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Inhibition of DNA methylation attenuates lung ischemia–reperfusion injury after lung transplantation

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Abstract

Objective: DNA methylation plays an important role in inflammation and oxidative stress. This study aimed to investigate the effect of inhibiting DNA methylation on lung ischemia–reperfusion injury (LIRI).

Methods: We adopted a completely random design for our study. Thirty-two rats were randomized into the sham, LIRI, azathioprine (AZA), and pluripotin (SC1) groups. The rats in the LIRI, AZA, and SC1 groups received left lung transplantation and intravenous injection of saline, AZA, and SC1, respectively. After 24 hours of reperfusion, histological injury, the arterial oxygen partial pressure to fractional inspired oxygen ratio, the wet/dry weight ratio, protein and cytokine concentrations in lung tissue, and DNA methylation in lung tissue were evaluated. The pulmonary endothelium that underwent hypoxemia and reoxygenation was treated with AZA or SC1. Endothelial apoptosis, chemokines, reactive oxygen species, nuclear factor-κB, and apoptotic proteins in the endothelium were studied.

Results: Inhibition of DNA methylation by AZA attenuated lung injury, inflammation, and the oxidative stress response, but SC1 aggravated LIRI injury. AZA significantly improved endothelial function, suppressed apoptosis and necrosis, reduced chemokines, and inhibited nuclear factor-κB.

Conclusions: Inhibition of DNA methylation ameliorates LIRI and apoptosis and improves pulmonary function via the regulation of inflammation and oxidative stress.

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Background

Hypoxemia and reoxygenation (H/R) during lung transplantation usually lead to lung ischemia–reperfusion injury (LIRI), resulting in transplanted lung dysfunction[1] and even an aggravated long-term outcome of patients.[2] Currently, approximately 20% of patients who undergo lung transplantation experience LIRI.[3] Moreover, local inflammation and the oxidative stress response play pivotal roles in the pathology of LIRI.[4] Indeed, once the genes related to inflammatory factors have been activated, the downstream cytokines of these genes are consistently synthetized and released. Therefore, effective inhibition of gene activation of inflammatory factors could be a treatment for LIRI.

Epigenetic regulation, especially DNA methylation, can dictate gene expression and cell fate, but does not change the gene order. Therefore, epigenetic regulation is involved in several diseases and the pathology of organ injury.[5,6] DNA methylation, which is mainly introduced by DNA methyltransferase (DNMT) and reversed by the ten-eleven translocation (TET) enzyme, regulates genomic function, including gene transcription, mutation, and stability.[5] Recently, DNA methylation has been shown to participate in lung inflammation in acute lung injury[7,8] and acute respiratory distress syndrome,[9] and inhibition of DNA methylation considerably ameliorates lung injury by modulating macrophages. A few studies have suggested that the hypoxic condition inhibits the activity of DNMT.[10] However, many studies[11] have indicated that DNA methylation contributes to ischemia–reperfusion injury and predicts the outcome of patients after kidney transplantation.[12] Additionally, inhibition of methylation or promotion of TET effectively reduces kidney[13] and brain injury in animal studies,[14] which is mainly attributed to the regulation of inflammation.[15–17] Considering the role of inflammation in LIRI, we speculate that the inhibition of DNA methylation can reduce LIRI after lung transplantation. In this study, we performed a left lung transplantation in rats and administered a DNMT inhibitor (5-aza-29-deoxycytidine [AZA]) or TET inhibitor (SC1). We detected the concentration of 5-mc (product of DNA methylation) produced by DNMT and 5-hmc (product of DNA demethylation) produced by TET to evaluate the effect of inhibition of DNA methylation or demethylation on LIRI after lung transplantation.

Methods

In vivo experiments

Animals. All male Sprague–Dawley rats (250–300 g) were purchased from the Animal Center of the Second Affiliated Hospital of Harbin Medical University. All rats were fasted for 12 hours but had free access to water. All of the procedures of this study were reported to the Ethics/Review Board and approved by the Institutional Animal Care and Use Committee of the Second Affiliated Hospital of Harbin Medical University (approval number: YJSDW2022-105). All of the procedures included in this

study were in accordance with the national guidelines for animal studies.

