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Nrf2/ARE pathway inhibits inflammatory infiltration by macrophage in rats with autoimmune myositis

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ABSTRACT

Background: Idiopathic inflammatory myopathies (IIM) are a group of autoimmune diseases characterized by muscle disorders. We conducted this study to detect whether NF-E2-related factor 2 (Nrf2) pathway inhibit inflammatory infiltration by macrophage in experimental autoimmune myositis (EAM) rat model.

Methods: CD163 levels were examined by immunohistochemistry (IHC), while serum creatine kinase (CK), reactive oxygen species (ROS), and serum monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) levels were determined by enzyme linked immunosorbnent assay (ELISA), both in IIM patients and EAM rat. We also detected MCP-1, TNF-α, IL-6, and Nrf2 levels by Realtime quantitative PCR (RT-PCR) in patients' muscles, and MCP-1, TNF-α, IL-6, and Nrf2, HO-1, NQO-1 levels by RT-PCR and Western blot in EAM rats' muscles. EAM macrophages were separated, and Nrf2 over-expression macrophages were constructed. ROS level and cell migration were detected by flow cytometer and transwell assay respectively. Then, levels of MCP-1, TNF-α, IL-6, Nrf2, Heme oxygenase-1 (HO-1) and NAD(P)H: quinine oxidoreductase 1 (NQO-1) were detected by RT-PCR and Western blot.

Results: Results showed that EAM rats were histopathologically inflammatory cell infiltration. Levels of CD163, serum CK and ROS, serum/muscle MCP-1, TNF-α and IL-6 increased and muscle Nrf2 level decreased in IIM patients and EAM rats. Cell migration ability and levels of ROS, MCP-1, TNF-α, IL-6, and plasma Nrf2 were down-regulated, and total/nucleus Nrf2, HO-1, NQO-1 were up-regulated notably when Nrf2 over-expressed.

Conclusion: Nrf2 inhibited EAM macrophage infiltration by activating Nrf2/ARE pathway which could induce ROS degradation and inhibit pro-inflammatory factors expression.

1. Introduction

Idiopathic inflammatory myopathies (IIM) are a group of systemic autoimmune diseases characterized by muscle disorders, with acute, subacute or chronic onset (Oldroyd et al., 2017). The clinical manifestations are muscle weakness, myalgia, muscle atrophy, creatine kinase (CK) elevation, with some visceral organs like lung, heart infected (Dimachkie et al., 2014) (Findlay et al., 2015). The serious ones could be life threatening. The incidence of IIM is 1–10 per million people. Polymyositis/dermatomyositis (PM/DM) are the main types of IIM (van der Kooi and de Visser, 2014) (Mammen, 2010; Mandel et al., 2017; Meyer et al., 2017). The muscle biopsy of PM/DM displays degeneration and necrosis of muscle fibers, along with inflammatory cell infiltration (Maoz et al., 1998) (Lundberg et al., 2016; Milone, 2017). The commonly used therapy methods for patients with immune-mediated inflammatory myopathies are corticosteroids and some immune-suppressant such as cyclophosphamide, methotrexate and so on (Tiniakou and Mammen, 2017) (Zong and Lundberg, 2011) (Dalakas, 2010; Inoue

Abbreviations: IIM, Idiopathic inflammatory myopathies; Nrf2, NF-E2-related factor 2; EAM, experimental autoimmune myositis; IHC, immunohistochemistry; CK, creatine kinase; ROS, reactive oxygen species; MCP-1, monocyte chemoattractant protein-1; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; ELISA, enzyme linked immunosorbnent assay; RT-PCR, Realtime quantitative PCR; HO-1, Heme oxygenase-1; NQO-1, NAD(P)H:quinine oxidoreductase 1; PM/DM, Polymyositis/dermatomyositis; ARE, antioxidant response element; NQO-1, NAD(P)H,quinine oxidoreductase 1; CFA, complete Freund's adjuvant; NS, normal saline; ACK, ammonium-chloride-potassium; RPMI, Roswell Park Memorial Institute; HE staining, Hematine and eosin staining; OD, optical density; RT-qPCR, Realtime quantitative Polymerase Chain Reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride; ECL, enhanced chemiluminescence; ANOVA, analysis of variance

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and Nishino, 2016). But the side effects of them are significant, such as relapse of IIM along with dose-reduction, or the resistance to the unspecific immunosuppression. So it is necessary to look for new effective therapy strategy for PM/DM. Identifying the key immune regulators of PM/DM would be a good way for its treatment.

The exact pathogenesis of PM/DM remains unclear so far. Some immune cells, chemokines and cytokines, autophagy are reported to be related to its generation. In PM, non-necrotic muscle fibers are invaded by the autophage of cytotoxic T cells and macrophages (De Paepe et al., 2007). Chemokines are critical mediators of inflammatory diseases by regulating leukocyte recruitment to the target sites of tissues. Cytokines, participating in inflammatory cells differentiation and activation, play key regulatory roles in immunoreactions including IIM (Canal et al., 2017). Monocyte chemoattractant protein-1 (MCP-1), interleukin-6 (IL-6) etc. are all important cytokines and correlated with IIM (Baird and Montine, 2008). Sanner H discovered, the level of MCP-1 in juvenile DM patients was higher than in healthy control, and correlated with disease duration and ages (Sanner et al., 2014). Serum IL-6 was detected higher in adult and juvenile DM too (Bilgic et al., 2009). In addition, oxidative stress is positive correlated with the inflammatory degree of diseases. As strong oxidative factors, reactive oxygen species (ROS) can activate a series of inflammatory factors, promoting inflammatory reaction (Signorelli and Katsiki, 2017).

