

Inhibition of MARCO ameliorates silica-induced pulmonary fibrosis by regulating epithelial-mesenchymal transition

Meng Yang^{a,c}, Xinlai Qian^b, Na Wang^a, Yingying Ding^a, Haibin Li^a, Yingzheng Zhao^a, Sanqiao Yao^{a,*}

^a School of Public Health, Xinxiang Medical University, Xinxiang, 453003, China

^b The third Affiliated Hospital, Xinxiang Medical University, Xinxiang, 453003, China

^c School of Public health, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, 430030, China

ARTICLE INFO

Keywords:

Silica
MARCO
EMT
Pulmonary fibrosis
Silicosis

ABSTRACT

Epithelial-mesenchymal transition (EMT) is linked to fibrosis following exposure to silica. The scavenger receptor, macrophage receptor with collagenous structure (MARCO) plays an important role in silica-induced inflammation, however, the effect of MARCO on silica-induced fibrosis has not been identified. We hypothesized that MARCO would regulate EMT and be involved in the development of silicosis. Herein, we found that MARCO was highly expressed in lung tissue after exposure to silica and a MARCO inhibitor PolyG could alleviate pulmonary fibrosis in vivo. Our results confirmed that the expression of epithelial marker such as E-cadherin decreased, while the expression of mesenchymal markers, including vimentin and α -SMA increased after silica treatment. Furthermore, PolyG administration efficiently blocked the mRNA and protein expression of EMT markers and decreased the level of fibrosis-related transcription factors and proteins, such as Col1a1, Col3a1, Collagen I and Collagen III in the lungs of silica-exposed rats. The findings demonstrate that the macrophage membrane receptor MARCO controls the fibrotic response through regulating EMT in experimental silicosis and suggest a novel target for preventive intervention.

1. Introduction

Silicosis, caused by long-term inhalation of free silica dust, is a major occupational disease worldwide, especially in developing countries (Leung et al., 2012). Alveolar epithelial cell injury and macrophage apoptosis, persistent inflammation and the subsequent formation of silicon nodules are common characteristics of silicosis (Chen et al., 2018). The mechanism of silicosis has not been fully elucidated, therefore it is necessary to find out potential preventive or therapeutic targets for the intervention of silicosis.

Inhaled silica particles can be cleared out partly by the alveolar macrophages (AMs), which are the primary immune phagocytic cells, through the mucociliary escalator and/or lymphatic systems (Hamilton et al., 2008). The balance between clearance and retention of silica particles in the lung by AM plays an important role in regulating the

inflammatory response and fibrosis (Thakur et al., 2009b). Previous studies have suggested that facilitating the clearance of silica particles from the alveolar and interstitial compartments alleviates the degree of pulmonary fibrosis. Furthermore, scavenger receptor MARCO has been proven to play a vital role as a predominant receptor in binding and uptake of silica particles and finally silica clearance (Thakur et al., 2008, 2009b).

Scavenger receptors (SRs), a 'superfamily' of membrane-bound receptors, have a high affinity for a wide variety of ligands. They play a range of physiological functions including intracellular transportation, lipid transportation and pathogen clearance (Zani et al., 2015). Recently, a number of studies focus on the mechanism of the class A scavenger receptor, especially MARCO, in the aspects of regulating inflammatory response and tissue remodeling (Maler et al., 2017). The MARCO expression of AMs is highly correlated with the amount of

Abbreviations: EMT, epithelial-mesenchymal transition; MARCO, macrophage receptor with collagenous structure; SR, scavenger receptor; Col1a1, collagen type I, alpha 1; Col3a1, collagen type III, alpha 1; PolyG, polyguanylic acid; PolyC, polycytidyllic acid; PolyU, polyuridylic acid; α -SMA, α -smooth muscle actin; AMs, alveolar macrophages; SD, Sprague-Dawley; CD36, cluster of differentiation 36; SRB1, scavenger receptor class B type 1; SCARA5, scavenger receptor class A member 5; BSA, bovine serum albumin; TBST, Tris-Buffered Saline Tween-20; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; qRT-PCR, quantitative reverse transcription PCR

* Corresponding author at: School of Public Health, Xinxiang Medical University, No.601 Jinsui Road, Xinxiang 453003, Henan province, China.

E-mail address: sanqiaoyao@126.com (S. Yao).

silica uptake. And the silica-induced cytotoxicity is completely inhibited by pretreatment of the murine AM with MARCO antibody, suggesting a particular role of MARCO in silica-induced cell death (Thakur et al., 2009a). Similarly, *in vitro* studies reveal that reducing the binding of silica to macrophages could decrease the levels of oxidative stress and apoptosis (Hamilton et al., 2008; Thakur et al., 2009a). Intriguingly, MARCO-mediated uptake could be inhibited by its ligand PolyG but not by polyribonucleotide PolyC and PolyU that are used as controls (Mukhopadhyay et al., 2011). In addition, it has been reported that the quadruplex structure of PolyG is a crucial structural element for recognition by SR (Huang et al., 2017).

Epithelial–Mesenchymal transition (EMT) is a process by which epithelial cells lose their cell polarity and cell-cell adhesion, and gain migratory and invasive properties to become mesenchymal cells (Lamouille et al., 2014). It has been reported that EMT is essential for numerous developmental processes including organ fibrosis, wound healing and initiation of metastasis in cancer progression (Bartis et al., 2014). Recent studies suggest that EMT may contribute to the development and the progression of silicosis, and it has been proved to be a valuable event occurred in alveolar epithelial cells (Heise et al., 2011; Yan et al., 2016). New research shows that pulmonary fibroblasts are among the sources for myofibroblast differentiation during the development of silicosis. Furthermore, the study also affirms the epithelial-to-mesenchymal or myofibroblast transition in the formation of silicosis (Deng et al., 2016).

