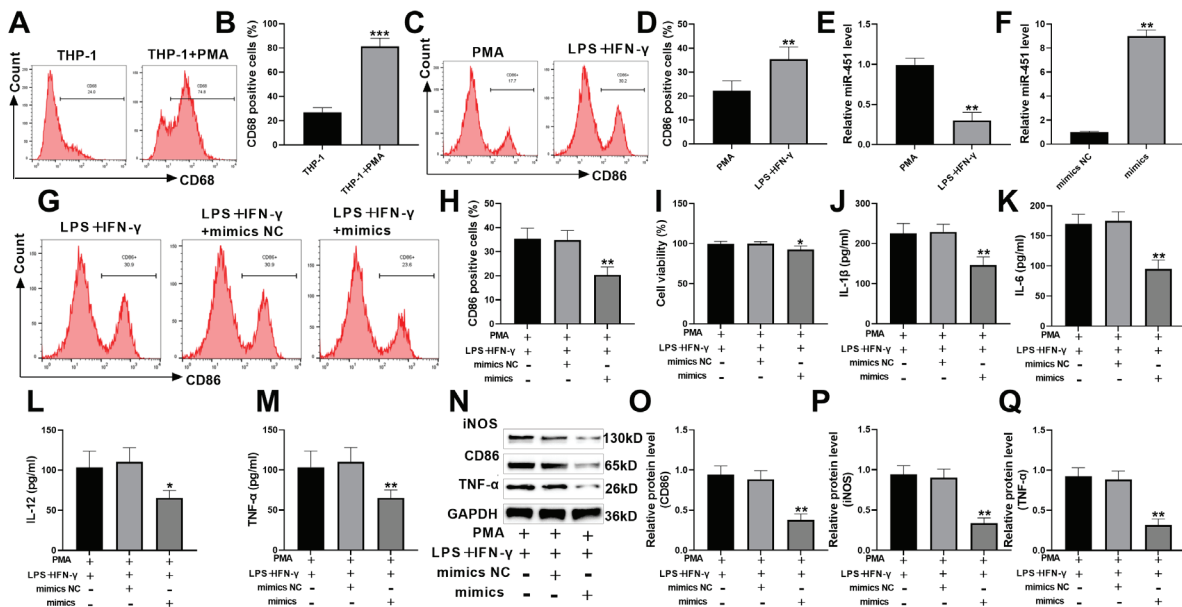


Fig. 2. miR-451 attenuates macrophage M1 polarization and inhibits the release of inflammatory factors.

A-B: M0 macrophage marker CD68 level was detected through FCM, and it was found that PMA could significantly increase CD68-positive cells.

C-D: M1 macrophage marker CD86 level was detected through FCM, and it was found that LPS and IFN- γ could significantly increase its expression.

E: miR-451 expression was discovered using qRT-PCR, and it was significantly decreased in M1 macrophages.

F: miR-451 mimics were transfected into M0 macrophages, and qRT-PCR detected the expression of miR-451, and its high expression was observed.

G-H: The transfected M0 macrophages were polarized into M1 macrophages, and FCM detected the expression of CD86. It was found that the expression of miR-451 was markedly reduced after overexpression. I: CCK8 was used to detect the proliferation of M1 macrophages, and the cell viability was significantly reduced after miR-451 overexpression.

J-M: Proinflammatory factors IL-1 β , IL-6, IL-12 and TNF- α contents in the culture medium supernatant were discovered by ELISA, and they were significantly reduced after miR-451 overexpression.

N-Q: M1 macrophage markers CD86, iNOS and TNF- α levels were detected by WB, and they were significantly reduced after miR-451 overexpression.

n=3, * p <0.05, ** p <0.01, *** p <0.001 vs LPS+IFN- γ +mimics NC group, one-way ANOVA was used for statistical analysis.

suggesting that miR-451 was significantly overexpressed, that is, transfection was successful. After transfection, the transfected M0 macrophages were polarized into M1 macrophages. It was found that after overexpression of miR-451, the M1 macrophage marker CD86-positive cells were significantly reduced (Fig. 2G-H), and the cell viability was also significantly reduced (Fig. 2I); that is, miR-451 can inhibit macrophage M1 polarization. At the same time, ELISA also found that proinflammatory factors IL-1 β , IL-6, IL-12 and TNF- α contents in the medium supernatant were significantly decreased after miR-451 overexpression (Fig. 2J-M), indicating that miR-451 inhibited the release of inflammatory factors. Finally, M1 macrophage markers CD86, iNOS, and TNF- α protein were also markedly reduced (Fig. 2N-Q). The above results indicate that

overexpression of miR-451 can attenuate macrophage M1 polarization and inhibit proinflammatory factor release.