NF-E2-related factor 2 (Nrf2), belonging to the family of CNC (Cap'n'Collar) transcription factor, plays important roles in cell defense process of resisting endogenous and exogenous oxidative stress (Zhou et al., 2017) (Shang et al., 2017). When Nrf2 is stimulated by chemicals, the ubiquitylation could be inhibited and more Nrf2 would be generated and accumulated. Then it would enter into cell nucleus to conjugate antioxidant response element (ARE) gene sequences, and activate AREs downstream anti-oxidative enzymes expression, such as Heme oxygenase-1 (HO-1) and NAD(P)H: quinine oxidoreductase 1 (NQO-1), inducing ROS degradation and cell protection from ROS damage(Sasaki et al., 2013) (Yamamoto et al., 2008). Nrf2 has been reported to display widely cell protection functions in immune system, with resistance to inflammation, tumor, atherosclerosis, ischemia-reperfusion injury, pulmonary fibrosis, nerve protection, etc.

In this study, after determining related elements on clinical PM/DM patients, we demonstrated the inflammatory infiltration and oxidative stress phenomena in experimental autoimmune myositis (EAM) rat model (Javadi and Sahebkar, 2017), which is a animal model similar to PM, and studied the molecular mechanism in vivo and in vitro, especially on some inflammatory factors and Nrf2/ARE pathway. It may help to identify the key immune regulators and provide new effective therapy strategy for PM/DM.

2. Materials and methods

2.1. Patients and tissue samples

24 PM/DM patients aged from 23 to 48 years old (median age: 41 years old) were enrolled in the study, with 10 male and 14 female patients. The course of disease was 3–16 months (median course of disease: 8 months). Meanwhile, 12 healthy people (5 male and 7 female) with no sign of myopathy, aging from 25 to 47 were enrolled as control group. All tissue-samples of patients were collected according to the procedures approved by the institutional review board of the independent ethics committee of The First Affiliated Hospital of Zhengzhou University. Informed consent was obtained before the study. The patients' information was presented in Table 1.

ELISA was performed to detect levels of serum CK (creatine kinase), ROS (Reactive oxygen species), MCP-1 (Mast cell protease-1), TNF-α, and IL-6, IHC was performed to detect CD163 expression, and RT-PCR was performed to measure mRNA levels of MCP-1, TNF-α, IL-6 and Nrf2 in muscle tissues of PM/DM patients.

2.2. Animal models

The EAM (experimental autoimmune myositis) animal model was adapted by multi-point injection of rabbit skeletal muscle homogenate accompanied by equal amount of complete Freund's adjuvant (CFA) (Sigma, USA). 12 female Wistar rats of 6–8 weeks, weighing 120–150 g, were randomly divided into two groups: control group and EAM group. The animals were purchased from Beijing Vital River company (Beijing, China), and raised at 25°C, with a shift of 12 h light/12 h dark. EAM rat model was multi-point injected with 0.4 mL (200 mg/mL) rabbit muscle homogenate, adding equal amount of CFA for 4 times, once a week, through back muscles and subcutaneous tissues. The control group was injected with normal saline (NS). The animals were intraperitoneal injected with 1% amobarbital anaesthetic before the separation and detection of tissues or blood. All procedures for animal care were approved by the Animal Management Committee of Zhengzhou University. All animal experiments were performed in compliance with the Guidelines for Proper Conduct of Animal Experiments, established by the Science Council.

2.3. Cell culture

Peritoneal macrophages were isolated from Wistar rats. The rats were injected phosphate buffered saline (PBS) with 3% Brewer's thioglycollate solution through peritonel cavity. Macrophage cells were harvested by PBS-lavaging peritoneal cavity 3 days later. Then cells were strained with 70 μm mesh and washed with ammonium-chloride-potassium (ACK) buffer, After that, cells were resuspended and cultured in Roswell Park Memorial Institute (RPMI) 1640 medium with 10% fetal bovine serum (FBS, Gemini, CA, USA) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, USA) at 37 °C in 5% CO_2 atmosphere. Cells of logarithm phase were used in our research.

2.4. Cell transfection

Cells were transfected with Nrf2 over-expression plasmid using transfection reagent lipofectamine2000 (Invitrogen, Carlsbad, USA), named Nrf2 group. The empty vector was transfected respectively as negative control (NC) group. Briefly, when cells were 90% confluent, plasmid and lipofectamine2000 (1:2) were mixed and added in gently. The culture media was changed into RPMI 1640 with serum after being cultured for 6 h at 37°C in 5% CO_2 incubator.

2.5. Hematine and eosin staining (HE staining)

Five weeks after immunization, the muscles of rat hind limbs were fixed with formaldehyde, paraffin-embedded and sliced before HE staining. The slices were stained with hematine and eosin staining reagents. After being dehydrated by ethyl alcohol of different concentrations and being transparentized by xylol, tissues were

Table 1
Patients information.

	Numbers	Gender (male/female)	Ages (years)	Course of disease (months)	Myasthenia (+/-)
PM	14	6/8	37.6 ± 5.5	8,6 ± 2.5	14/0
DM	10	4/6	36.9 ± 6.4	4.7 ± 1.2	10/0

immobilized with wood gum and dried later. The infiltration of inflammatory cells and muscle filers were observed under the light microscope. The Kohyama histo-pathologic grades were evaluated.

2.6. Enzyme linked immunosorbnent assay (ELISA)

The quantities of CK, ROS and pro-inflammatory cytokines (MCP-1, TNF-α, IL-6) in patients and animal models were determined by ELISA kits (R&D, Minneapolis, USA), following the manufacturer's instructions. Samples and standard substances were added into wells of 96-well plate and incubated for 90 min at 37°C, and biotinylated antibodies were added into the wells and incubated for another 60 min. ABC (Avidin peroxidase complex) was added in and incubated for 30 min before TMB (tetramethylbenzidine) coloration. Finally, optical density (OD) values were read at 450 nm by a microplate reader (Thermo, USA), and samples quantities were calculated by standard curve.