Recent advances in molecular pathways that control the association between inflammation and organ fibrosis suggest that EMT is a common link in the progression of these devastating diseases. (Lopez-Novoa and Nieto, 2009). Several studies have focused on the role of SR in silica-induced silicosis, especially inflammation effects *in vitro* and *in vivo*. Compared with WT mice, SRA-I/II deficient mice exhibit little to no deposition of collagen, yet they demonstrate diminished clearance of silica from the lung and enhanced accumulation of inflammation cells (Beamer and Holian, 2005). However, MARCO^{-/-} mice exposed to silica show enhanced both acute and chronic inflammation, in addition, the development of fibrosis as measured by hydroxyproline content is slightly increased (Thakur et al., 2009b). Thus, the previous results are controversial, and whether MARCO are involved in the fibrosis response is still unknown. Our previous study demonstrated that the level of reactive oxygen species and apoptosis decreased in silica-exposed rats after treatment with PolyG (a MARCO inhibitor) (Zhang et al., 2014), while it is not well understood whether PolyG alleviates silica-induced fibrosis and deserves further studies.

Interestingly, the latest research shows that scavenger receptor MARCO is also expressed on lung epithelial cells and mediates the uptake of amorphous silica nanoparticles (Lara et al., 2018). Additionally, scavenger receptors such as CD36, SRB1 and SCARA5 could participate in regulating EMT in diabetic nephropathy and hepatocellular carcinoma (Hou et al., 2015; Liu et al., 2013; Yu et al., 2015). Here, we hypothesized that MARCO would be also involved in regulating EMT, and the MARCO inhibitor PolyG might halt the progression of pulmonary fibrosis response in the silicosis model of rats. For this purpose, we investigated the mechanism of silicosis via inhibiting the expression of MARCO using PolyG and further explored the potential role of MARCO in the prevention of silicosis.

2. Material and methods

2.1. Crystalline silica

Crystalline silica particulates (approximately 97% between 1 and 5 μm diameter; Frederick, MD, USA) were ground for 3 h with agate mortar, weighed and suspended in sterile saline (50 mg/ml) and suspensions were sonicated for 10 min prior to instillation (Li et al., 2016).

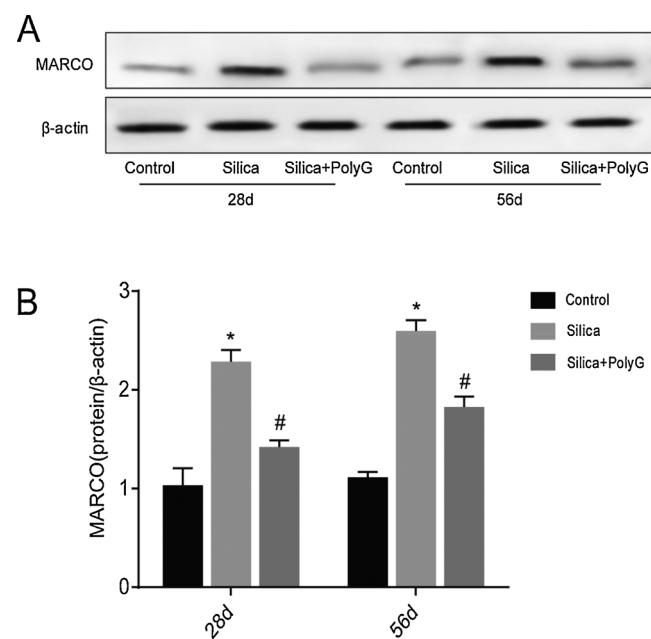


Fig. 1. Silica-induced MARCO expression was inhibited by PolyG in rat silicosis model. (A). Expression of MARCO protein level in rat lungs, measured by Western blotting. (B) MARCO protein level were quantified using Image J Software. Data were presented as mean \pm SD ($n = 8$). * $P < 0.05$ vs. control group, # $P < 0.05$ vs. silica group.

2.2. Animals

Forty-eight adult male Sprague-Dawley (SD) rats (purchased from Beijing Weitong Lihua Experimental Animal Technology Co. Ltd, Beijing, China) weighing 180–220 g were housed in a pathogen-free facility at North China University of Science and Technology (Tangshan, China). Rats were housed under specific pathogen-free conditions (room temperature 20–24°C; relative humidity 50–60%; 12 h/12 h light/dark cycle) and provided with food and water ad libitum. This study was carried out in accordance with the Animal Care and Use Committee at North China university of Science and Technology and all animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.3. Silica-induced silicosis in rats

Experimental silicosis model was performed as follows: SD rats were anesthetized with pentobarbital sodium (0.3 ml/100 g) (Sigma-Aldrich, St. Louis, USA) and then were given 50.0 g/L silica suspension 1 mL by a method of non-surgical intratracheal instillation (Rayamajhi et al., 2011).

2.4. Experimental design

All rats were randomly divided into 3 groups: saline group ($n = 16$), in which the rats were instilled with saline; silicosis model group ($n = 16$), in which the rats were instilled with crystalline silica as described before; PolyG group ($n = 16$), in which the rats were instilled with crystalline silica and given a single intravenous injection of PolyG (2.5 mg/Kg body weight) at the same time as silica exposure. PolyG (Sigma-Aldrich, St. Louis, USA) was dissolved in sterile saline to a concentration of 1 mg/mL. Eight rats in each group were sacrificed at 28 days and 56 days after silica instillation.