miR-451 can target ATF2 expression

The target gene of miR-451 was screened by various databases as ATF2, ENCORI/starBase targeting score ≥ 1 (Fig. 3A). The expression of the ATF2 gene and ATF2 protein was notably decreased after overexpression of miR-451. After knocking down miR-451, ATF2 gene expression and ATF2 protein level were significantly increased (Fig. 3B-D), indicating that miR-451 can downregulate ATF2. When miR-451 was overexpressed, WT-ATF2 luciferase activity was markedly reduced, but MUT-ATF2 luciferase activity was not affected (Fig. 3E), and its binding site is shown in Fig. 3F. This further confirmed the binding of miR-451 to ATF2. Finally, ATF2 protein level in M1 macrophages was significantly increased (Fig. 3G-H).

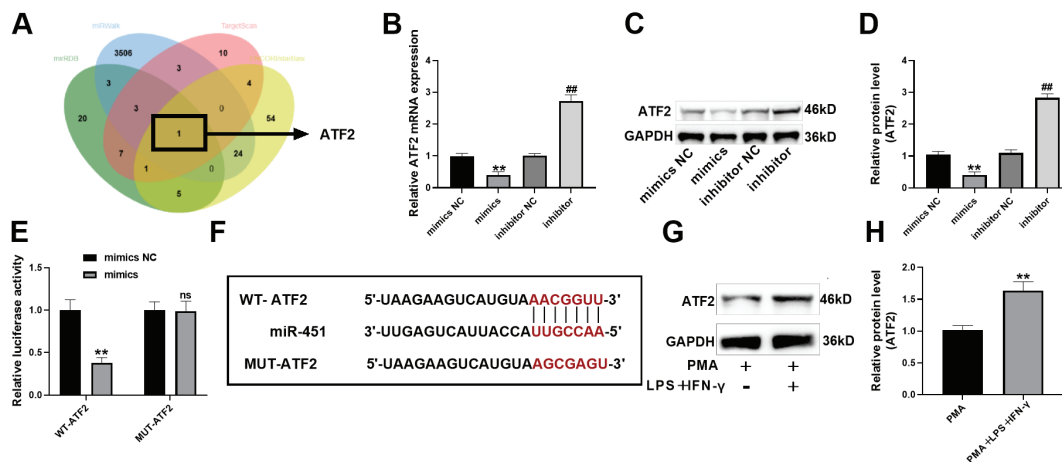


Fig. 3. miR-451 can target ATF2 expression.

A: The potential target gene ATF2 of miR-451 was screened by miRWalk, TargetScan, mirRDB and ENCORI/starBase databases.

B: The expression level of the ATF2 gene was detected by qRT-PCR, which was decreased after miR-451 overexpression and increased after miR-451 knockdown.

C-D: WB detected ATF2 protein level, which declined after miR-451 overexpression and elevated after miR-451 knockdown.

E: Dual luciferase assay showed that overexpression of miR-451 reduced WT-ATF2 luciferase activity.

F: The binding site of miR-451 and ATF2.

G-H: ATF2 level in M1 macrophages was discovered through WB, and it was significantly increased.

n=3, ** $p < 0.01$ vs mimics NC group; ## $p < 0.01$ vs inhibitor NC group, one-way ANOVA was used for statistical analysis.

miR-451 inhibits macrophage M1 polarization and proinflammatory cytokine release by inhibiting ATF2

Next, ATF2 was overexpressed in this study, and ATF2 gene expression and ATF2 protein level were significantly overexpressed in M0 macrophages (Fig. 4A-C), indicating that ATF2 was successfully overexpressed. Then we overexpressed miR-451 and ATF2 in M0 macrophages and polarized them into M1 macrophages using LPS and IFN- γ . Compared with miR-451 overexpression, miR-451 and ATF2 overexpression increased CD86-positive cells (Fig. 4D-E), and the protein levels of CD86, iNOS and TNF- α were also notably raised (Fig. 4F-I). IL-1 β ,