Lung transplantation. Thirty-two recipient rats were randomized into the sham, LIRI, AZA, or SC1 groups (n = 8 in each group). The rats in the sham group received only thoracotomy and mechanical ventilation, and were intravenously injected with saline. The rats in the LIRI, AZA, or SC1 groups that received left lung transplantation were injected with saline, AZA (Sigma, St Louis, MO, USA) or SC1 (Cayman Chemical, Ann Arbor, MI, USA).

Left lung transplantation was performed according to our previous study.[18–20] The donor rats were anesthetized (intraperitoneal injection of 3% pentobarbital sodium, 30 mg/kg) and intubated with a 10 mL/kg tidal volume and 50 breaths/min respiratory rate (50% O_2 + 50% N_2). The rats received 300 U/kg heparin for heparization and thoracotomy. Cold saline (4°C) was injected via the pulmonary artery at 20 cm H_2O pressure. The pulmonary bronchus, artery, and vein were trimmed and then fixed to the cuff tube. The isolated lung was preserved at 4°C for 60 minutes. In this study, the cold ischemia time of the transplanted lung was determined according to previous studies.[21–23] The recipients received the same anesthesia, intubation, and mechanical ventilation as the donors. The right femoral artery and vein were cannulated to maintain anesthesia, monitor hemodynamic changes, and perform arterial blood analysis. After lateral thoracotomy at the third to fourth intercostal region, the left pulmonary bronchus, artery, and vein were dissociated. The bronchus, artery, and vein were transiently blocked with a vascular clamp and then anastomosed with the donor lung using the cuff technique. The tidal volume was adjusted to 6 mL/kg and returned to 10 mL/kg after reperfusion. After lung transplantation, the thoracotomy was closed in the recipients, and they were extubated after recovery of spontaneous breathing.

Immediately after reperfusion, the rats in the sham and LIRI groups received 0.5 mL of saline. The rats in the AZA or SC1 groups received AZA (1 mg/kg[7]) or SC1 (10 nM[24]). During the transplantation procedure, the temperature was maintained within 37°C to 39°C, and postoperative analgesia was provided by 1% ropivacaine for infiltration.

Arterial blood analysis and blood samples were collected at baseline and 24 hours after transplantation. Furthermore, the transplanted lung was harvested and prepared for further analysis.

Evaluation of DNA methylation and demethylation. To detect DNA methylation and demethylation in transplanted lung tissue, we detected concentrations of the product of DNA methylation induced by DNMT (5-mc) and demethylation induced by TET (5-hmc) in lung tissue with enzyme-linked immunoassay (ELISA) kits (Abcam, Cambridge, MA, USA) according to the manufacturer's instructions.

Evaluation of the arterial oxygen partial pressure to fractional inspired oxygen ratio, protein concentrations, and the wet/dry weight ratio. The arterial oxygen partial pressure to fractional inspired oxygen (PaO_2/FiO_2) ratio, protein concentrations, and the wet/dry weight ratio of transplanted lungs were calculated. PaO_2 was analyzed by a Bayer Rapidlab 348 analyzer (Bayer Diagnostics, Leverkusen, Germany). The wet/dry ratio of transplanted lung tissue was tested (weighed first, dried for 48 hours at 60°C, and weighed again). The protein concentrations in lung tissue were investigated using the Bradford method.[25]

Inflammation in lung tissue. Local inflammation was evaluated by measuring cytokines in lung tissue. Part of the left lung was

homogenized with 4°C saline at a ratio of 1:9 (w/v). The homogenates were centrifuged ($1000 \times g$ for 15 minutes), and the supernatant was collected. The cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and IL-10 in the left lung were measured with ELISA kits (Wuhan Boster Bioengineering, Wuhan, China).

Oxidative stress assay. We collected part of the transplanted lung and homogenized lung tissue with nine volumes (w/v) of saline. The homogenization was centrifuged at $1000 \times g$ at 4°C for 15 minutes, and the supernatants were recovered. The superoxide anion, malondialdehyde (MDA) content, and xanthine oxidase (XO) activity were analyzed using commercial assay kits (Nanjing Jiancheng, Jiangsu, China).