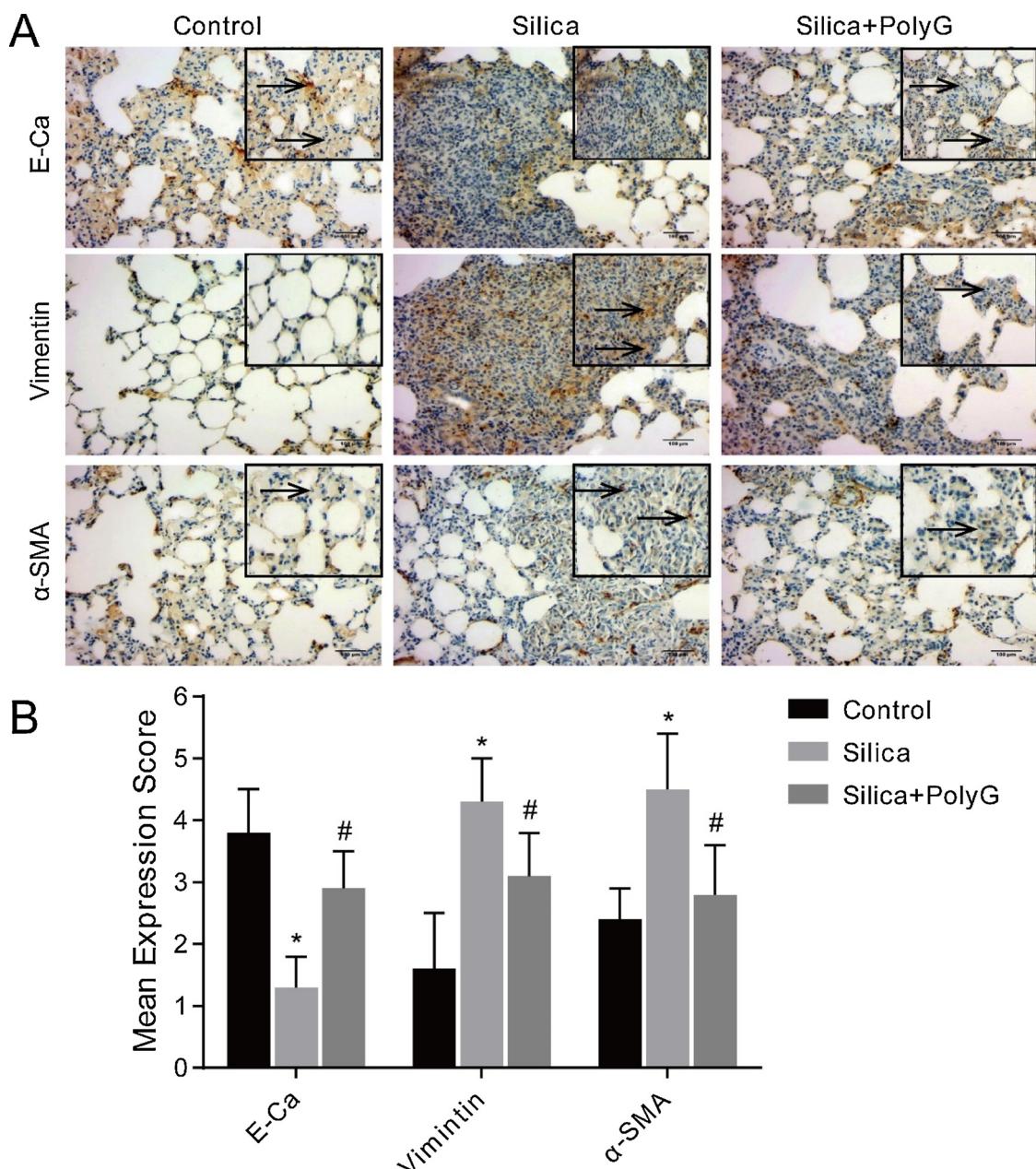


Fig. 2. Effects of inhibition of MARCO on the expression of EMT markers by immunohistochemical analysis at 28 days ($\times 200$). (A) Representative images of immunohistochemical staining for Protein location of E-cadherin, vimentin and α -SMA of rat lungs. Insets: Higher magnification showing the positive cells. (B) The positively stained nuclei were analyzed, and the mean staining intensity was scored by Image-Pro Plus software. Data were presented as mean \pm SD ($n = 8$). * $P < 0.05$ vs. control group, # $P < 0.05$ vs. silica group.

2.5. HE and Masson staining

Briefly, the middle lobe of the right lung was fixed with 10% formalin for 48 h and embedded in paraffin. Sections (4- μ m thick) were cut and stained with hematoxylin and eosin (H&E) and Masson trichrome (M-T) staining for evaluation of histopathological changes in the lung, and then the sections were observed using light microscope (Motic®, model AE2000, China). The collagen deposition was scored according to the density of Masson trichrome staining by Image-Pro Plus 6.1 software (Media Cybernetics, Inc., Rockville, MD, USA), a digitalized immunohistochemistry scoring program (Chen et al., 2012).

2.6. Immunohistochemical analysis

An SP immunohistochemistry reagent kit (Wuhan BOSHIDE

Biological Engineering, Co., Ltd., China) was used to perform immunohistochemistry according to the manufacturer's instructions. Briefly, paraffin-embedded sections (as described in 2.5) were blocked with 5% bovine serum albumin (BSA), and then incubated with the following primary antibodies: E-cadherin (Santa Cruz Biotechnology, Santa Cruz, CA), vimentin and α -SMA (Cell Signaling Technology Inc, Beverly, MA, USA). The sections were incubated with a biotinylated secondary antibody and images were acquired by microscope (Motic®, model AE2000, China). Quantitative image analysis was processed by Image-Pro Plus 6.1 software (Wang et al., 2009).

2.7. Quantitative real-time RT-PCR

Total RNA was isolated from left lung tissues using Trizol Reagent (Thermo Fisher Scientific, Waltham, MA) with the commercialized