IL-6, IL-12 and TNF- α contents in the supernatant were significantly increased (Fig. 4J-M). ATF2 mitigated the inhibitory effect of miR-451 on macrophage M1 polarization. Combined with bioinformatics and miR-451 overexpression experiments, it was demonstrated that miR-451 could target ATF2 to attenuate macrophage M1 polarization and inflammation.

miR-451 inhibits macrophage M1 polarization by inhibiting ATF2 and regulating AMPK/mTOR signaling pathway

miR-451 can regulate cell function through the AMPK/mTOR signalling path-

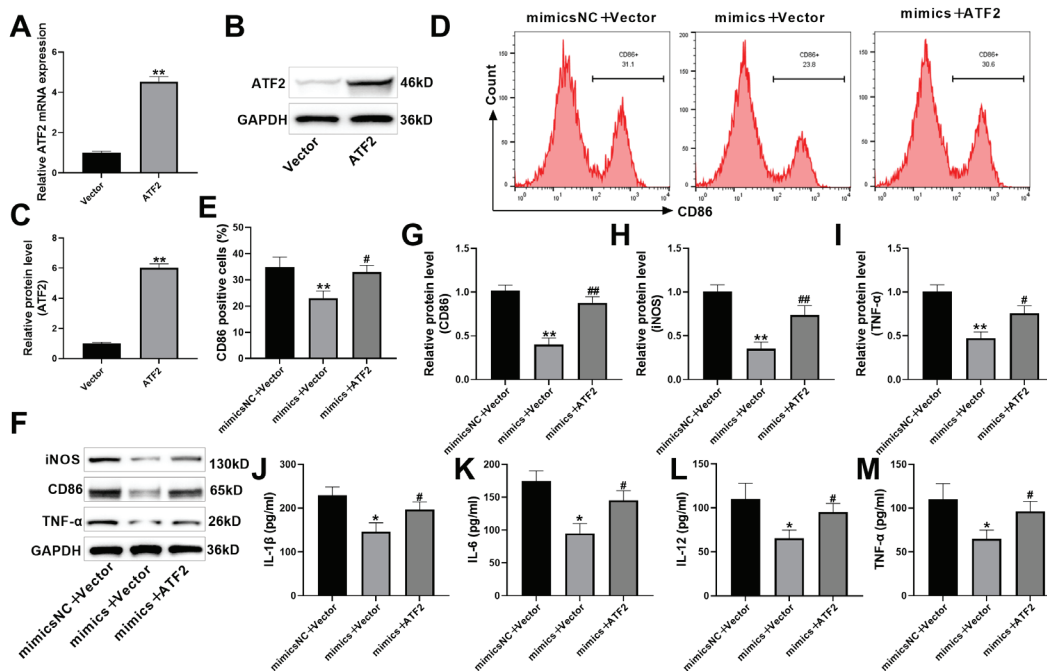


Fig. 4. miR-451 inhibits macrophage M1 polarization and proinflammatory cytokine release by inhibiting ATF2.

A-C: The overexpression level of ATF2 was detected by qRT-PCR and WB, and the gene expression and protein level of ATF2 were significantly increased.

D-E: The transfected M0 macrophages were polarized into M1 macrophages, and the expression of CD86 was detected by FCM, which was significantly increased upon ATF2 overexpression.

F-I: CD86, iNOS and TNF- α levels were discovered through WB, and they were significantly increased after ATF2 overexpression.

J-M: IL-1 β , IL-6, IL-12 and TNF- α contents in the medium supernatant were discovered by ELISA, and they were significantly increased after ATF2 overexpression.

n=3, * p <0.05, ** p <0.01 vs mimics NC+Vector group; # p <0.05, ## p <0.01 vs mimics+Vector group, one-way ANOVA was used for statistical analysis.

way. In this study, AMPK inhibitor compound C was used for intervention experiments. p-AMPK protein levels were elevated, and p-mTOR protein expression declined when miR-451 was overexpressed. On this basis, p-AMPK expression lessened and p-mTOR expression increased after overexpression of ATF2 and application of compound C (Fig. 5A-C), indicating that miR-451 regulates AMPK/mTOR signalling pathway by inhibiting ATF2. At the same time, CD86, iNOS and TNF- α levels were markedly raised after compound C intervention (Fig. 5D-G), indicating that inhibition of the AMPK/mTOR pathway could promote macrophage M1 polarization. In conclusion, miR-451 attenuates macrophage M1 polarization by targeting ATF2 to regulate the AMPK/mTOR axis.