Examination of histopathological injury. The lung tissue sections were stained with hematoxylin and eosin to estimate the lung histological injury. The histological injury score was estimated (Table 1) by an independent pathologist who did not participate in this study, and five variables were included as follows: lung hemorrhage, peribronchial infiltration of inflammatory cells, pulmonary interstitial edema, pneumocyte hyperplasia, and intra-alveolar infiltration of inflammatory cells. The lung injury score ranged between 0 and 10.[26]

In vitro experiments

Cell culture. Rat primary pulmonary microvascular endothelial cells were purchased from PriCells (Wuhan, Hubei, China) and cultured according to the manufacturer's protocol. Briefly, the cells were cultured in endothelial cell growth medium (PriCells) containing 10% fetal calf serum, 100 U/mL penicillin, 100 mg/mL streptomycin, and 1% endothelial cell growth factors under standard cell culture conditions (21% O_2, 5% CO_2, and 74% N_2).

Table 1. Lung injury evaluation variables.

Parameters	Score
Hemorrhage	0 or 1
Peri-bronchial infiltration	0 or 1
Interstitial edema	0 to 2
Pneumocyte hyperplasia	0 to 3
Intra-alveolar infiltration	0 to 3

Allocations of the endothelium. The normal endothelium was divided into the normoxia, H/R, AZA, or SC1 groups. The cells in the normoxia group were cultured under normal circumstances. The cells in the H/R, AZA, and SC1 groups underwent hypoxemia for 1 hour and reoxygenation for 24 hours (according to our preliminary study). The normoxia and H/R groups received phosphate-buffered saline, and cells in the AZA and SC1 groups received AZA (50 nM[7]) and SC1 (10 nM[27]), respectively.

Induction of hypoxemia and reoxygenation. All endothelial cells were cultured under normal conditions (5% CO_2, 21% O_2, and 95% N_2) with fresh fetal bovine serum. The cells in the H/R, AZA, and SC1 groups were replaced with fresh fetal bovine serum and glucose-deprived culture medium in a hypoxic chamber (5% CO_2 and 95% N_2; hypoxia chamber; BioSpherix, Lacona, NY, USA) for 1 hour. All of the cells were then washed, and the medium was replaced with normal fresh bovine serum. The cells were reoxygenated under normal conditions (5% CO_2, 21% O_2, and 95% N_2) and treated with AZA and SC1 for 24 hours.

Tube formation of the endothelium. To evaluate the function of the endothelium, approximately 2×10^4 endothelial cells were seeded onto Matrigel (50 μL/well) (BD Biosciences, Bedford, MA, USA)-coated plates at 37°C. The cells were treated with endothelial basal medium containing 0.5% fetal bovine serum. After incubation for 24 hours, the

tube networks were observed and evaluated by the length of the tube in four random fields.

Cell proliferation. The proliferation and viability of the endothelium were evaluated by cell counting kit-8 (Seven BioTech, Beijing, China) and measured according to the manufacturer's protocol. Briefly, four groups of endothelial cells at a density of 1×10^4 cells/well were plated in 96-well plates. After 1 day of incubation, 100 μL of 10% cell counting kit-8 solution was added to the 96-well plate. The absorbance of the solution was detected at 450 nm with a microplate reader (Quant Bio Tek Instruments, Winooski, VT, USA) after an incubation of 1 hour.

Apoptosis and necrosis of the endothelium. An Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (BestBio, Shanghai, China) was purchased and used to investigate the apoptotic and necrotic endothelium with flow cytometry (FACScan; Becton Dickinson, Franklin Lake, NJ, USA). The entire endothelium was washed with phosphate-buffered saline and cultured. The cells were suspended in annexin binding buffer and stained with annexin V-FITC and PI-phycoerythrin for 30 minutes in a dark room. The endothelial cells were measured by flow cytometry (Beckman Coulter, Brea, CA, USA) to distinguish the apoptotic cells (annexin V-positive and PI-negative) from necrotic cells (annexin V- and PI-positive).