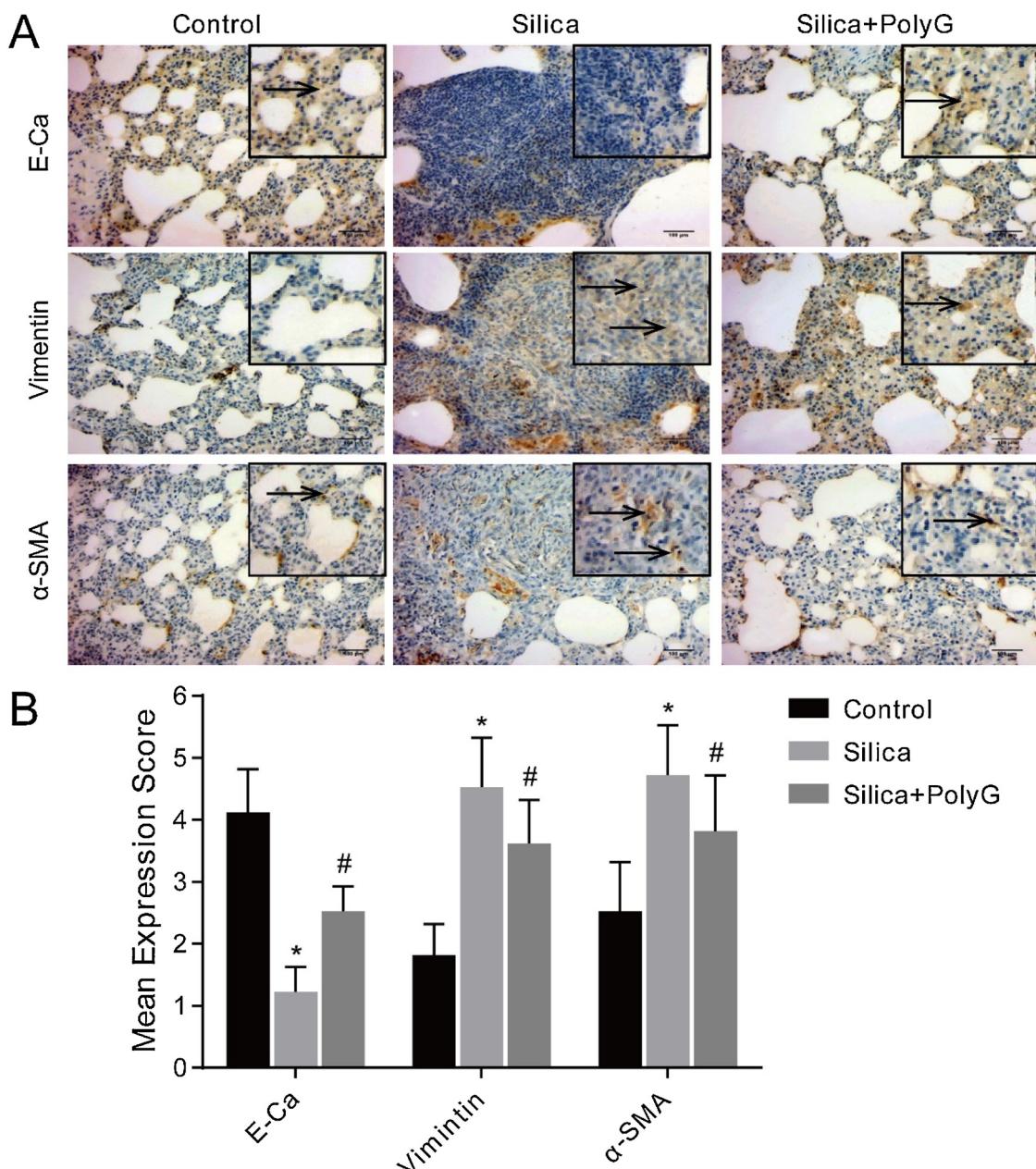


Fig. 3. Effects of inhibition of MARCO on the expression of EMT markers by immunohistochemical analysis at 56 days ($\times 200$). (A) Representative images of immunohistochemical staining for E-cadherin, vimentin and α -SMA of rat lungs. Insets: Higher magnification showing the positive cells. (B) The positively stained nuclei were analyzed, and the mean staining intensity was scored by Image-Pro Plus software. Data were presented as mean \pm SD ($n = 8$). * $P < 0.05$ vs. control group, # $P < 0.05$ vs. silica group.

protocol. The RNA was reverse transcribed to cDNA using the TransScript First-Strand cDNA Synthesis SuperMix (Invitrogen). The sequences of primers are described below: E-cadherin, forward (5'-GATTACAAGTTCCCGCCATC-3') and reverse (5'-CTTGACCCGGTTC TCCTC-3'); vimentin, forward (5'-AGGAACAGCATGTCCAATCG-3') and reverse (5'-AAGGGCATCCACTTCAGCTG-3'); α -SMA, forward (5'-CACCATCGGGATAACGCTTC-3') and reverse (5'-CTGTCAGCAA TGCCTGGGTA-3'); Col1a1, forward (5'CAATCGGCTAAAGAAGTCT GTC-3') and reverse (5'-AGGTGGGTACACTGTAGCCT-3'); Col3a1, forward (5'-AGGTGGGTACACTGTAGCCT-3') and reverse (5'-GATCGCAT AGGTGACAGGTGTT-3'); GAPDH, forward (5'-GTGATTCTTGCACCG GCAG-3') and reverse (5'-CAGGAGCATGGTTGGAGT-3'). cDNA was analyzed by SYBR-Green qPCR Kit (Thermo Fisher Scientific, Waltham, MA) with an ABI 7900 HT (Applied Biosystems, Foster City, CA). $2^{-\Delta\Delta CT}$ values was used to evaluate the relative mRNA expression and

GAPDH was used as an internal control for normalization.

2.8. Western blot analysis

Equal amounts of protein (60 μ g) were separated by 10% sodium dodecyl sulfate polyacrylamide gels electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane (Millipore, Billerica, MA, USA). Nonspecific binding was blocked with 5% non-fat milk for 1 h and then incubated at 4°C overnight with the primary antibodies: vimentin, α -SMA, and β -actin (Cell Signaling Technology Inc, Beverly, MA, USA), Collagen I and Collagen III (Affinity Biosciences, OH, USA), E-cadherin and MARCO (Santa Cruz Biotechnology, Santa Cruz, CA). After washed three times with Tris-Buffered Saline Tween-20(TBST), the membranes were incubated with HRP-conjugated goat anti-rabbit (Cell Signaling Technology, Beverly, MA, USA) for 1 h and then washed