miR-451 regulates transfusion immunity in mice infused with traumatic shock

Finally, this study established a mouse model of traumatic hemorrhagic shock, performed blood exchange operations, and injected agomir-451 intravenously. In Model mice, the number of CD86-positive cells increased, suggesting that macrophage M1 polarization occurred. After miR-451 overexpression, CD86-positive cells decreased (Fig. 6A-B), indicating that miR-451 can also inhibit macrophage M1 polarization *in vivo*. At the same time, it was also found in the blood that the contents of TNF- α , IL-6, iNOS, IL-18, IL-1 β , CCL22, and TGF- β increased in Model mice, and significantly decreased after miR-451 overexpression (Fig. 6C-I), indicating that miR-451 can also inhibit inflammation *in vivo*. In summary, miR-451 can regulate

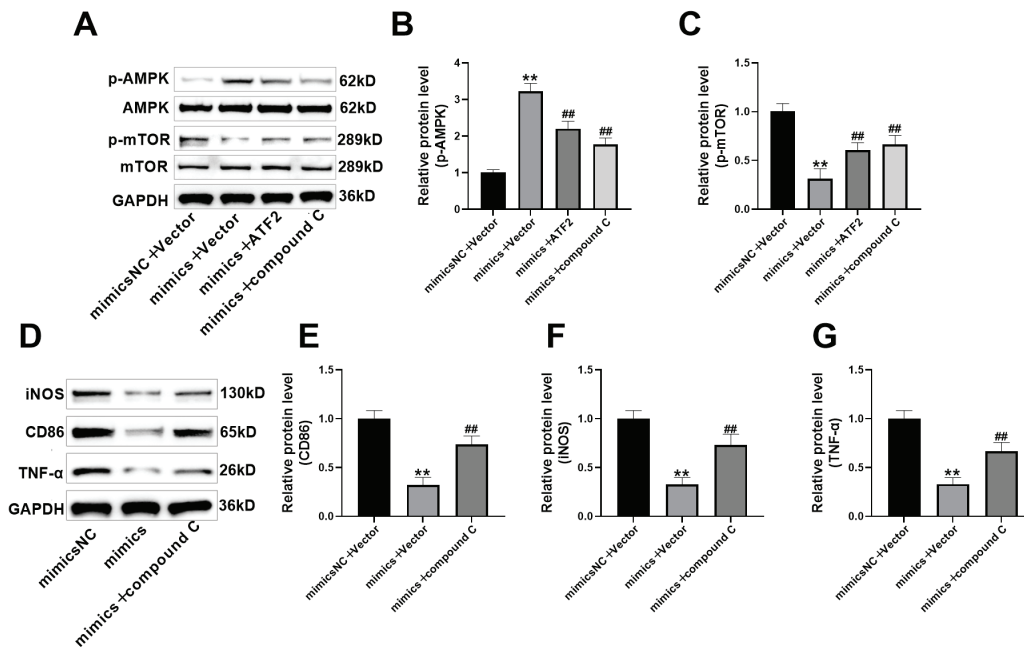


Fig. 5. miR-451 inhibits macrophage M1 polarization by inhibiting ATF2 and regulating the AMPK/mTOR signalling pathway.

A-C: WB was used to detect the expression of AMPK/mTOR pathway proteins. It was found that the level of p-AMPK increased and p-mTOR protein decreased when miR-451 was overexpressed. On this basis, ATF2 overexpression and application of compound C were significantly reversed.

D-G: CD86, iNOS and TNF- α levels were discovered through WB, and they were significantly increased after compound C intervention.

n=3, ** p <0.01 vs mimics NC+Vector group; ## p <0.01 vs mimics+Vector group, one-way ANOVA was used for statistical analysis.

