# Autologous transfusion of "old" red blood cells-induced M2 macrophage polarization through IL-10-Nrf2-HO-1 signaling complexes

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## Abstract

**Background.** Red blood cell (RBC) transfusion is associated with systemic inflammation and immune suppression as adverse outcomes.

**Objectives.** To investigate the immunomodulatory function of the transfused autologous RBC in altering pro-inflammatory and immunosuppressive effects.

**Material and methods.** A total of 24 Sprague Dawley male rats were randomly divided into 3 groups (n = 8 in each group). Group 1 did not receive blood transfusions, while the other 2 groups of rats separately received transfusion of RBC stored for 14 days (group 2) and 35 days (group 3). The rats were treated with HO-1 inhibitor, HO-1 inducer and nuclear factor erythroid 2-related factor 2 (Nrf2) activator after they separately received autologous transfusion of RBC that were cryopreserved for 14 days or 35 days. The blood samples of the rats were collected 12 h after the transfusion, and the macrophage phenotype of M1 and M2 were analyzed with flow cytometry (FCM). Also, the surface protein expression of CD68 and CD200R in macrophages were analyzed and the inflammatory signals in the serum were measured with enzyme-linked immunosorbent assay (ELISA). Moreover, the location and expression of proteins heme oxygenase 1 (HO-1), arginine 1 (Arg-1) and nitric oxide synthase 2 (NOS2) in macrophage were detected with immunofluorescence (IF).

**Results.** Autologous transfusion of long-time stored ("old") RBC promoted macrophage polarization to M2 phenotype and upregulated the expression of its surface proteins CD68 and CD200R. The pro-inflammatory cytokines tumor necrosis factor α (TNF-α), interleukin (IL)-6, IL-1β, and IL-18 were inhibited, and the secretion of NOS isoforms (iNOS) in serum was reduced with blood transfusion; contrarily, the production of IL-10 and CCL22 was increased. Additionally, HO-1, Arg-1 and NOS2 proteins were located in the cytoplasm, and HO-1 and Arg-1 proteins were highly expressed in macrophage, while the expression of protein NOS2 was low. Moreover, Nrf2, HO-1 and Arg-1 proteins were upregulated in macrophage after receiving "old" RBC transfusion.

**Conclusions.** Autologous transfusion of "old" RBC drove the macrophage phenotype toward M2 macrophages and induced immunosuppressive effects through the IL-10–NRF2–HO-1 signals.

**Key words:** immunosuppressive treatment, red blood cell transfusion, M2 macrophage, IL-10–NRF2–HO-1 signals

# Introduction

Blood transfusion is widely used in clinical practice; it is estimated that the transfusion of red blood cells (RBC) is the major type, accounting for nearly 50% of transfusions.[1] The obtained RBC cryopreserved at 4 ±2°C for 35–42 days were recognized as safe[2]; however, in recent years, more adverse effects have occurred after blood transfusions,[3–5] which is why the storage lesions of RBC have attracted more attention. Even though low-temperature storage slows down the metabolism of RBC, studies have revealed that the structure and physiological and immunological characteristics of the long-time stored RBC (also named "old" RBC) have changed. The research of Karon et al. showed that the levels of RBC membrane 2,3-diphosphoglycerate (2,3-DPG) had decreased during storage.[6] The proteomic analysis identified the membrane structural proteins in RBC, such as spectrin, band 3 and band 4.1, which were changed.[7] Moreover, the stored RBC induced hemolysis and the release of free heme, hemoglobin and free iron, which play important roles in immunomodulation.[8] The free heme was not only sequestered by plasma haptoglobin, but also initiated the Fenton reaction to induce the release of iron[9]; then, the accumulation of iron induced the generation of reactive oxygen species (ROS) and radical chains, which thus led to tissue damage and inflammation.[10,11] Additionally, the free heme act as a pro-inflammatory factor engaged in the immune response of monocytes, macrophages, Tregs, and endothelial cells.[12,13] A study showed that free heme mediated macrophage polarization and shaped the expression of M1 and M2 markers.[14] More interestingly, the microvesicles were also found in the stored "old" RBC, which participated in the activity of endothelial activation,[15] blood coagulation[16] and immunomodulation.[17] Hence, the variability of stored RBC is closely associated with systemic proinflammatory and immunosuppressive effects in blood post-transfusion.