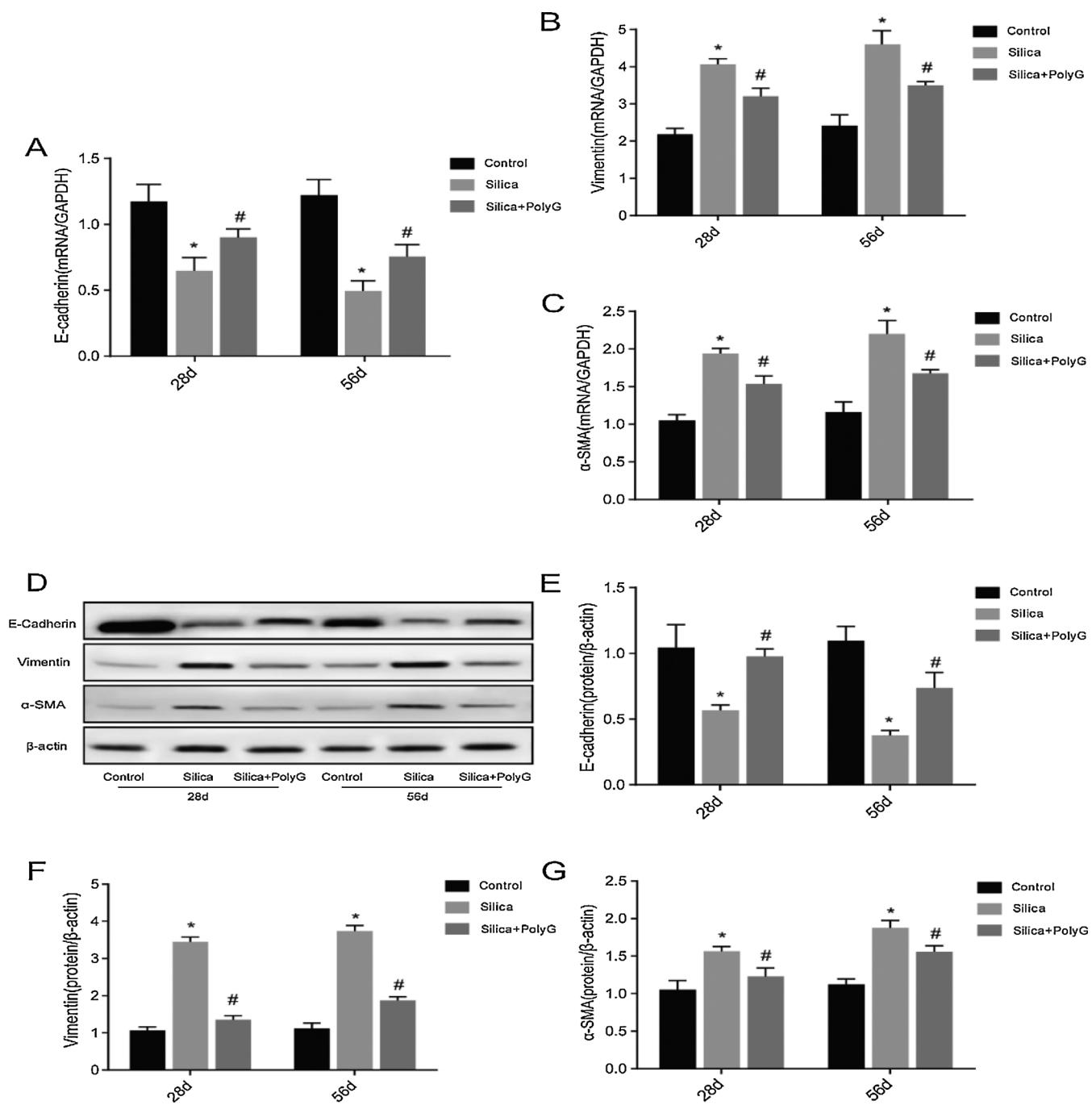


Fig. 4. Inhibition of MARCO expression reduced silica-induced EMT in rat silicosis model. (A–C) Expression of EMT markers mRNA levels in rat lungs, measured by qRT-PCR. (D) Expression of EMT markers protein levels in rat lungs, measured by Western blotting. (E–G) Protein level were quantified using ImageSoftware. Data were presented as mean \pm SD ($n = 8$). * $P < 0.05$ vs. control group, # $P < 0.05$ vs. silica group.

three times with TBST. Each protein image was acquired by Amersham Imager600 (GE Healthcare) and analyzed with Image J Software (BIO-RAD, California, USA).

2.9. Statistical analysis

All data were analyzed using SPSS17.0 software (SPSS Inc, Chicago, IL) for Windows. Continuous variables with normal distribution were presented as mean \pm standard deviation. The difference among multiple groups were assessed by one-way ANOVA analysis and the comparison between any two groups was adjusted using Tukey method. A P -value less than 0.05 was considered statistically significant.

3. Results

3.1. PolyG reduced MARCO expression in rat silicosis model

To confirm the effect of PolyG on MARCO expression in rat lung tissues, total protein was extracted from all three groups and analyzed by Western blotting. As shown in Fig. 1, compared with the control group, the protein level of MARCO was increased significantly in silica group both at 28 days and 56 days, while the elevation was reduced in PolyG treatment group. It indicated that PolyG could block the expression of MARCO.