Inflammation. Intercellular adhesion molecule-1 (ICAM-1) and monocyte chemotactic protein-1 (MCP-1) in the cellular medium were detected using ELISA kits (Wuhan Boster Bioengineering). Moreover, the activity and expression of NF-κB in the endothelium were detected using the Western blot and Transcription Factor Assay Kit (Abcam, Toronto, Canada).

Western blot analysis. After 24 hours of reoxygenation, the endothelium was collected for further analysis. The proteins in the endothelium were extracted as previously described.[28] Briefly, the cells were lysed and centrifuged at 12,000 × g for 15 minutes, and then the supernatant was collected. The protein concentrations were calculated by the Bradford method.[25] Equal amounts of protein from each sample were injected into the gel and transferred onto polyvinylidene fluoride membranes. The polyvinylidene fluoride membranes were blocked with 5% dry milk and then incubated with primary antibodies (NF-κB) (Cell Signaling Technology, Boston, MA, USA). After washing with phosphate-buffered saline, the membranes were incubated with horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Blots were then developed with enhanced chemiluminescence developing solutions and quantified with ImageJ software (NIH).

The *in vivo* portion of the study was replicated two times, and the *in vitro* portion was repeated eight times.

Statistical analysis. All data are presented as the mean ± standard deviation. Differences among groups were analyzed by one-sided analysis of variance and analyzed by Bonferroni correction using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA). A P value of <0.05 was considered statistically significant.

Results

5-mc and 5-hmc in lung tissue after transplantation

After 24 hours of reperfusion, 5-mc concentrations were significantly higher in the LIRI group than in the sham group (both $P < 0.001$). In contrast, 5-hmc concentrations were significantly lower in the LIRI group than in the sham group (both

$P < 0.001$) (Figure 1). This result suggested that the DNA methylation level was significantly increased, but DNA demethylation was significantly decreased after reperfusion.

Inhibition of DNA methylation improves the PaO_2/FiO_2 ratio, protein concentrations, and the wet/dry weight ratio of transplanted lungs

After reperfusion, the PaO_2/FiO_2 ratio, protein concentrations, and the wet/dry weight ratio in rats that received lung transplantation had deteriorated compared with those in the sham group (all $P < 0.05$). The AZA group showed a significantly improved PaO_2/FiO_2 ratio (Figure 2a), protein concentrations (Figure 2b), and wet/dry weight ratio compared with the LIRI group (Figure 2c) (all $P < 0.05$). The SC1 group showed a significantly worsened PaO_2/FiO_2 ratio, protein concentrations, and wet/dry weight ratio compared with the LIRI group (all $P < 0.05$).

Inhibition of DNA methylation inhibits inflammation

After lung transplantation, TNF-α, IL-1β, IL-6, and IL-10 concentrations in transplanted lungs were significantly higher than those in sham lungs (all $P < 0.05$). TNF-α, IL-1β, and IL-6 concentrations were significantly lower, but IL-10 concentrations were significantly higher, in the AZA group than in the LIRI group. The SC1 group showed a significant opposite anti-inflammatory effect to that in the AZA group (all $P < 0.05$) (Figure 3a–d).

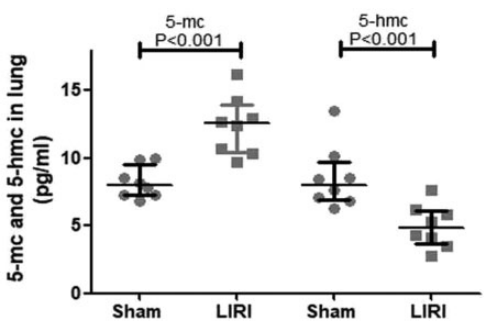

Figure 1. DNA methylation and demethylation in lung tissue. The concentrations of 5-hmC were significantly higher, but those of 5-hmC were significantly lower in the LIRI group than in the sham group. LIRI, lung ischemia–reperfusion injury.