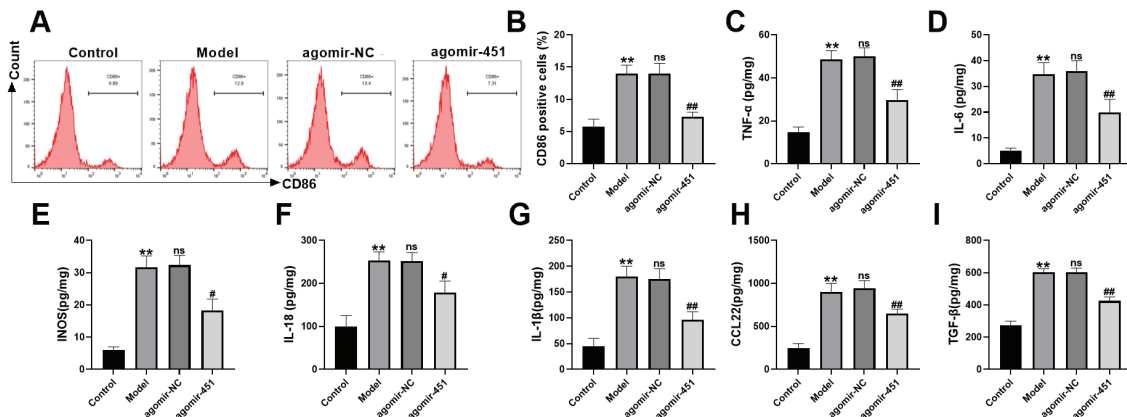


Fig. 6. miR-451 infusion regulates transfusion immunity in mice with traumatic shock.

A-C: The expression of CD86 was detected by FCM, which was increased in Model mice and decreased after miR-451 overexpression.

D-G: The blood inflammatory cytokines TNF- α , IL-6, iNOS, IL-18, IL-1 β , CCL22 and TGF- β expressions were detected by ELISA. It can be seen that they increased in Model mice and decreased after miR-451 overexpression.

n=6, ** p <0.01 vs Control group; ## p <0.01 vs Model group, one-way ANOVA was used for statistical analysis.

blood transfusion immunity in mice infused with stored RBCs by inhibiting macrophage M1 polarization and inflammation.

DISCUSSION

In recent years, with the continuous improvement of medical level and surgical volume, the demand for clinical blood is increasing. At present, the primary source of blood transfusion is still allogeneic stored RBC. In recent years, RBC storage damage has been the focus of blood transfusion studies. RBC is one of the most widely used blood components in clinical practice. However, RBC in the middle and late stages of infusion storage is not without risk²⁵. Because RBC is stored at sub-physiological pH and temperature, a series of metabolic, oxidative and physiological changes occur in RBC, which are called RBC storage lesions. Many of these changes increase with time, affecting the overall quality standards, functional integrity and *in vivo* survival of RBC infusion. Some changes in the early storage period (about 14 days) are reversible, but with

storage time extension, the damages are irreversible²⁶.

Studies have confirmed that after RBC is isolated, the low-temperature storage environment will lead to electrolyte imbalance, metabolite accumulation, oxidative stress, membrane damage, and ultimately lead to storage damage²⁷. These changes damage RBC metabolism, function, and viability. However, the mechanism of RBC storage damage is still unclear. The gene chip study revealed that 109 miRNAs increased and 102 genes decreased during storage, when RBCs were stored for 20 days compared with the fresh group. Sarachana et al.²⁹ found four microRNAs related to RBC storage damage. RT-PCR analysis showed that these four miRNAs were differentially expressed on day 14 and day 28. Bioinformatics analysis identified the potential targets and biological functions of these miRNAs. Overexpression of miR-196a in human RBC cell lines confirmed its protective effect on cell death and ATP loss²⁹. It has been found that miR-451 is the most expressed miRNA in mature RBCs. At the same time, some studies have

reported that RBC microparticles can mediate the transmission of miR-451 to endothelial cells and affect the results after RBC transfusion³⁰, suggesting that miR-451 may mediate RBC storage damage and may also affect blood transfusion receptors through microparticles, exosomes and other ways to cause adverse transfusion events.

Studies have shown that the old RBCs with a long storage period (≥ 2 weeks) release the most abundant heme content and the most obvious damage-related molecular pattern. Heme can amplify the LSP-induced inflammatory response, and a large number of old RBCs are infused into special patients, such as those with traumatic hemorrhagic shock and sepsis. The free heme will exceed the body's metabolic capacity and promote the body's inflammatory damage. It was found that IL-1 β , IL-6, IL-12 and TNF- α were highly expressed in long-term stored whole blood³¹. Infusion of suspended, less white RBCs can cause an increase in inflammatory factors and initiate neutrophil activity.³² Therefore, it can be speculated that infusion of long-term stored RBCs may cause explosive release of inflammatory factors, and ultimately cause human anti-RBC allogeneic immune response.