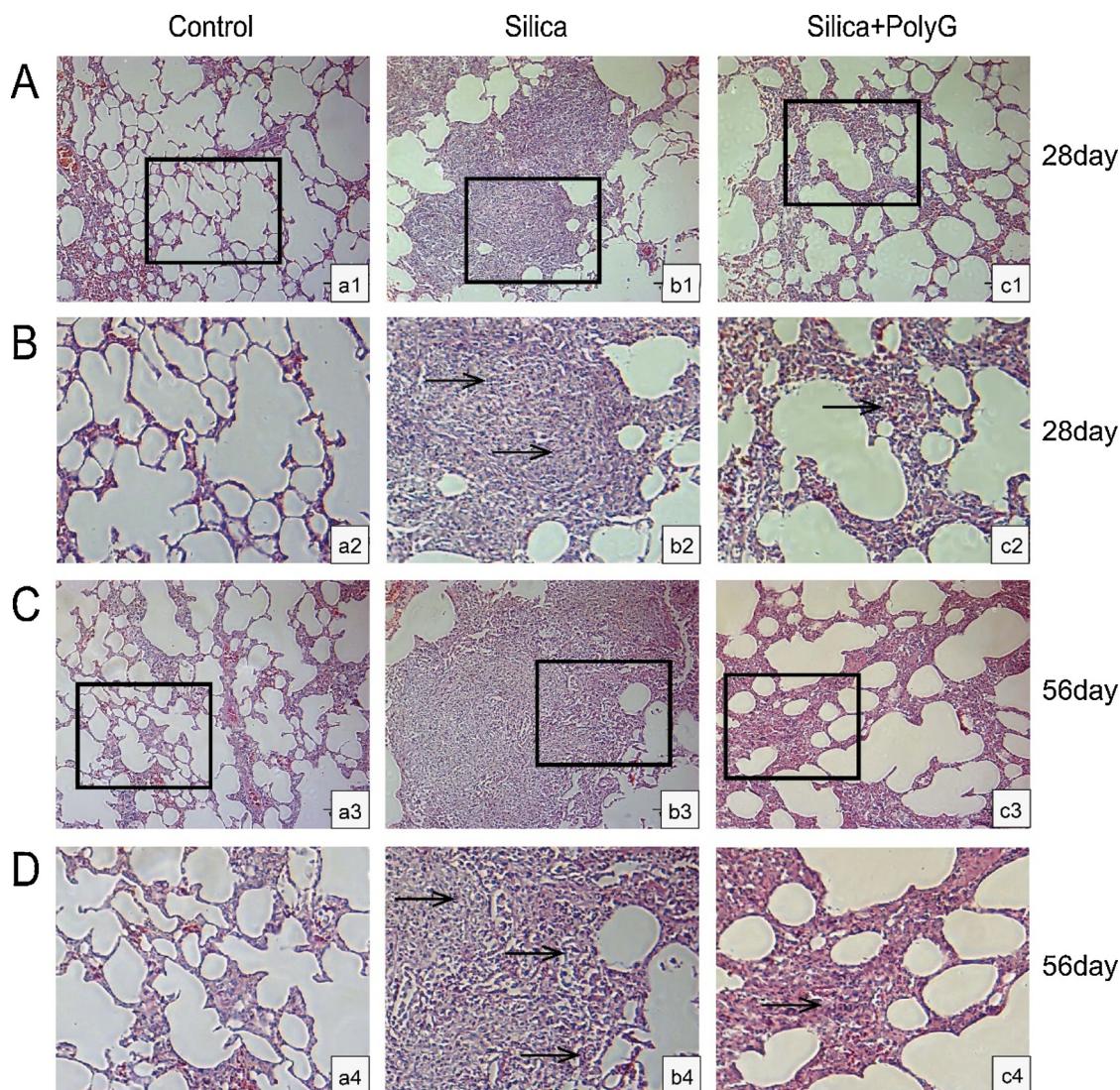


Fig. 5. HE staining of lung tissue in each group at 28 days and 56 days after instillation. A&C, magnification at $\times 100$; B&D, a higher magnification of the boxed areas in top panels. (a1-a4) control group; (b1-b4) silica group; (c1-c4) silica plus PolyG group. Arrows point out pathological changes of lung tissue.

3.2. Inhibition of MARCO prevented silica-induced EMT in rat silicosis model

Furthermore, we tested whether inhibition of MARCO could modulate silica-induced EMT in rats. E-Cadhein-positive cells were on the surface of the alveolar wall cavity in control group. In contrast, fewer E-Cadhein-positive cells were observed in silica group, whereas this decrease was reversed in PolyG treatment group (Figs. 2A and 3 A). In addition, immunohistochemistry analysis revealed that the positive cells of mesenchymal markers α -SMA and vimentin were not observed in control group, while α -SMA and vimentin were mainly expressed in silicon nodules and interstitial fibrotic areas in silica group, and the positive regions of vimentin and α -SMA were obviously reduced after treatment with PolyG (Figs. 2A and 3 A).

3.3. Inhibition of MARCO regulated the expression of silica-induced EMT markers in rat silicosis model

Moreover, we determined the effect of inhibition of MARCO on EMT markers expression at the molecular level. Results showed that silica exposure led to downregulation of E-Cadhein (Fig. 4A and E), and upregulation of vimentin (Fig. 4B and F) and α -SMA (Fig. 4C and F) both at mRNA and protein levels when compared with the control group.

After treatment with PolyG, expression of E-Cadhein was significantly increased while vimentin and α -SMA expression decreased when compared with silica group at both time points.

3.4. Inhibition of MARCO mitigated the lung pathology changes in rat silicosis model

HE staining (Fig. 5) showed that the alveolar structure of rats from the control group were intact with a little of inflammatory cells infiltration. Lung alveolar structures of rats from the silica group were severely damaged with a large amount of inflammatory cell infiltration, which mainly were lymphocyte and macrophage cells. Thickening of the pulmonary alveolar and small vessel walls, as well as silicon nodules containing macrophages and fibroblasts were observed in rats of silica group. In the PolyG treatment group, the number of inflammatory cells and scattered fusion of silicon nodules were reduced compared to the silica group. The results of Masson staining (Fig. 6) showed that the normal collagen fiber stent were observed in the lung tissues of the control group. On the contrary, a large amount of collagen fibers were deposited in the pulmonary mesenchyme, especially around bronchi and vessels in the silica group (Fig. 6E). The more inflammatory cells and silicon nodules were observed after silica exposure for 56 days, however, compared with the silica group, the area of collagen