2.7. Immunohistochemistry (IHC)

Muscle tissues were immobilized with cold 4% paraformaldehyde (PFA), reacted with CD163 primary antibody (BD, San Jose, USA) overnight at 4 °C, and incubated with HRP-conjugated (horse radish peroxidase-conjugated) goat-anti-rabbit IgGs (secondary antibody) (Abcam, Camb, UK) for 30 min. Then, samples reacted with coloring agent DAB (Diaminobenzidine) (Sigma, St, Louis, USA) and was analyzed by Image-Pro Plus software. No specific labeling was observed in the absence of a primary antibody.

2.8. Reactive oxygen species (ROS) detection

ROS was detected by oxygen-sensitive fluorescence probe DCTH-DA assay. 10 μmol/L DCFH-DA was added in the well of 6-well plate and incubated for 20 min at 37°C. Then cells were washed with phosphate buffer for three times and collected to be analyzed by a flow cytometer (BD, San Diego, CA). Cell Quest software was used to analyze ROS levels.

2.9. Transwell assay

Cell migration abilities were detected by 24-well Transwell chambers attached with 8 μm-pore polycarbonate filters (Corning, NY, USA). Briefly, cells were collected and resuspended in serum-free medium, transferred to the diluted chambers (5×10^4 cells/well), and incubated for 24 h with the bottom chambers filling with culture medium with 10% FBS before examination. The penetrated cells on the lower surface, having passed through the filter, were fixed by 95% ethanol and stained by 0.1% crystal violet for 30 min. Finally, migrated cells were calculated in five randomly selected high power fields under a microscope (Olympus, Japan).

2.10. Realtime quantitative polymerase chain reaction (RT-qPCR)

The mRNA expression levels of MCP-1, TNF-α, IL-6, Nrf2, HO-1, and NQO-1 were measured by RT-PCR. Total RNA was extracted by TRIzol reagent (Invitrogen, Carlsbad, USA) and reversely transcribed to cDNA with PrimeScript RT Master Mix Perfect Real Time kit (Takara, Japan), according to the manufacture's protocol. PCR amplification was performed for 2 min at 95 °C, followed by 40 cycles: denaturation at 95 °C for 12 s, annealing/extension at 62 °C for 20 s in ABI 7300 Thermocycler (Applied Biosystems, Foster City, USA) using the SYBR Premix Ex Taq kit (Takara, Japan). The quantification was identified by method of $2^{-\Delta\Delta Cq}$ (Livak and Schmittgen, 2001). The primer sequences were listed in Table 2.

Table 2
Primers used in RT-PCR.

	Name	Type	Sequence
human	β-actin	Forward	TGGCATCCACGAAACTACCT
		Reverse	CATCTGCTGGAAGGTGGACA
	MCP-1	Forward	TAGCAGCCACCTTCATTCCC
		Reverse	GGTGGTCCATGGAATCCTGA
	TNF-α	Forward	CTGAAAGCATGATCCGGGAC
		Reverse	TTAGAGAGAGGTCCCTGGGG
	IL-6	Forward	AGACAGCCACTCACCTCTTC
		Reverse	TTTCACCAGGCAAGTCTCCT
	Nrf2	Forward	ATGCCCTCACCTGCTACTTT
		Reverse	AGGCCAAGTAGTGTGTCTCC
rat	β-actin	Forward	CGTAAAGACCTCTATGCCAACA
		Reverse	TAGGAGCCAGGGCAGTAATC
	MCP-1	Forward	AGGTGTCCCAAAGAAGCTGT
		Reverse	ACAGAAGTGCTTGAGGTGGT
	TNF-α	Forward	GAAACCTGCTGCTCACCTTG
		Reverse	GGGGTACTGGGAGGAAAACA
	IL-6	Forward	CCACCCACAACAGACCAGTA
		Reverse	ACTCCAGAAGACCAGAGCAG
	Nrf2	Forward	TGCCCACATTCCCAAACAAG
		Reverse	TTGCTCCATGTCCTGCTGTA
	HO-1	Forward	TTCAGAAGGGTCAGGTGTCC
		Reverse	CTGTGTGGCTGGTGTGTAAG
	NQO-1	Forward	GCCTGAGCCCGGATATTGTA
		Reverse	TGCAGAGAGTACATGGAGCC

2.11. Western blot analysis

The protein levels of MCP-1, TNF-α, IL-6, Nrf2, HO-1, and NQO-1 in animal tissue and cells were determined by Bradford assay. Then proteins were denatured and obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane (Amersham, Amersham, UK). Following blockage with 5% skim milk in PBS for 1 h, the blotting membranes were incubated overnight with the primary antibodies, including rabbit anti-MCP-1 (Abcam, Ab25124, 1:2000), anti-TNF-α (Abcam, Ab6671, 1:2000), anti-IL-6 (Abcam, Ab208113, 1:1000) and anti-Nrf2 (Abcam, Ab137550, 1:2000), anti－HO-1 (Abcam, Ab13243, 1:2000), anti-NQO-1 (Abcam, Ab34173, 1:1000), anti-Lamin B1 (Abcam, Ab16048, 1:5000), and anti-β-actin (Abcam, Ab8227, 1:2000) respectively at 4 °C, then they were probed with HRP-conjugated secondary antibody, Goat anti rabbit IgG H&L (HRP) (Abcam, Ab205718, 1:5000), for 1 h at room temperature. The PVDF membrane was exposed to X-ray film and immunoreactive bands were detected with enhanced chemiluminescence (ECL) reagents (Amersham, Arlington Heights, USA). Lab Works Image Acquisition and Analysis Software (UVP, Upland, USA) were used to quantify band intensities. β-actin was analyzed as endogenous control. Antibodies were purchased from Abcam (Camb, UK) company.

2.12. Statistical analysis

All results were expressed as mean ± standard deviations of three independent experiments. Statistical analysis was conducted using a SPSS 11.0 statistical package and data were analyzed by Dunnett's *t*-test $P < 0.05$ was considered significant, $P < 0.01$ was considered especially significant.