The "old" RBC transfusion also burdened the phagocytic ability of monocytes and macrophages. A study conducted by Dinkla et al. identified that the storage-induced autoantibodies in RBC membrane accelerated phagocytosis by macrophages.[18] This correspondingly resulted in heme accumulation in macrophages, which in turn shaped the macrophages toward M2 phenotype through upregulating the heme oxygenase 1 (HO-1) and promoted the secretion of interleukin (IL)-10.[19] Therefore, the stored RBC transfusion induced adverse effects, owing to systemic inflammation and immune suppression. The aim of this study was to investigate the immunomodulation effects on macrophage of the "old" RBC transfusion in animal study and its related mechanism.

# Material and methods

## Material

A total of 24 Sprague Dawley male rats, 6–7 weeks old, 205.45 ±20.55 g (Shanghai Lab Animal Research Center, Shanghai, China); animal blood-taking needles (G-5mm, Braintree Scientific, USA); uncoated vacutainer tube with sodium citrate (Greiner Bio-One International GmbH, Kremsmünster, Austria); peripherally inserted central catheter (PICC; Bard Access Systems, Inc., Salt Lake City, USA); blood transfusion tube needle (Bard Access Systems, Inc.); plastic storage bag prefilled with mannitol adenine phosphate (MAP) storage solution (Sichuan Nigale Biomedical Co. Ltd., Chengdu, China); HO-1 inducer-cobalt protoporphyrin (CoPP, catalog No. ALX-430-076-M025; Jiangxi Haoran Bio-Pharma Co., Ltd., Nanchang, China); HO-1 inhibitor-zinc protoporphyrin IX (ZnPP IX, catalog No. 282820-50MG; Jiangxi Haoran Bio-Pharma Co,. Ltd.); and Nrf2 activator-oltipraz (catalog No. MB2316; Dalian Meilun Biotech Co., Ltd., Dalian, China).

This study conformed to the standards of the animal ethics committee.

## Sample collection

Following transfusion, the fresh whole bloods were collected from the aorta abdominalis after the 10 rats were anesthetized with 2% isoflurane and sacrificed. The obtained blood was leukocyte-depleted with automatic leukocyte filter (catalog No. PXL8Y; Shanghai Jiading Photoelectric Instrument Co., Ltd., Shanghai, China) The left RBC units were then all stored in a plastic storage bag and separately stored at 4°C.

## Transfusion model

The 24 Sprague Dawley male rats were maintained in adapted cultivation at 22–25°C, in a 50–70% relative humidity environment with a 12-hour light/dark cycle for 2 weeks. Then, they were divided into 3 groups: group 1 did not receive blood transfusions, while the other 2 groups of rats separately received transfusion of RBC stored for 14 days (group 2) and 35 days (group 3). The collected RBC samples stored for 14 and 35 days were separately transported in a blood transfusion tube needle connected with a PICC, which was injected into the vein. After a six-hour blood transfusion, the rats were anesthetized with 2% isoflurane and sacrificed; then, the blood samples were collected from the eyeball with an uncoated vacutainer tube with sodium citrate.

## Flow cytometry

The blood samples collected from the eyeball were diluted 1:3 with phosphate-buffered saline (PBS) containing heparin, and 3 mL of Ficoll-Hypaque solution (Tianjin

Haoyang Commercial Co., Ltd., Tianjin, China) was slowly added into the tube, which was then centrifuged twice at 3,000 × g for 15 min at 4°C. Afterward, the supernatant in the middle class was transferred into a new tube, where its density was adjusted at $5 \times 10^5 - 3 \times 10^6$ with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). Later, we firstly analyzed the percentage of macrophages. The steps were as follows: the monocytes were stained with allophycocyanin (APC)-conjugated monoclonal antibody against mouse CD16 (catalog No. 17-0168-41; eBioscience, Thermo Fisher Scientific, Waltham, USA); subsequently, the phycoerythrin (PE)-conjugated monoclonal antibody against mouse CD68 (catalog No. MA5-23572; Invitrogen, Carlsbad, USA) and CD200R (catalog No. 12-9201-42; eBioscience) were separately stained with the monocytes for 1 h at 4°C. Afterward, the cells were washed with PBS and the second antibody – anti-mouse immunoglobulin G (IgG) – was added. Finally, the monocytes were fixed with paraformaldehyde for 30 min and analyzed with FACSCalibur™ (eBioscience). We obtained the monocyte medium and stained the primary conjugated antibody as above. The Fc was blocked and the isotypic antibodies were used as a control; similarly, the surface proteins CD68 and CD200R were analyzed with FACSCalibur.