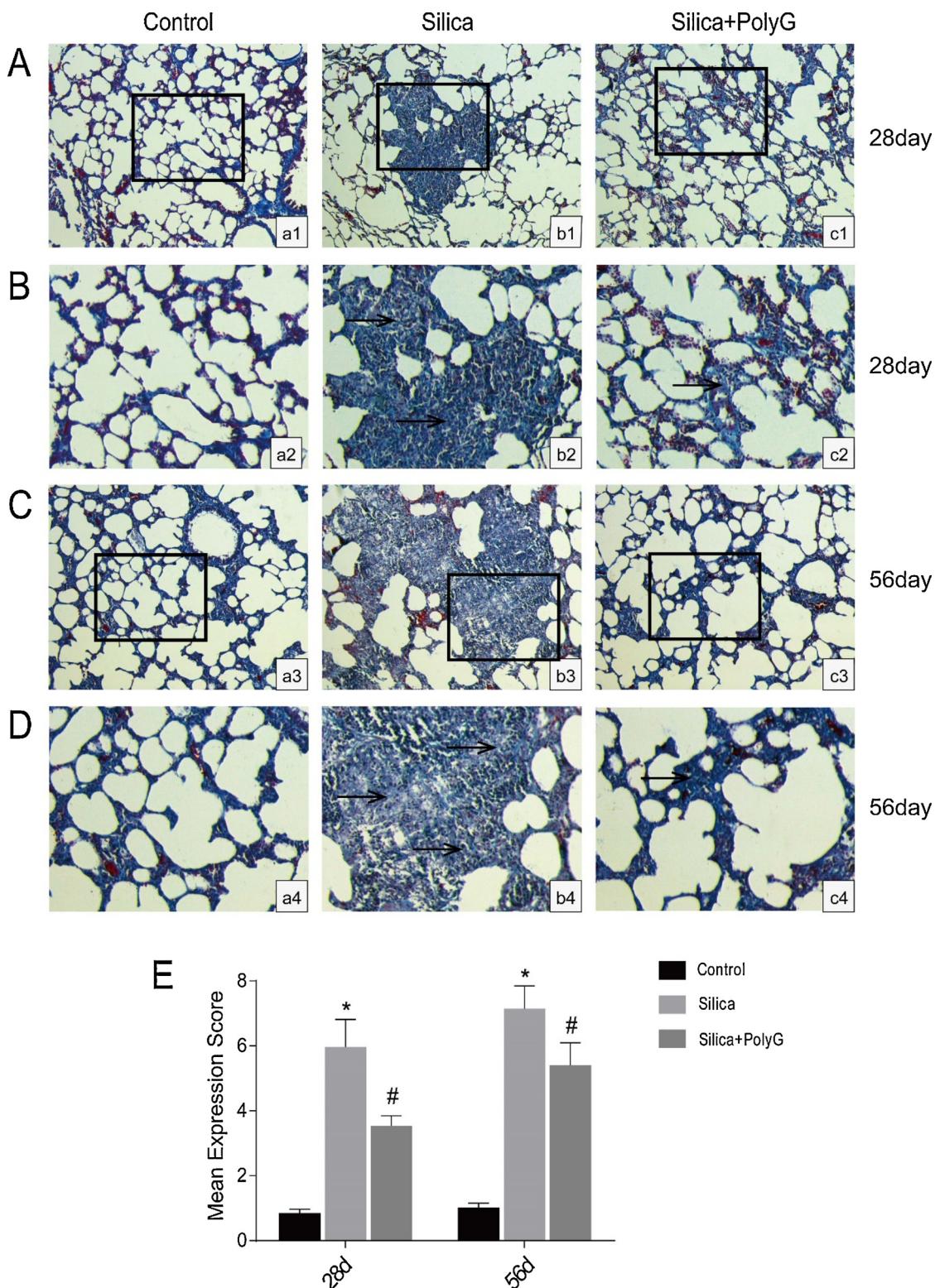


Fig. 6. Masson staining of lung tissue in each group at 28 days and 56 days after instillation. A&C, magnification at $\times 100$; B&D, a higher magnification of the boxed areas in top panels. (a1-a4) control group; (b1-b4) silica group; (c1-c4) silica plus PolyG group. (E) The mean staining intensity of deposited collagen was scored by Image-Pro Plus software. Arrows point out pathological changes of lung tissue.

deposition in PolyG treatment group was also decreased.

3.5. Inhibition of MARCO alleviated silica-induced fibrosis in rat silicosis model

After silica exposure for 28 days and 56 days, we found that the

levels of procollagen (Col1a1 and Col3a1) (Fig. 7A-B) and Collagen subtypes (ColI and Col III) (Fig. 7C-E) in lung tissues measured by qRT-PCR and Western blotting, respectively, were significantly higher than the control group. In rats with intravenous injection of PolyG, the levels of fibrotic indicators were markedly decreased compared to those in the silica group.