Inhibition of DNA methylation reduces the oxidative stress response

After 24 hours of reperfusion, superoxide anion and MDA concentrations and XO activity were significantly higher in transplanted lung tissue than in sham lung tissue (all $P < 0.05$). Superoxide anion and MDA concentrations and XO activity were significantly lower in the AZA group than in the LIRI group (all $P < 0.05$). In contrast to the AZA group, the oxidative stress response was aggravated in the SC1 group compared with the LIRI group (all $P < 0.05$) (Figure 4a–c).

Inhibition of DNA methylation ameliorates lung injury

No histological injury was observed in the sham group. The rats that underwent lung transplantation showed considerable histological changes, such as severe alveolar and mesenchymal edema and infiltration of inflammatory cells, including neutrophils and macrophages, compared with the sham rats. In addition, broken alveoli, thickened alveolar walls, and hemorrhage were found in rats that received lung transplantation (Figure 5a). The histological injury score was lower in the AZA group, but higher in the SC1 group, than in the LIRI group (Figure 5b, all $P < 0.05$).

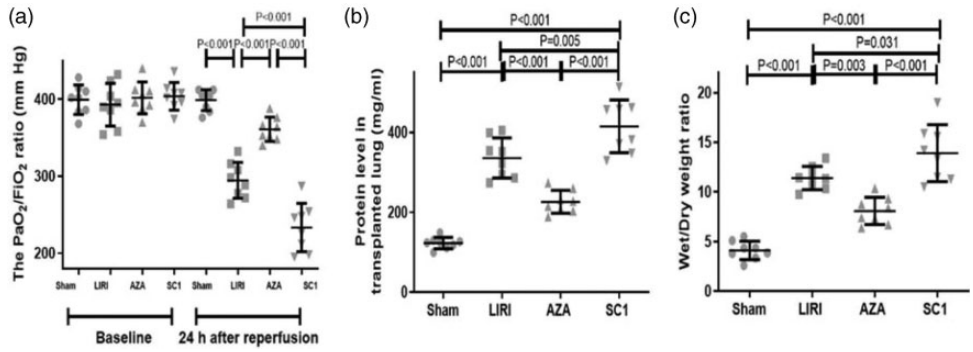

Figure 2. Inhibition of DNA methylation improves the PaO_2/FiO_2 ratio and reduces protein concentrations and the wet/dry weight ratio after lung transplantation. After 24 hours of reperfusion, the PaO_2/FiO_2 ratio was significantly lower in the LIRI group than in the sham group. However, the PaO_2/FiO_2 ratio was improved by AZA and deteriorated by SC1 (a). Protein concentrations (b) and the wet/dry weight ratio (c) were higher in the LIRI group than in the sham group, but these increases were reduced by AZA and worsened by SC1 (●, sham group; ■, LIRI group; ▲, AZA group; ▼, SC1 group).
PaO_2/FiO_2, arterial oxygen partial pressure to fractional inspired oxygen; LIRI, lung ischemia–reperfusion injury; AZA, azathioprine; SC1, pluripotin.

Inhibition of DNA methylation improves tube formation and viability of endothelial cells after reoxygenation

Tube formation and endothelial viability were significantly lower in the H/R group than in the normoxia group (both $P < 0.001$). Endothelial viability and tube formation were improved in the AZA group, but aggravated in the SC1 group, compared with the H/R group (all $P < 0.05$) (Figure 6a–c).

Inhibition of DNA methylation inhibits cell apoptosis and necrosis

After reoxygenation, the numbers of apoptotic and necrotic endothelial cells were significantly higher in the H/R group than in the normoxia group ($P < 0.05$). The ratios of apoptotic cells and necrotic cells were significantly downregulated in the AZA group and upregulated in the SC1 group compared with the H/R group (Figure 7a). These differences between the groups were significant (all $P < 0.05$), except for the apoptotic index between the H/R and SC1 groups (Figure 7b).