## ELISA assay

The blood samples collected from the eyeball were centrifuged twice at 3,000 × g for 15 min at 4°C; then, the supernatants were transferred into a new tube and analyzed for the inflammatory-associated molecules of tumor necrosis factor α (TNF-α; catalog No. 70-ab35884-050; MultiSciences Biotech Co., Ltd., Hangzhou, China), IL-1β (catalog No. 70-ab33591-050; MultiSciences Biotech Co., Ltd.), IL-6 (catalog No. 70-ab36529-050; MultiSciences Biotech Co., Ltd.), IL-10 (catalog No. 70-ab33577-050; MultiSciences Biotech Co., Ltd.), nitric oxide synthase isoform (iNOS; catalog No. 93-E4649-100; BioVision Inc., Milpitas, USA), IL-18 (catalog No. 70-ab33588-050; MultiSciences Biotech Co., Ltd.), and CCL22 (catalog No. 70-ab2243-050; MultiSciences Biotech Co., Ltd.) with enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions.

## Immunofluorescence

The blood samples collected from the eyeball were diluted 1:3 with PBS containing heparin, and 3 mL of Ficoll-Hypaque solution (Tianjin Haoyang Commercial Co., Ltd.) was slowly added into the tube, which were then centrifuged twice at 3000 × g for 15 min at 4°C. Afterward, the supernatants in the middle class were transferred into a new tube, and the monocytes were cultivated with DMEM containing 10% FCS and and cell slides were made in each group.

Afterwards, the cell slides were permeabilized with 0.1% TritonX-100 for 5 min, and then they were blocked with 10% bovine serum albumin (BSA) for 1 h at room temperature. After that, the primary antibodies HO-1 (catalog No. ab13248; Abcam, Cambridge, UK), arginine 1 (Arg-1; catalog No. 43933; Cell Signaling Technology, USA) and nitric oxide synthase 2 (NOS2; catalog No. ab15323; Abcam) were separately immunoblotted in the cells for 12 h, 4°C; then, the cells were incubated with anti-mouse IgG antibody for 1 h at 37°C. Thereafter, the cell slides were washed with PBS 3 times, and then the cell nucleus were dyed with 4',6-di-amidino-2-phenylindole (DAPI) and mounted on the cell slide with anti-fade mounting medium; finally, the stained images were examined under confocal microscopy (Leica Camera AG, Wetzlar, Germany).