3. Results

3.1. Expression levels of inflammatory or oxidative stress related factors in IIM patients

IHC was conducted to verify positive CD163 levels. ELISA was performed to determine levels of serum CK, ROS and pro-inflammatory factors (MCP-1, TNF-α, IL-6). RT-PCR was performed to measure mRNA

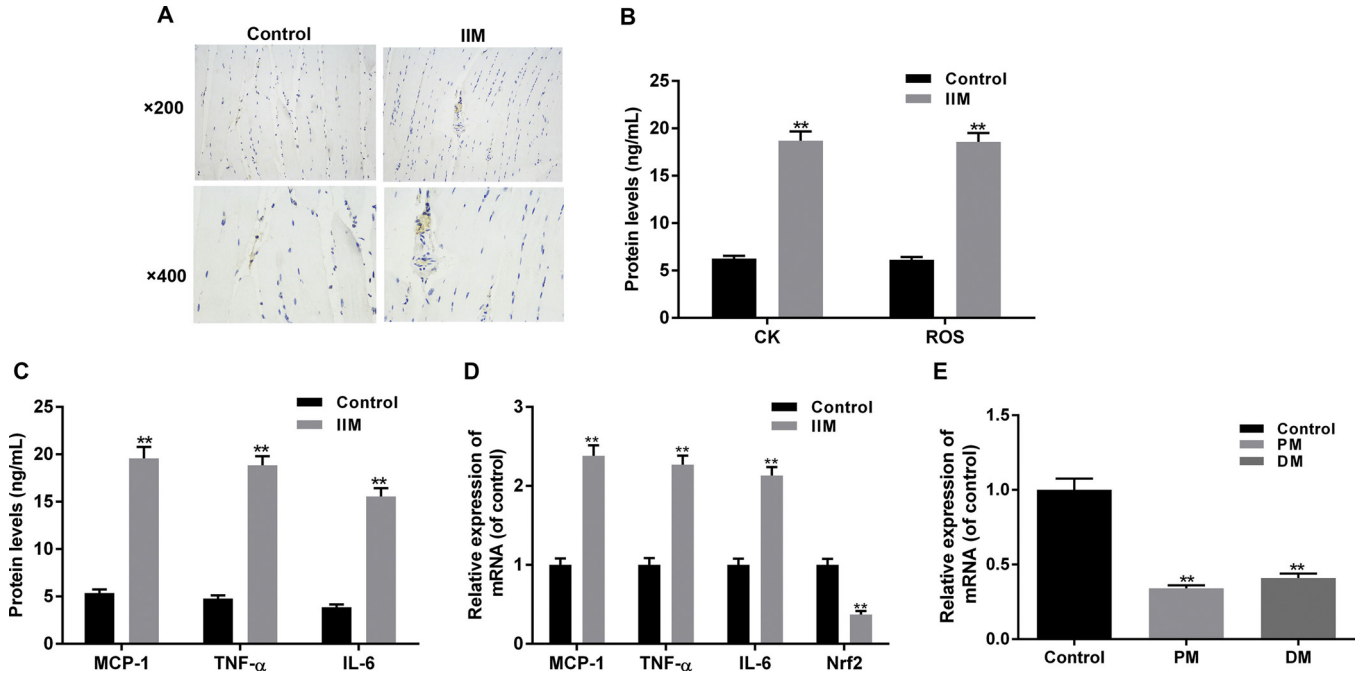

Fig. 1. Expression levels of oxidative stress related factors and inflammatory factors in IIM patients. (A) IHC was conducted to verify significantly increased CD163 in IIM patients compared with control. (B) ELISA was performed to determine levels of serum CK and ROS, finding much higher levels of serum CK and ROS in IIM patients ($P < 0.01$). (C) ELISA was performed to determine levels of serum pro-inflammatory factors (MCP-1, TNF-α, IL-6), finding remarkably increased levels in IIM patients ($P < 0.01$). (D) RT-PCR was performed to measure mRNA levels of MCP-1, TNF-α, IL-6 and Nrf2 in muscle tissues. The mRNA expression levels of MCP-1, TNF-α, IL-6 in IIM patient muscles were promoted and Nrf2 was inhibited notably ($P < 0.01$). (E) RT-PCR was performed to compare mRNA levels of Nrf2 between PM and DM patients, finding no significant difference. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control group.

levels of MCP-1, TNF-α, IL-6 and Nrf2 in muscle tissues. The results showed that, no CD163 expression was observed in the muscle samples of healthy people, and the CD163 level was increased significantly in PM/DM patients compared with control, positive CD163 staining was observed among mononuclear inflammatory cells, especially macrophages (Fig. 1A). As detected, serum CK and ROS levels were much higher in patients too (Fig. 1B). In addition, levels of pro-inflammatory cytokines (MCP-1, TNF-α, IL-6) were increased remarkably in patients, both in serum protein and muscle mRNA manners ($P < 0.01$) (Fig. 1 C and D), while the mRNA expression of Nrf2 in patient muscles was inhibited notably ($P < 0.01$) (Fig. 1 D). In addition, we compared the expression level of Nrf2 between PM and DM patients, and found no statistically significant difference (Fig. 1 E).

3.2. Histo-pathologic changes in the muscle samples from EAM rat models

The histo-pathologic changes in EAM rats were identified by HE staining and observed by light microscope. As shown in Fig. 2 A, the muscle fibers in control group were of multi-angle shape and in the same type, while cell nucleus mostly located at the periphery of muscle fibers. The muscle fibers of typeI (dark staining) and typeII (light staining) were both apparent to see. Six rats in EAM model group showed different degrees of muscle pathological changes in the fifth week after immunization, HE staining showed interstitial edema, vascular endothelial swelling, multiple inflammation changes in muscle fiber reflected by muscle fiber necrosis and degeneration, striation disappeared, internal nuclei and fiber splitting, uneven staining, mononuclear cells Infiltration around the non-necrotic muscle fibers and interstitial blood vessels. Above performances are similar to human polymyositis skeletal muscle lesions. The muscle inflammatory grades were judged by Kohyama international standard with 4 grades. As shown in Fig. 2 B, the average histo-pathologic grades of rats' limbs in EAM rats were evaluated as grade 3 (lesions affect the entire muscle bundle), while normal muscle fiber in control group was defined as grade 0. There was statistical difference between EAM group and control group ($P < 0.01$).