Inhibition of DNA methylation reduces inflammation of endothelial cells

The release of proinflammatory factors in the medium by endothelial cells was significantly induced in the H/R group compared with the normoxia group (all $P < 0.05$). ICAM-1 and MCP-1 concentrations in the medium were significantly lower in the AZA group, but higher in the SC1 group, than in the LIRI group (all $P < 0.05$) (Figure 8a). Moreover, the activity and phosphorylation of NF-κBp65 were inhibited in the AZA group, but promoted in the SC1 group, compared with the H/R group (all $P < 0.05$) (Figure 8b).

Discussion

This study suggested that DNA methylation was involved in the pathology of LIRI. The inhibition of DNA methylation by TET2 significantly attenuated LIRI by regulating inflammation and the oxidative stress response, improving endothelial cell function, and reducing endothelial cell apoptosis.

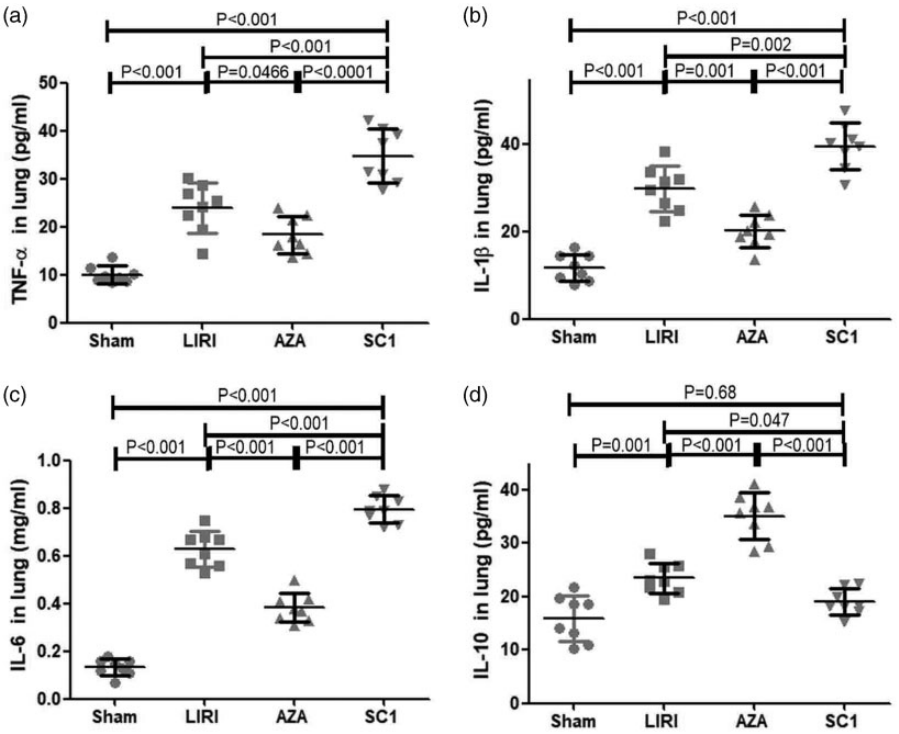

Figure 3. Inhibition of DNA methylation reduces local inflammation after lung transplantation. At 24 hours after reperfusion, TNF-α (a), IL-1β (b), IL-6 (c), and IL-10 (d) concentrations in transplanted lungs were significantly higher in the LIRI group than in sham group. TNF-α, IL-1β, and IL-6 concentrations were lower in the AZA group, but IL-10 concentrations were higher in the AZA group than in the LIRI group. SC1 showed the opposite effect to AZA on local inflammation (●, sham group; ■, LIRI group; ▲, AZA group; ▼, SC1 group).
TNF-α, tumor necrosis factor-α; IL, interleukin; LIRI, lung ischemia–reperfusion injury; AZA, azathioprine; SC1, pluripotin.

LIRI is the major complication after lung transplantation and plays a vital role in the outcome of patients. During LIRI, H/R induce an oxidative stress response and severe inflammation in the endothelium,[19,29,30] which further deteriorates endothelial function and proliferation.[31] Moreover, during the ischemic period, hypoxia triggers lung vascular remodeling and endothelial dysfunction[32,33] via activation of hypoxia-inducible factors through the promotion of histone acetylation[34] and methylation.[35] Recently, DNA methylation has also been shown to regulate cytokine expression in different organ injury models.[36–39] On the basis of the important role of inflammation in LIRI and the direct regulation of DNA methylation in inflammation, we postulated that the inhibition of DNA methylation could ameliorate LIRI.