## Western blot

The 60 rats after adapted cultivation were divided into 3 groups: group 1 (n = 20) did not receive a blood transfusion, while the rats in the other 2 groups (group 2: n = 20 and group 3: n = 20) separately received a transfusion of RBC stored for 14 days (group 2) and 35 days (group 3). Meanwhile, the rats from each of the 3 parts were divided into 4 subgroups (n = 5 in each subgroup) – control subgroup, HO-1 inhibitor administration (ZnPP IX) subgroup, HO-1 inducer administration (CoPP) subgroup, and Nrf2 activator administration (oltipraz) subgroup. After 12 h, the rats were anesthetized with 2% isoflurane and sacrificed. The blood samples were collected from each rat from the aorta abdominalis and then diluted 1:1 with PBS; subsequently, they were centrifuged and the monocytes were isolated with Ficoll-Hypaque solution. Thereafter, the total proteins of the monocytes were extracted with radioimmunoprecipitation assay (RIPA) buffer containing phenylmethylsulfonyl fluoride (PMSF), and then they were denatured and the protein concentration was evaluated. Afterward, the proteins were isolated with sodium dodecyl sulfate (SDS) polyacrylamide gels and the gels were transferred to polyvinylidene difluoride (PVDF) membranes. They were then blocked with 5% fat-free milk for 1 h; subsequently, the protein membranes were separately incubated with primary antibodies: anti-HO-1 (catalog No. ab13248; Abcam), anti-Arg-1 (catalog No. 43933, Cell Signaling Technology, Danvers, USA) and anti-Nrf2 (catalog No. ab89443; Abcam) for 12 h at 4°C. Afterward, the protein membranes were incubated with anti-mouse IgG antibody for 1 h. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein was used as control. Finally, the electrochemiluminescence (ECL) detection reagents were added into the protein bands, which were visualized with a bandscan instrument (Cytiva, Marlborough, USA), while the gray intensity of the proteins was analyzed with ImageJ software (National Institutes of Health, Bethesda, USA).
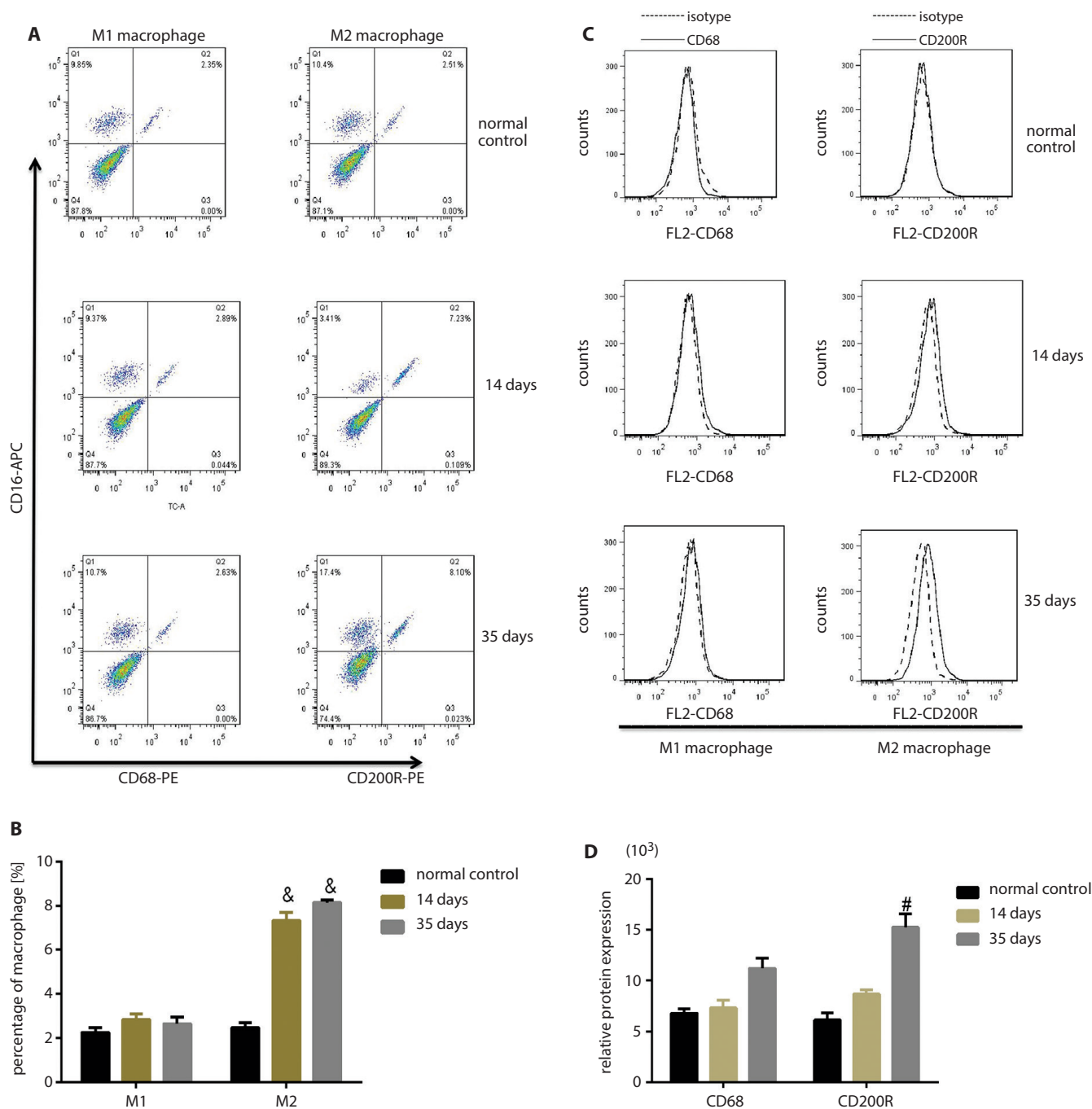
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**Fig. 1.** Flow cytometry (FLC) sorted the macrophage phenotype (A) and detected the surface proteins expression of CD68 and CD200R (C). Blood transfusion shifted the macrophage to M2 phenotype – &p < 0.05 of M2 percentage with blood transfusion vs without blood transfusion (B). The surface protein CD200R was significantly upregulated – #p < 0.05 with a transfusion of blood stored 35 days vs with a transfusion of blood stored 14 days and without a blood transfusion (D)