3.3. Expression levels of CD163 in muscle tissues of EAM rats

IHC was performed to detect CD163 levels in muscles of EAM rats, to reflect the inflammatory macrophage infiltration levels. It was found that CD163 levels in muscles of EAM rats increased significantly, compared with control group, positive CD163 staining was observed among mononuclear inflammatory cells, especially macrophages (Fig. 2 C).

3.4. Expression levels of oxidative stress related factors and inflammatory factors in EAM rat model

ELISA was performed to determine levels of serum CK, ROS, and pro-inflammatory factors (MCP-1, TNF-α, IL-6). RT-PCR and Western blot were performed to measure mRNA and protein levels of muscle MCP-1, TNF-α, IL-6 and Nrf2, HO-1, NQO-1. The results showed that, compared with control model, protein levels of serum CK and ROS both increased significantly in EAM rats ($P < 0.01$)(Fig. 3A). Levels of pro-inflammatory factors (MCP-1, TNF-α, IL-6) increased remarkably in EAM rats too, in serum protein and muscle mRNA/protein manners ($P < 0.01$) (Fig. 3B, C and D). In addition, the mRNA expression and protein levels of Nrf2, HO-1, NQO-1 in EAM rats muscles were inhibited notably. ($P < 0.01$)(Fig. 3 E and F).

3.5. Over-expression of Nrf2 decreased ROS levels and inhibited cell migration of EAM macrophages

Flow cytometer was performed to detect ROS levels in macrophages separated from EAM rats (EAM group), EAM macrophages transfected with empty vectors (NC group) and EAM macrophages transfected with Nrf2 over-expression plasmids (Nrf2 group). As shown in Fig. 4 A, ROS

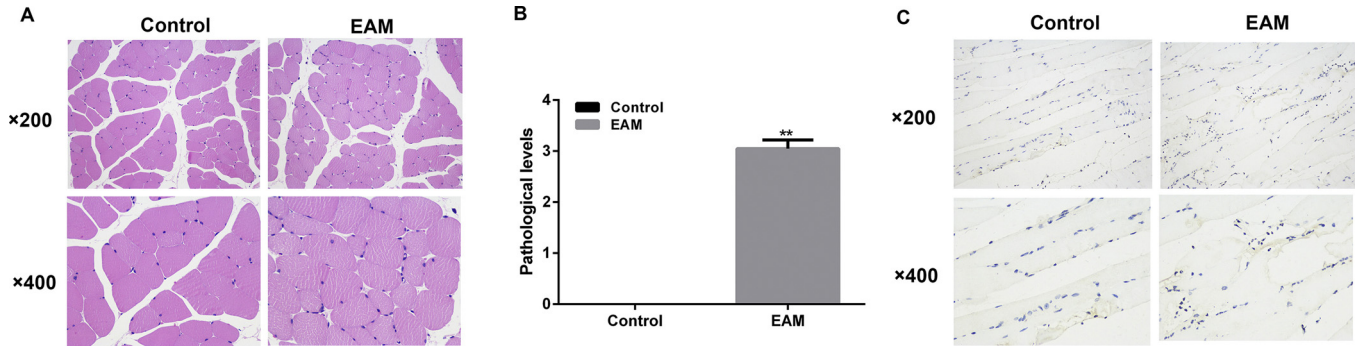

Fig. 2. Histo-pathologic changes and CD163 expression level in EAM rat model muscles.
(A) The histo-pathologic changes in EAM rats were identified by HE staining. The muscle fibers in control group were of multi-angle shape and of the same type, while cell nucleus mostly located at the periphery of muscle fibers. In EAM rats, there were inflammatory cell infiltration, quantities of muscle cells necrosis, internal nuclei and fiber splitting, uneven staining, mononuclear cells Infiltration around the non-necrotic muscle fibers and interstitial blood vessels. (B) The muscle inflammatory grades were evaluated by Kohyama international standard. The average histo-pathologic grade of rats' limbs was grade 3 in EAM rats ($P < 0.01$). (C) IHC was performed to detect CD163 levels in muscles of EAM rats. CD163 levels in EAM rats increased significantly. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control group,