In rat experiments, we found that 5-hmC concentrations were significantly increased, but 5-hmC concentrations were decreased, in transplanted lung tissue. This result suggested that DNA methylation was activated, and that demethylation was depressed by ischemia/reperfusion. This result is consistent with that found in previous

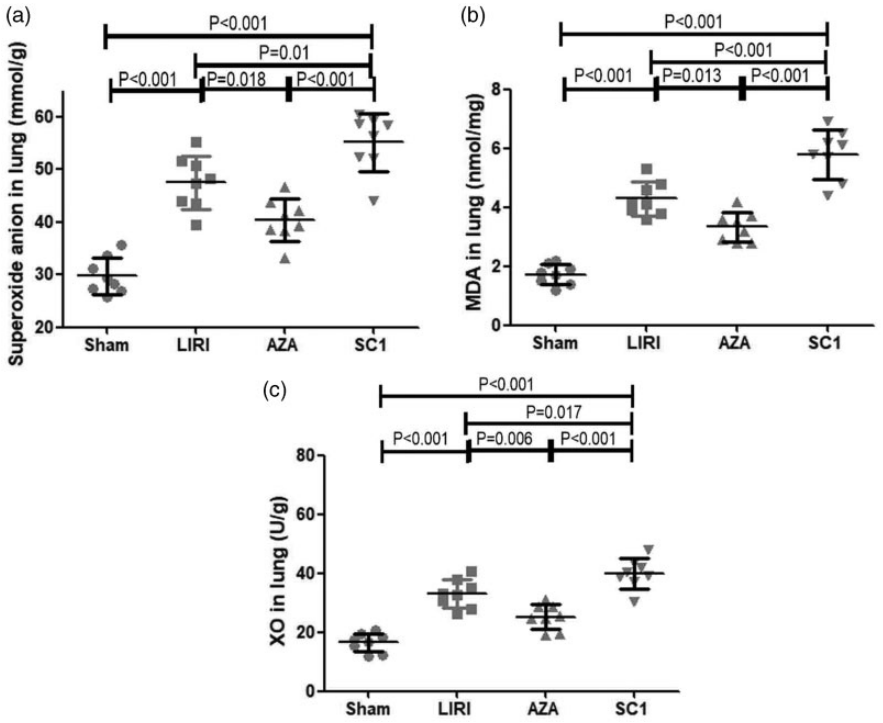

Figure 4. Inhibition of DNA methylation reduces the oxidative stress response. The concentrations of superoxide anion (a), XO (b) and MDA (c) were notably induced by reperfusion injury in the LIRI group compared with the sham group. The oxidative stress response was inhibited in the AZA group, but aggravated in the SC1 group compared with the LIRI group (●, sham group; ■, LIRI group; ▲, AZA group; ▼, SC1 group).
MDA, malondialdehyde; OX, xanthine oxidase; LIRI, lung ischemia–reperfusion injury; AZA, azathioprine; SC1, pluripotin.

studies.[12,40,41] On the basis of this result, we administered AZA and SC1 to inhibit the activity of DNMT and TET, which induce DNA methylation or demethylation, respectively, to determine whether epigenetic regulation is involved in LIRI. We found that AZA significantly mitigated LIRI and improved pulmonary function induced by lung transplantation. In contrast to AZA, the inhibition of DNA demethylation worsened LIRI. Furthermore, cytokine concentrations in lung tissue also indicated that the inhibition of DNA methylation reduced local inflammation, and that inhibition of demethylation exacerbated inflammation.