## Statistical analysis

The data was analyzed with the IBM SPSS Statistics for Windows software, v. 19.0 (IBM Corp., Armonk, USA), and the one-way analysis of variance (ANOVA) method was applied for analyzing the group difference. A p-value of <0.05 was considered the statistically significant difference. The graphs were depicted with GraphPad Prism v. 6.0 software (GraphPad Software Inc., San Diego, USA), and presented as means ± standard error of means (M ±SEM).

# Results

## Autologous transfusion of "old" RBC induced M2 macrophage polarization

After the rats received transfusion of RBC stored for 14 days or 35 days, the flow cytometry (FCM) results showed that the percentage of M2 phenotype macrophage was obviously increased after receiving a blood transfusion (Fig. 1A,B). The surface proteins CD68 in M1 phenotype macrophage and CD200R in M2 phenotype macrophage were also varied. Results revealed that the protein CD200R was highly expressed on M2 macrophage, while the protein CD68 was also upregulated, but the extent was not as evident as in the case of CD200R (Fig. 1C,D); hence, as a whole, the RBC transfusion shifted the macrophage toward to M2 phenotype.

## Autologous transfusion promotes the secretion of pro-inflammatory factors, including IL-6, IL-1β, TNF-α, iNOS, IL-10, IL-18, and CCL22

The pro-inflammatory cytokines TNF-α, IL-6, IL-1β, and IL-18 were decreased with transfusion of blood stored for 35 days, while its amount was increased with a transfusion of RBC stored for 14 days. The variation tendency of iNOS was similar to the pro-inflammatory cytokines, which was consistent with the activity of iNOS involved in inflammation and enhancing the synthesis of pro-inflammatory mediators (Fig. 2). Conversely, the secretion of anti-inflammatory cytokine IL-10 and chemokine CCL22 was increased in blood after transfusion (Fig. 2). The exent of anti-inflammatory cytokine IL-10
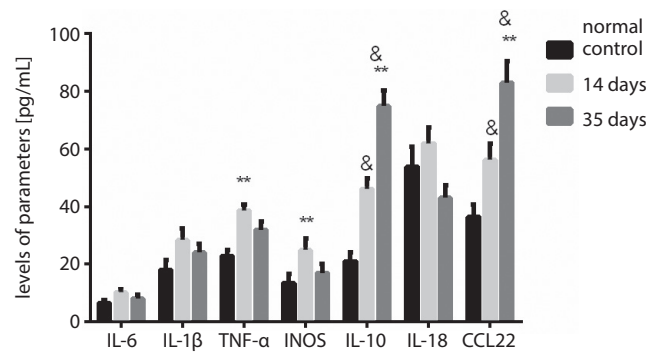


Fig. 2. The ELISA assay detected the amount of inflammatory molecules – **p < 0.05 of TNF-α, iNOS, IL-10, and CCL22 with a transfusion of blood stored 14 days vs without a blood transfusion; &p < 0.05 of IL-10 and CCL22 with a transfusion of blood stored 35 days vs with a transfusion of blood stored 14 days

and chemokine CCL22 secretion was more obvious with a transfusion of RBC stored for 35 days. These results demonstrated that autologous transfusion of blood could increase the inflammatory response.