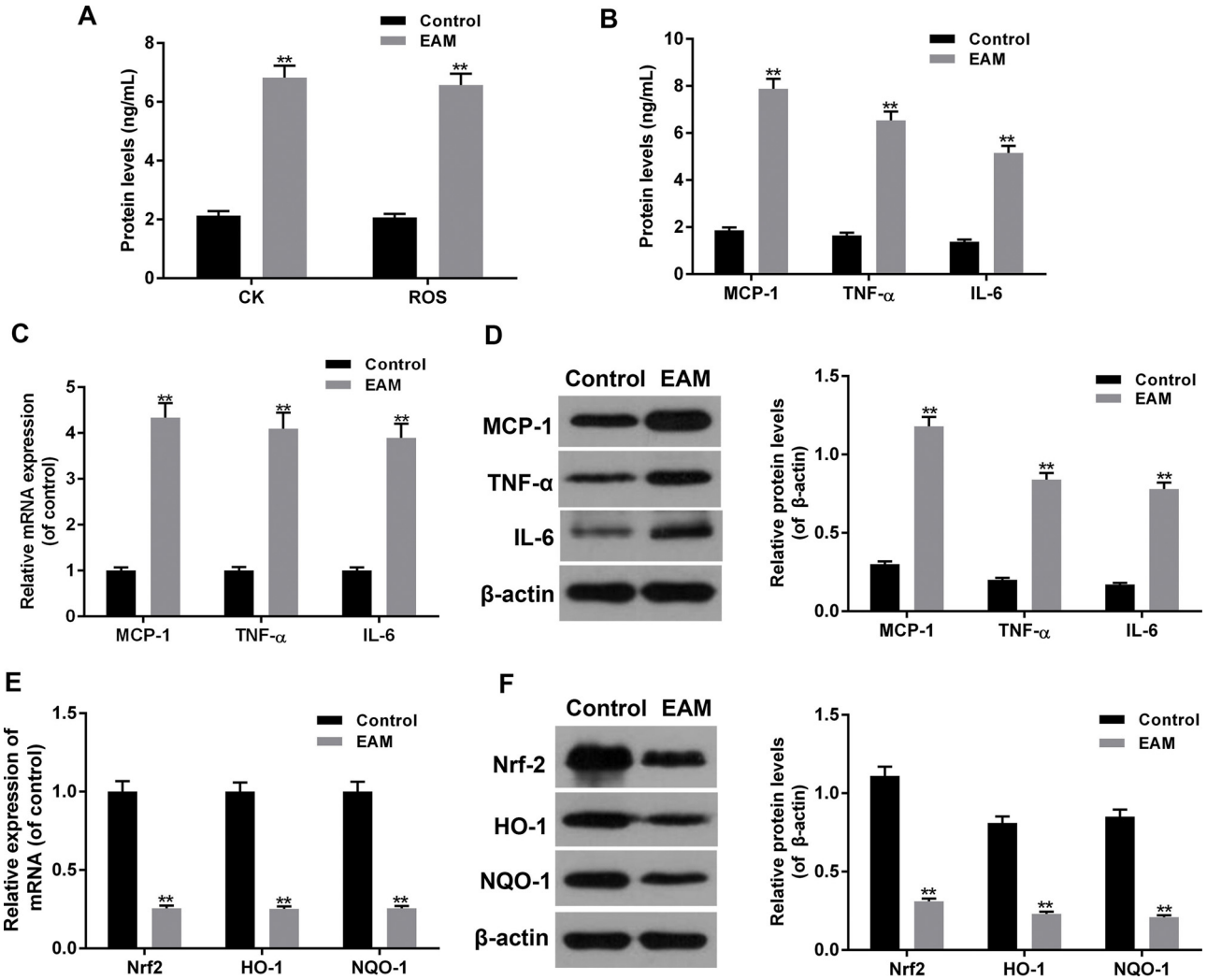

Fig. 3. Expression levels of oxidative stress factors and inflammatory factors in EAM rat model. (A) ELISA was performed to determine levels of serum CK and ROS, finding significantly increased serum CK and ROS in EAM rats ($P < 0.01$). (B) ELISA was performed to determine levels of serum pro-inflammatory factors (MCP-1, TNF-α, IL-6), finding remarkably increased serum MCP-1, TNF-α, IL-6 in EAM rats ($P < 0.01$). (C) RT-PCR was performed to measure mRNA expression levels of muscle MCP-1, TNF-α, IL-6, which increased significantly in EAM rats ($P < 0.01$). (D) Western blot was performed to measure protein levels of muscle MCP-1, TNF-α, IL-6, which increased significantly in EAM rats ($P < 0.01$). (E) RT-PCR was performed to measure mRNA expression levels of muscle Nrf2, HO-1, NQO-1, which decreased significantly in EAM rats ($P < 0.01$). (F) Western blot was performed to measure protein levels of muscle Nrf2, HO-1, NQO-1, which decreased significantly in EAM rats ($P < 0.01$). $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control group.

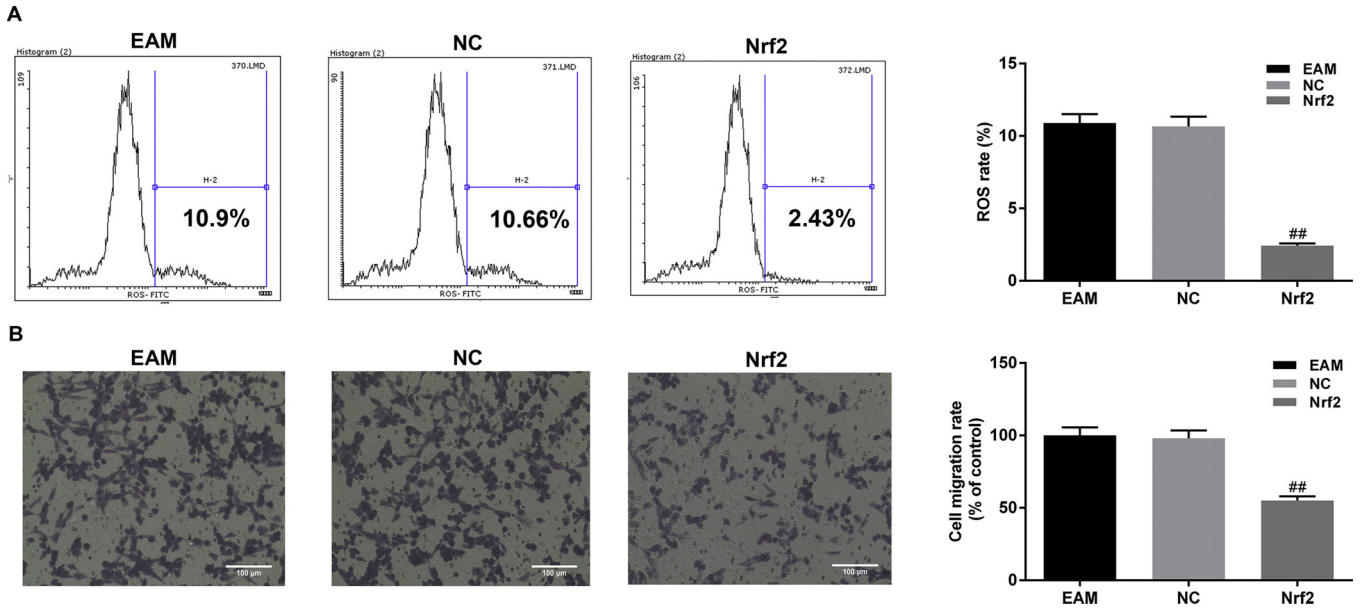

Fig. 4. Over-expression of Nrf2 decreased ROS levels and inhibited cell migration of EAM macrophages. (A) Flow cytometer was used to detect ROS levels in macrophages of EAM group, NC group and Nrf2 group. ROS levels decreased dramatically in Nrf2 group ($P < 0.01$). (B) Transwell assay was performed to determine the migration ability of different groups, finding the migration ability of Nrf2 group was inhibited significantly ($P < 0.01$). $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. EAM model group.