In addition to the role of oxygen supplementation, RBCs also have apparent immunomodulatory effects^{33, 34}. Studies have found that both RBC metabolites and surface molecules have immunomodulatory effects. For example, the metabolite heme molecules can stimulate macrophages to induce inflammatory responses and interfere with RBC function³⁵. The CD47 molecule on the surface of red blood cells acts as a 'do not eat me' signal, which is directly involved in the recognition and phagocytosis of red blood cells by macrophages.³⁶ RBC immune factor is one of the critical reasons for ineffective infusion. At this stage, storage of RBC can affect the inflammatory expression of macrophages. The immune effect of RBC has attracted attention in recent years, and RBC can produce a variety of immunomodulatory

substances during storage²⁶. Its molecular mechanism is complex and has not been fully elucidated.

Macrophages are key cell populations associated with the body's innate immune response, which not only affect the body's immune function, but also affect the metabolism of RBC³⁷. Macrophages can differentiate into M1 and M2 types, which are two types of cell populations with different functions. This process is called macrophage polarization^{38, 39}. M1 macrophages (mainly expressing cell membrane proteins such as CD86, CD80 and MHC-II) secrete proinflammatory response factors, while M2 macrophages (mainly expressing cell membrane proteins such as CD206 and CD103) secrete anti-inflammatory cytokines to exert anti-inflammatory effects⁴⁰. M1 macrophages can increase IL-1 β and TNF- α contents, aggravate host cell damage and hinder tissue repair. In the inflammatory environment, red blood cell distribution width (RDW) increased compared with a typical environment, and inflammation had a particular effect on RBCs, maintaining the normal morphology⁴¹. In summary, the polarization of macrophages presents different immune functions, and M1 and M2 types may affect the survival and proliferation of RBC.

This study found that IL-1 β , IL-6, IL-12 and TNF- α contents were significantly elevated in whole blood samples stored for 21 and 35 days. However, the miR-451 level was significantly decreased. The Pearson correlation analysis showed that there was a correlation between miR-451 and M1 macrophages, suggesting that miR-451 may be involved in RBC storage damage by regulating macrophage polarization-mediated inflammatory response. To this end, this study first used PMA to induce THP-1 cells into M0 macrophages, and then polarized M0 macrophages into M1 macrophages by LPS and IFN- γ , and the miR-451 expression was significantly reduced in M1 macrophages. Next, this study transfected miR-451 mimics into M0 macrophages and then polarized them

into M1 macrophages. It was found that the activity of M1 macrophages was declined, and the markers CD86, iNOS expression and inflammatory factors IL-1 β , IL-6, IL-12, and TNF- α contents were significantly reduced. In summary, the overexpression of miR-451 can attenuate macrophage M1 polarization and restrain the release of proinflammatory factors.

In this study, ATF2 was identified as a potential target gene of miR-451. Overexpression of miR-451 reduced ATF2 levels, while knockdown of miR-451 increased ATF2 levels. The two have binding sites, indicating that miR-451 can target and downregulate ATF2. When harmful substances stimulate macrophages, G protein-coupled receptors and non-G protein-coupled receptors are activated, leading to increased phosphorylation of MAPK protein 42. This MAPK then enters the nucleus and activates the nuclear transcription factor ATF2, which promotes the secretion and release of proinflammatory cytokines IL-6, IL-1 β , and TNF- α 43. Simultaneously, this study also found that the level of ATF2 protein in M1 macrophages was notably increased. After overexpressing ATF2 in M0 macrophages, they were polarized into M1 macrophages. Compared to the overexpression of miR-451 alone, ATF2 overexpression increased the levels of M1 macrophage markers CD86, iNOS, and inflammatory factors IL-1 β , IL-6, IL-12, and TNF- α . Overall, miR-451 targets ATF2 to reduce macrophage M1 polarization and inflammation.