## Autologous transfusion directs the subcellular distribution of HO-1, Arg-1 and NOS2 to the cytoplasm

A few studies reported that HO-1 could mediate the anti-inflammatory phenotype of macrophage through regulating NRF2.[20] In this study, the proteins HO-1, Arg-1 and NOS2 were found to be located in the cytoplasm of macrophage; HO-1 and Arg-1 had high expression (Fig. 3). Therefore, we proposed that autologous transfusion of blood exerted its function through the redistribution of HO-1, Arg-1 and NOS2.
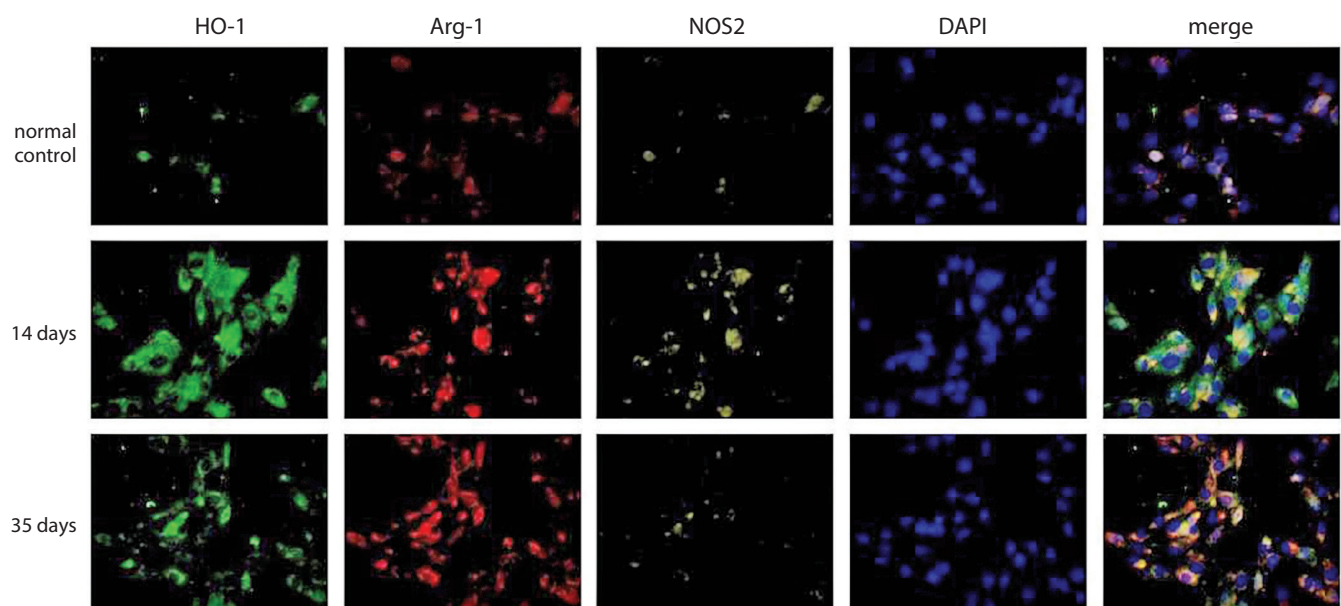