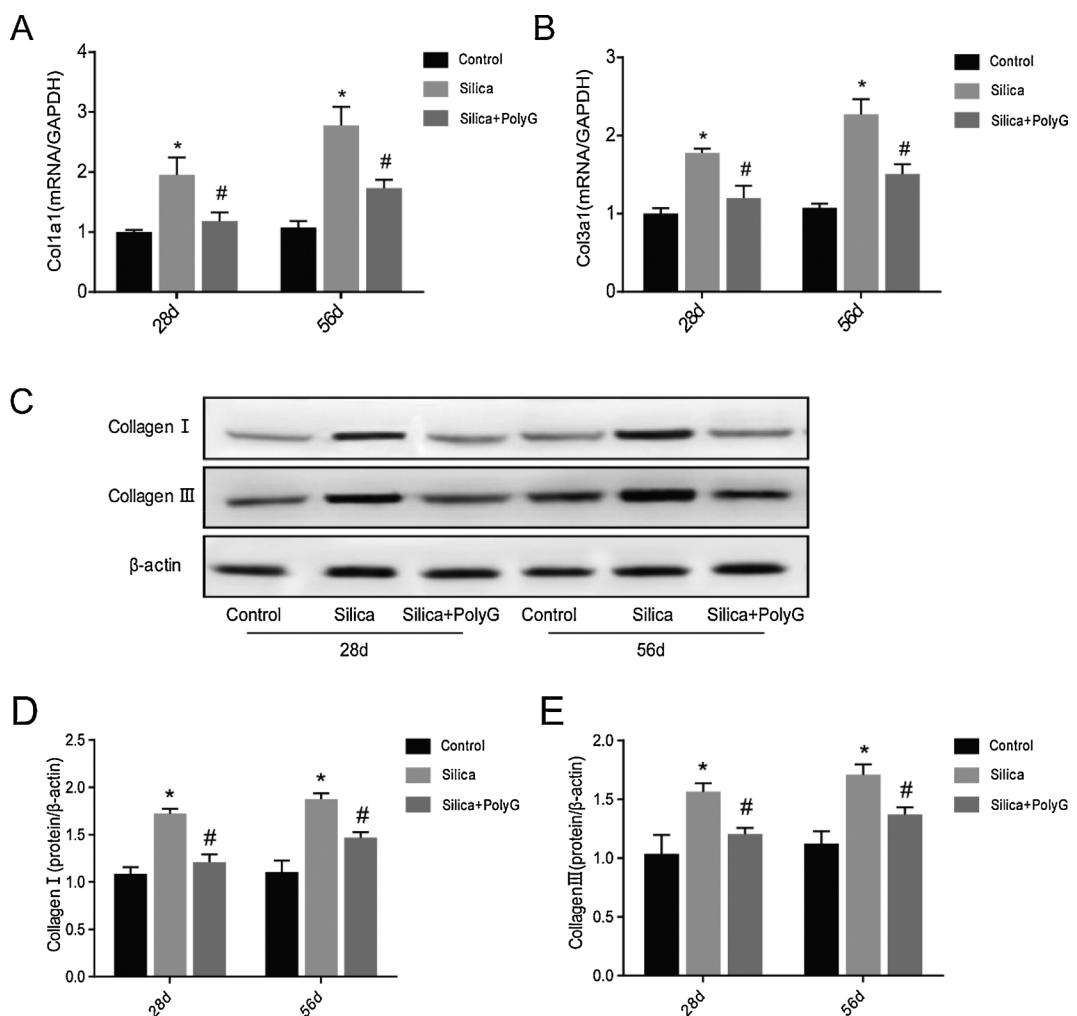


Fig. 7. Inhibition of MARCO expression attenuated silica-induced lung fibrosis in rats. (A–B) Expression of Col1a1 and Col3a1 mRNA in rat lungs, measured by qRT-PCR. (C) Expression of Collagen I and Collagen III protein levels in rat lungs, measured by Western blotting. (D–E) The relative expression of proteins were quantified using Image J Software. Data were presented as mean \pm SD ($n = 8$). * $P < 0.05$ vs. control group, # $P < 0.05$ vs. silica group.

4. Discussion

In this study, we demonstrated that MARCO was involved in silica-induced pulmonary fibrosis and provided new evidence for the anti-fibrosis strategy of using MARCO inhibitors.

AMs play a central role in silica-induced inflammation and subsequent pulmonary fibrosis. The balance between clearance and retention of silica particles in the lung is maintained by AM and unsuccessful clearance of silica may result in persistent inflammation (Thakur et al., 2009b). MARCO, one of the most important class A scavenger receptor, is the predominant scavenger receptor for recognition and binding silica particles by AMs. Previous work has shown that the phagocytosis of silica particles mediated by MARCO is inhibited by PolyG, a MARCO ligand, in a competitive manner. Comparing with the phosphate buffer and titanium dioxide treatment groups, mice exposed to silica exhibit a significantly increased expression of MARCO. MARCO^{-/-} mice exposed to silica show stronger inflammatory reaction and lung injury, but the degree of fibrosis is not increased (Thakur et al., 2009b). Recently, Murthy, S et al. demonstrate that the degree of pulmonary fibrosis induced by crocidolite is significantly reduced in MARCO^{-/-} mice than wild-type mice (Murthy et al., 2015). Consistent with these results, our present study found that an up-regulated expression of MARCO at day 28 and 56 in the silica exposed group, and this increase was inhibited after PolyG treatment. Simultaneously, preventive administration of PolyG could attenuate the

ROS generation and macrophage apoptosis through inhibiting mitochondrial-dependent apoptosis pathway (data not shown). Here, we demonstrated that PolyG effectively inhibited the expression of MARCO, low level of MARCO might have a beneficial effect in lung tissue during pulmonary fibrosis.

Recently, more evidence is given that the transition of epithelial cells to myofibroblasts (EMT) plays an important role in silica-induced pulmonary fibrosis (Liang et al., 2016). EMT is a process that an extreme form of cell plasticity (a normal epithelial morphology transition to a spindle-shaped morphology) characterized by loss of epithelial markers and acquisition of mesenchymal markers (Kalluri and Weinberg, 2009). E-cadherin is expressed on most epithelial cells, and functions in establishing cell polarity and maintaining normal tissue structure (Rodriguez-Boulan and Macara, 2014). The loss of E-cadherin expression has been known to be a universal marker of EMT changes in epithelial cells. Vimentin is normally expressed in mesenchymal cells, and comprises the cytoskeleton along with tubulin-based microtubules and actin-based microfilaments (Wickstead and Gull, 2011). In general, it is often used as a marker of mesenchymally-derived cells or cells undergoing an EMT during both normal development and metastatic progression. α-SMA can be found in vascular smooth muscle cells and myoepithelial cells in normal condition (Merkulova-Rainon et al., 2012). It is often used to identify pathologic fibroblasts, and EMT is also associated with cells that eventually express α-SMA as myofibroblasts.

Therefore, the immunohistochemical profiles of E-cadherin,

vimentin and α -SMA were observed in order to investigate the mechanism of EMT suppression after inhibition of MARCO in rats with inhaled silica suspension. From a biological point of view, the upregulation in vimentin and α -SMA expression and reduction of E-cadherin expression in lung tissues suggest that EMT is occurring. Consistent with several previous studies, our results showed EMT was involved in the development and progression of silicosis. Additionally, our study found that the process of EMT in PolyG treatment group were significantly inhibited compared with the silica group. Quantitative real-time PCR was used to confirm reduced E-cadherin and increased vimentin and α -SMA in experimental silicosis model at the molecular level. Similarly, loss of E-cadherin and gain of vimentin and α -SMA were further validated by Western blotting, there was good agreement in the overall trend between Western blotting and qRT-PCR results. However, the EMT process was still occurred in PolyG treatment group compared with the control group, and it indicated that other receptors or mechanism may be involved in the EMT. Given the evidence that some scavenger receptors played an important role in the regulation of EMT in different diseases, our results showed that MARCO could also participate in the EMT.

Pulmonary fibrosis is characterized by the accumulation of excess fibrous connective tissue, with increased collagen gene expression and abnormal collagen deposition in the lungs (Wynn and Ramalingam, 2012). To observe the distribution of inflammatory cells and detect the collagen deposition in lung interstitium, we used HE staining and Masson staining to assess differences in the progression of pulmonary fibrosis between groups. Simultaneously, the collagen deposition was further confirmed on the molecular level, the mRNA expression of Col1a1 and Col3a1 was examined by qRT-PCR, the protein expression of Collagen I and III was tested by Western blotting. We found aggregated inflammatory infiltrate and intense collagen deposition in the lung, accompanied by severe distortion of structure and consolidated areas of fibrosis in rats exposed to silica suspension. Furthermore, prolonged observation time revealed that the degree of fibrosis in the PolyG treatment group was still increased, however, the severity of fibrosis was milder than the model group at the corresponding time points. Thus, the data from this study indicated that inhibition of MARCO alleviated lung fibrosis in silica exposed rats.

Taken together, our findings showed that inhibition of MARCO leading to a suppression of EMT, which resulted in the reduction of collagen deposition in the lung parenchyma. Therefore, the scavenger receptor MARCO could possibly lead to the development of a preventive target for silicosis.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

This work was supported by grants of National Natural Science Foundation of China (No. 81273017).

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