levels decreased dramatically in Nrf2 group, compared with NC group and EAM group ($P < 0.01$). Transwell assay was performed to determine the migration ability of different groups, finding the migration ability of Nrf2 group was inhibited significantly, compared with NC group and EAM group ($P < 0.01$) (Fig. 4 B).

3.6. Over-expression of Nrf2 inhibited pro-inflammatory factors and promoted oxidative stress inhibitors expression of EAM macrophages

RT-PCR and Western blot were performed to detect mRNA and protein levels of pro-inflammatory factors (MCP-1, TNF-α, IL-6) and oxidative stress inhibitors (Nrf2, HO-1, NQO-1) in EAM macrophages, NC group and Nrf2 over-expression group. Both the mRNA and protein levels of MCP-1, TNF-α, IL-6 were decreased significantly ($P < 0.01$) (Fig. 5 A and B). The mRNA and protein levels of total Nrf2, HO-1, NQO-1 were promoted notably. In addition, more Nrf2 transfered from plasma to nucleus, with increased nucleus Nrf2 protein level and decreased plasma Nrf2 protein level, detected by Western blot ($P < 0.01$) (Fig. 5 C and D).

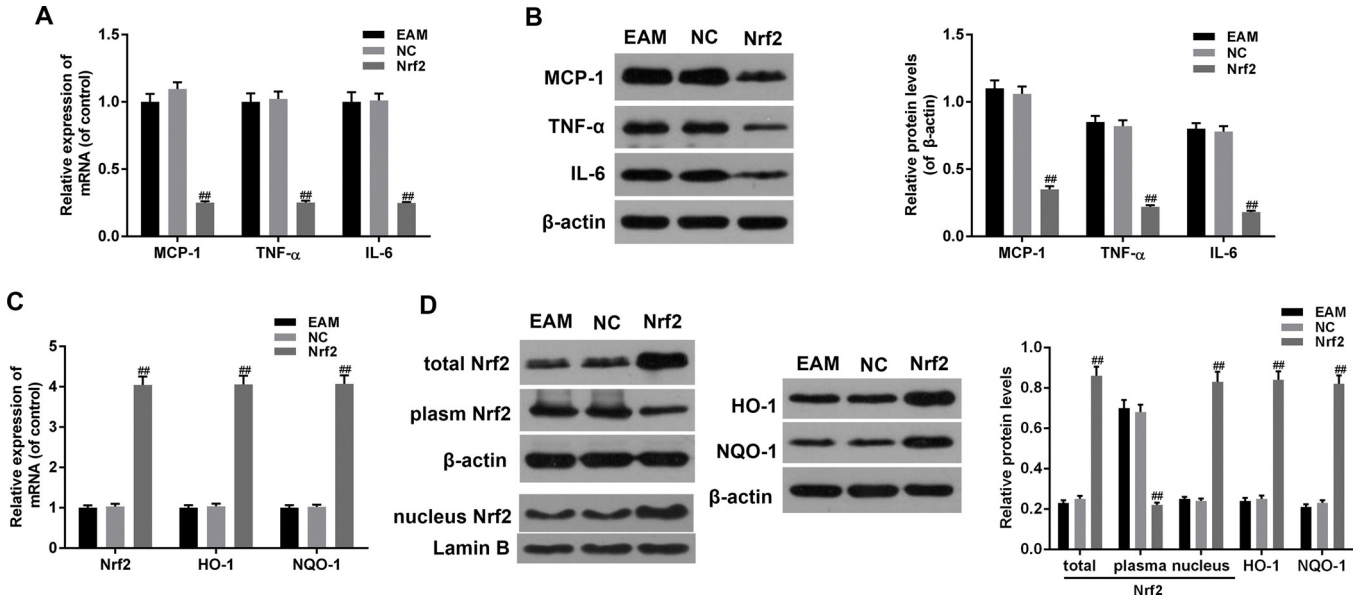

Fig. 5. Over-expression of Nrf2 inhibited the expression of pro-inflammatory factors and promoted the expression of oxidative stress inhibitors in EAM macrophages. (A) RT-PCR was performed to detect mRNA expression levels of pro-inflammatory factors (MCP-1, TNF-α, IL-6) in EAM macrophages, NC group and Nrf2 over-expression group ($P < 0.01$). (B) Western blot was performed to detect protein levels of pro-inflammatory factors (MCP-1, TNF-α, IL-6) ($P < 0.01$). (C) RT-PCR was performed to detect mRNA expression of oxidative stress inhibitors (Nrf2, HO-1, NQO-1) ($P < 0.01$). (D) Western blot was performed to detect protein levels of oxidative stress inhibitors (Nrf2, HO-1, NQO-1). The protein levels of total Nrf2, HO-1, NQO-1 were inhibited notably. In addition, more Nrf2 transfered from plasmid to nucleus, with increased nucleus Nrf2 and decreased plasmid Nrf2 ($P < 0.01$). n = 3, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. EAM model group.