Fig. 3. Immunofluorescence (IF) identified the proteins HO-1, Arg-1 and NOS2. The proteins HO-1, Arg-1 and NOS2 located in the cytoplasm of macrophage and proteins HO-1 and Arg-1 with a high expression, while protein NOS2 did not have a high level of expression
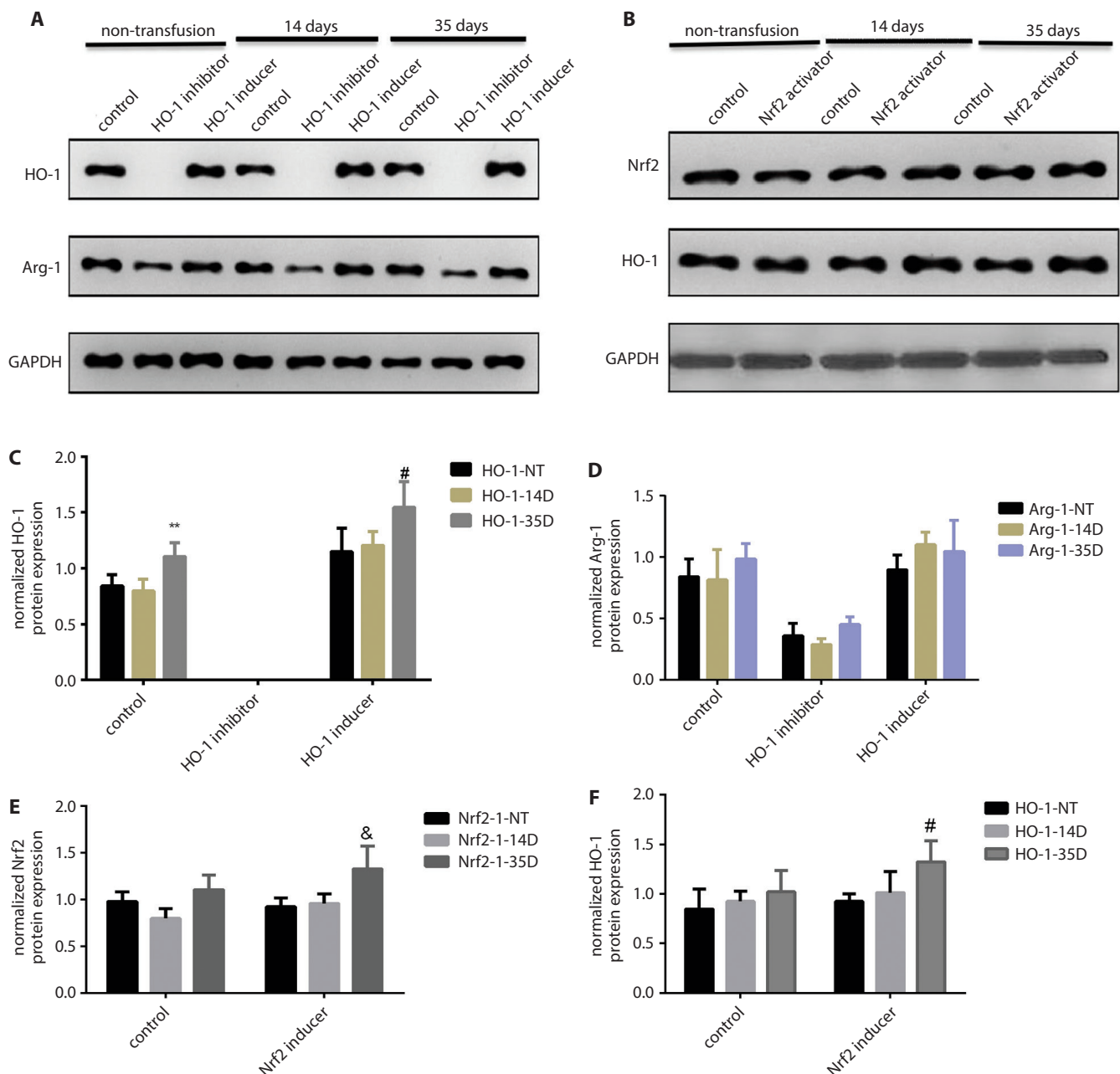
Fig. 4. A and B. The expression of proteins Nrf2, HO-1 and Arg-1 in macrophages of rats with or without blood transfusion analyzed with western blot. The HO-1 inhibitor (ZnPP IX), HO-1 inducer (CoPP) and Nrf2 activator (oltipraz) were all administrated in rats with or without blood transfusion. C. The upregulated HO-1 with a transfusion of blood stored 35 days – **$p < 0.05$ vs with a transfusion of blood stored 14 days; #$p < 0.05$ vs without a blood transfusion. D. With HO-1 inhibitor (ZnPP IX) administration, the protein Arg-1 downregulated. E and F. The upregulated Nrf2, HO-1 with a transfusion of blood stored 35 days; &, #$p < 0.05$ of proteins Nrf2, HO-1 vs without a blood transfusion

## HO-1 inhibitor significantly repressed the protein expression of HO-1 while promoting the expression of Arg-1, but NRF2 activator has no effects on NRF2 and HO-1 after autologous transfusion

With the HO-1 inhibitor administration (ZnPP IX), HO-1 inducer administration (CoPP) and Nrf2 activator administration (oltipraz), we found that the expression of proteins Nrf2, HO-1 and Arg-1 was significantly changed with a transfusion of RBC stored for 35 days, compared with the rats who did not receive blood transfusion or received a transfusion of RBC stored for 14 days (Fig. 4A,B). The inhibition of HO-1 affected the expression of protein Arg-1 (Fig. 4C,D), and to some extent, the activation of HO-1 and Nrf2 promoted the expression of proteins Nrf2, HO-1 and Arg-1 with a transfusion of RBC stored for 35 days (Fig. 4E,F). Hence, an autologous transfusion of "old" RBC induced immunosuppressive effects through

the IL-10-Nrf2-HO-1 signals. These results indicated that an autologous transfusion of blood functioned through HO-1 and Arg-1, but not through Nrf2.