As a cell energy sensor, AMPK is responsible for regulating cell energy and material metabolism. mTOR is mainly involved in cell growth, proliferation and metabolism. AMPK is also an important upstream regulator of mTOR 44. AMPK is an enzyme complex that is crucial for macrophage polarization and inflammation. Activation of the AMPK signalling pathway can not only reduce inflammatory response in adipose tissue of obese mice, but also inhibits macrophage M1 polarization 45,46. It is known that protein kinase B and the downstream signalling mol-

ecule mTOR play the key roles in regulating macrophage polarization. mTOR is activated by phosphorylation and exists in the form of mTOR complex mTORC1 and mTORC2 in cells. Inhibition of mTOR causes macrophages to polarize to the proinflammatory phenotype M1 47. Activation of mTORC1 may lead to a decrease in the anti-inflammatory phenotype M2 polarization 48. The M1 polarization of alveolar macrophages is considered to be essential for the pathogenesis of transfusion-related acute lung injury 49. Macrophage cells undergo functional changes and severe reactions upon ingesting transfusion RBC microparticles 50, suggesting that miR-451 may regulate macrophage polarization by modulating the mTOR signalling pathway, thereby mediating inflammatory responses and contributing to the promotion of post-transfusion complications. This study showed that p-AMPK protein levels rose and p-mTOR protein expression lowered when miR-451 was overexpressed. When ATF2 was overexpressed and the AMPK inhibitor compound C was applied, the protein level of the AMPK/mTOR pathway was significantly reversed. In addition, this study also found that M1 polarization of macrophages increased significantly after compound C intervention. Overall, miR-451 could target ATF2 to regulate the AMPK/mTOR axis to attenuate macrophage M1 polarization and participate in the immune response of stored RBCs.

Clinical practice shows that both immune-related and non-immune-related factors during RBC transfusion can cause adverse reactions to blood transfusion. These adverse reactions are closely related to RBC transfusion. RBC storage damage is closely associated with the incidence of adverse consequences of blood transfusion. Infusion of suspended RBC with 'storage damage' will increase the incidence of adverse transfusion reactions and mortality 51. Infusion of stored RBCs not only damages the oxygenation capacity of tissues, but also the RBCs entering the body react with macrophages

to become injury or risk-related molecules, which further activate the innate immune response and lead to the damage of corresponding normal tissues or organs⁵². Finally, this study found that M1 macrophages and inflammatory factors were significantly increased in mice undergoing infusion, while miR-451 overexpression reduced M1 macrophages and inflammatory factor levels.

In summary, the data indicated that the inhibition of ATF2 by targeting miR-451 regulates the AMPK/mTOR pathway, which suppresses the polarization of macrophages to the M1 phenotype and reduces the inflammatory response. This process, in turn, diminishes the damage to red blood cells (RBCs) during storage and lessens the immune response triggered by the infusion of stored RBCs (see Fig. 7). This study offers a new approach and methodology for decreasing RBC storage damage. However, research on RBCs should extend beyond their physiological roles; they should not be viewed solely as cells responsible for supplying oxygen. The immune regulation of stored RBCs may be a key factor in adverse infusion reactions, and changes in macrophage function are a significant aspect of this immune regulation. Focusing on the immune function of macrophages is a vital direction for studying the immune mechanisms underlying adverse infusion reactions. Nevertheless, considering the complexity and diversity of immune responses following infusion, our research team will further design and explore relevant research paths.

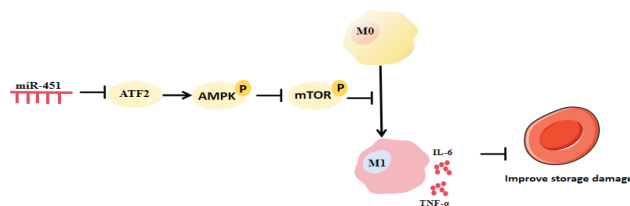


Fig. 7. miR-451-targeted inhibition of ATF2 to regulate AMPK/mTOR pathway, inhibit macrophage M1 polarization, and improve RBC storage damage and transfusion immunity.

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None.

Consent to publish

The manuscript has neither been previously published nor is it under consideration by any other journal. The authors have all approved the content of the paper.

Consent to participate

We secured a signed informed consent form from every participant.

Ethic approval

The Jiaxing First Hospital Committee approved this study.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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Author contribution

XW: Edited and refined the manuscript with a focus on critical intellectual contributions. XC, HZ: Developed and planned the

study, performed experiments, and interpreted results. LC, HW: Participated in collecting, assessing, and analyzing the data. Made significant contributions to data interpretation and manuscript preparation. XW, HQ: Provided substantial intellectual input during the drafting and revision of the manuscript.

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