4. Discussion

IIM is a group of systemic autoimmune diseases characterized by muscle disorders and creatine kinase (CK) elevation. The muscle biopsy of PM/DM displays degeneration and necrosis of muscle fibers, along with inflammatory cell infiltration (Wangkaew et al., 2016). The commonly used therapy methods for patients are of many side effects. Identifying the key immune regulators of PM/DM would be a good way for its treatment(Malik et al., 2016) (Rayavarapu et al., 2011). Nrf2 has been reported to display widely cell protection function in immune reaction, and resistance to inflammation, tumor, atherosclerosis, ischemia-reperfusion injury, pulmonary fibrosis, nerve protection etc.. It may contribute to the treatment of IIM.

In the present study, we examined levels of CD163, serum CK, ROS, pro-inflammatory factors and factors in Nrf2/ARE pathway and so on, both in clinical PM/DM patients and EAM rat models which is an animal model exactly similar to PM patients. The EAM rat model in our study was well built and verified by histopathologic detection. Six rats in EAM model group showed different degrees of muscle pathological changes in the fifth week after immunization, HE staining showed interstitial edema, vascular endothelial swelling, multiple inflammation changes in muscle fiber reflected by muscle fiber necrosis and degeneration, striation disappeared, internal nuclei and fiber splitting, uneven staining, mononuclear cells Infiltration around the non-necrotic muscle fibers and interstitial blood vessels. Above performances are similar to human polymyositis skeletal muscle lesions. The average histo-pathologic grade of rats' limbs in EAM rats was evaluated as grade 3 (lesions affect the entire muscle bundle), while normal muscle fiber in control group was defined as 0 grade. As we know, IIM is characterized by CK elevation. CK is related to muscle atrophy, ATP regeneration and so on. In our study, serum CK levels increased significantly both in PM/DM patients and EAM rat model. CD163 is a transmembrane protein expressed by macrophage and functions on macrophage to participate in immune activity regulation, as a scavenger receptor protein to recognize and clear away some endogenous or exogenous products like peroxide (29) (30). It has been verified to be a marker of the lineage of monocyte and macrophage. Serum CD163 levels were reported elevated and correlated with PM/DM severity in patients previously (31). In our study, CD163 was detected increased dramatically in PM/DM patients and EAM rats too, reflecting more macrophages were produced when PM/DM developed.

NF-E2-related factor 2 (Nrf2), plays important roles in cell defense process of resisting endogenous and exogenous oxidative stress. When Nrf2 deletion or activation obstacle occur, cells will be in oxidative stress status, opt to induce the extension of inflammation repair time, tumor and so on. Although there is a limitation of not detecting Nrf2 levels in specific cells like macrophages in patients, the expression levels of Nrf2 were found inhibited in muscle tissues of PM/DM patients and EAM rats. Meanwhile, the Nrf2 levels were of no statistically significant difference between PM and DM patients. To determine the mechanism of Nrf2 in EAM, we constructed a Nrf2 over-expression model using macrophages separated from EAM rats. As we all know, cell migration is always found in tumor generation and development, which also plays important roles in macrophage inflammatory infiltration in IIM. In our study, cell migration ability was down-regulated notably in Nrf2 over-expressed EAM macrophages (Nrf2 group), compared with EAM group, reflecting Nrf2 could inhibit macrophage infiltration by down-regulating cell migration ability.

When Nrf2 was over-expressed in EAM macrophages, the accumulation make it enter into cell nucleus (Lawal, 2017) (Jain et al., 2017). Consistent with it, increased concentration of Nrf2 was found in cell nucleus and decreased in cell plasmid. In cell nucleus, Nrf2 could conjugate with ARE gene sequence and activate ARE downstream antioxidative enzymes HO-1 and NQO-1, with promoted levels, inducing ROS degradation and cell protection from ROS damage. ROS and free radicals possess could enhance oxidative ability, being harmful to protein, nucleic acids and lipids. There is just very little ROS in normal biologic systems in physiological status, which participates in cell signaling transduction or cellular oxido-reductive reaction as an electron carrier (Lightfoot et al., 2015). In addition, ROS also participates in a variety of diseases such as cardiovascular disease, tumor, liver diseases (Di Filippo et al., 2016). In our study, ROS was found increased in PM/DM patients, and decreased in macrophages from EAM rats when Nrf2 over-expressed. In addition, the degree of oxidative stress is positively correlated with the degree of inflammation. ROS can activate a serious of inflammatory factors.

Cytokines/chemokines are reported to regulate differentiation and activation of inflammatory cells. As a chemokine, MCP-1 induces monocytes differentiate to macrophages, and pro-inflammatory macrophage will secrete more chemokines to produce more macrophage infiltration and initiate inflammation process (Mansour et al., 2017). TNF-α and IL-6 are pro-inflammatory factors with direct or indirect pro-inflammatory functions (Szodoray et al., 2010) (Notarnicola et al., 2015) (Park and Lee, 2017). They directly effect on inflammatory cells or stimulate the release of other inflammatory factors too. Over-expression of pro-inflammatory factors breaks the inflammation balance and induces chronic inflammation and injures. PM/DM patients and EAM rat model were detected with increased levels of MCP-1, TNF-α and IL-6, both in serum and muscle tissues of PM/DM patients and EAM rats, while over-expression of Nrf2 inhibited their expression. It indicated that a large number of chytokines like MCP-1, TNF-α and IL-6 were produced and released from muscle to blood to function in the whole body, representing Nrf2 could inhibit macrophage inflammatory infiltration by inhibiting the expression of pro-inflammatory factors.

5. Conclusions

Over-expression of Nrf2 could inhibit EAM macrophage inflammatory infiltration by activating Nrf2/ARE signaling pathway, which could induce ROS degradation and inhibit the expression of pro-inflammatory factors, such as MCP-1, TNF-α and IL-6. The study would take a great step on PM/DM mechanism research, and give novel light to the diagnosis and treatment of PM/DM. For the heterogeneity of human IIM, it is difficult to imitate all subtypes of IIM in one animal model, future study is required to elucidate the effect of Nrf2 on IIM in human beings.

Competing interests

The authors declare no conflicts of interest.

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