## Discussion

The mechanism responsible for the adverse outcomes of systemic inflammation and immune suppression was controversial. In our study, we found that the transfusion of "old" RBC in rats induced the polarization of macrophages to M2 phenotype and upregulated their surface marker CD200R expression. The levels of anti-inflammatory signals IL-10 and CCL22 were also enhanced. More interestingly, the pro-inflammatory signals TNF-α, iNOS, IL-6, IL-1β, and IL-18 were increased after a transfusion of RBC stored for 14 days, while their levels were decreased after a transfusion of RBC stored for 35 days. This feature might be caused by the regulation of both pro-inflammation and anti-inflammation response. However, a RBC transfusion in a human study showed that there was no increase in pro-inflammatory cytokines, except for a small increase in non-transferrin-bound iron (NTBI).[21] However, a rat model with of blood transfusion suggested that a pro-inflammatory cytokine storm and an increase of NTBI occurred.[22] Hence, the debatable results may be caused by species difference and the limitation of study samples. The transfusion of stored blood resulting in increased hemolysis and erythrophagocytosis features were increasingly confirmed, which lead to a significant increase in serum iron, free heme and bilirubin levels.[23–25] The free heme was suggested as a pro-inflammatory signal, involved in the activity of IL-1 and TNF-a, and ROS generation.[26,27] Hence, this may be the reason that the pro-inflammatory signals TNF-α, iNOS, IL-6, IL-1β, and IL-18 were increased with the transfusion blood stored for 14 days. Moreover, HO-1 was suggested as a free heme scavenger, which had anti-inflammatory effects with a reduction of TNF-a, IL-8 and macrophage inflammatory protein (MIP)-1β levels and upregulation of IL-12.[28] This evidence was in line with our study that the pro-inflammatory signals were decreased with a transfusion of blood stored for 35 days. On the other hand, studies also showed that HO-1 was involved in the activity of polarizing M2 phenotype[29,30]; hence, this may be potentially involved in polarizing M2 phenotype.

With further studies, we found that HO-1, Arg-1, NOS2, and Nrf2 proteins were indeed engaged in M2 polarization. The HO-1, Arg-1 and Nrf2 proteins were obviously expressed in macrophages. This finding was consistent with the conclusion of Park et al.[31] that Nrf2 was an essential regulator of HO-1 participating in antioxidative stress-induced inflammatory activity. Moreover, the results of M1 subtype marker molecule – NOS2 and M2 marker – Arg-1[32] further confirmed our finding that "old" RBC transfusion induced M2 phenotype polarization. Hence, the immunomodulation mechanism of our study was closely associated with Nrf2-HO-1 signals. Additionally,

in our study, the secretion of CCL22 was also increased after transfusion. The CCL22 played roles in the trafficking of activated/effector T-lymphocytes. The study also showed that heme could drive the expansion of Tregs by inducing HO-1 expression in non-classical monocytes,[33] so there is a sign that Tregs lymphocytes may also be involved in the immunosuppressive activity.

On the other hand, numerous studies have identified the cluster of differentiation (CD) molecules that are closely associated with the phagocytosis ability. The CD47, an integrin-associated protein of RBC, was proved to be a self-marker in deciding its phagocytosis by binding to the signal regulatory protein α (SIRPα) of the macrophage.[34,35] Furthermore, the microvesicles of CD47, PS, CD55, and CD59 also shaped the phagocytosis of RBC.[36,37] Therefore, we should further investigate the changes in RBC, such as the change in membrane proteins, the secretion of microvesicles or spontaneous hemolysis, so as to more clearly reveal the mechanism of "old" RBC transfusion. Our study showed that the transfusion of "old" RBC in rats induced the polarization of macrophages to M2 phenotype through the IL-10-NFR2-HO-1 signals.

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