To detect the possible mechanism of this phenomenon, we cultured the endothelium and administered hypoxia and reoxygenation to mimic LIRI. We found that AZA and SC1 applied to the endothelium did not affect endothelial viability under normal conditions. After LIRI, cell viability and tube formation were significantly decreased. However, AZA and SC1 notably improved and deteriorated endothelial viability and tube formation capacity, respectively. These results suggest that DNA demethylation can protect the biological function of the endothelium after LIRI. These results are consistent with those from a previous study, which showed

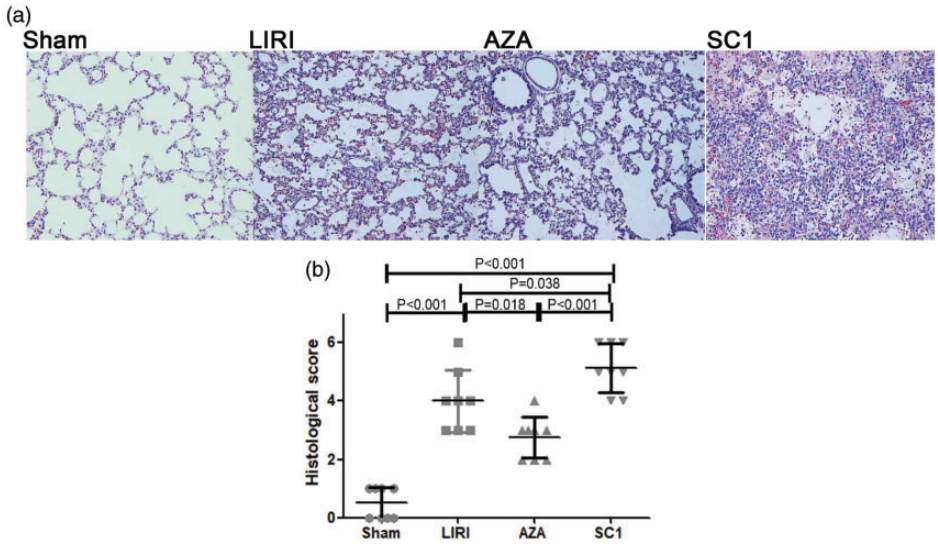

Figure 5. Inhibition of DNA methylation attenuates histological lung injury after lung transplantation. After reperfusion, typical histological changes were observed in the LIRI group, such as infiltration of inflammatory cells, edema, and thickening and breakage of alveoli. Pathological injury was alleviated in the AZA group, but aggravated in the SC1 group compared with the LIRI group (a) and The histological injury score represents the protective effect of DNA methylation on lung injury (b) (●, sham group; ■, LIRI group; ▲, AZA group; ▼, SC1 group).
LIRI, lung ischemia–reperfusion injury; AZA, azathioprine; SC1, pluripotin.

endothelial cell protection through preventing DNA methylation in the LIRI model.[42]

In addition to endothelial function, we also investigated DNA methylation in endothelial injury after LIRI. After reoxygenation, apoptosis and necrosis were significantly higher in the LIRI group than in the sham group. Apoptosis and necrosis of the endothelium play crucial roles in the pathology of LIRI. The number of apoptotic and necrotic cells directly determines the function of transplanted lungs and patients' outcomes.[43,44] Apoptosis and necrosis were significantly reduced by AZA and worsened by SC1. These results suggest that DNA methylation is a damaging factor for LIRI, and that DNA demethylation is a protective factor for LIRI. We speculate that the protective effect of AZA on LIRI may be attributed to its anti-inflammatory and antioxidative effects.[45]

During LIRI, because of a lack of oxygen, anaerobic metabolism directly affects the production of adenosine triphosphate and releases superoxide anions. This process mainly occurs in the mitochondria and results in endothelial cell injury and increased permeability of the endothelium under the activation of XO.[46] Superoxide anion not only directly causes endothelial injury, but also activates the intrinsic apoptosis pathway. In this study, we found that AZA reduced the release of superoxide anions and XO activity after LIRI. This inhibition of the oxidative stress response by AZA may be due to the protection of DNA demethylation in the mitochondria.[47] The product of DNA demethylation by TET (5-hmC) can localize in the mitochondria,[24] protect the permeability of the mitochondrial membrane, and reduce the release[47] of superoxide anions.

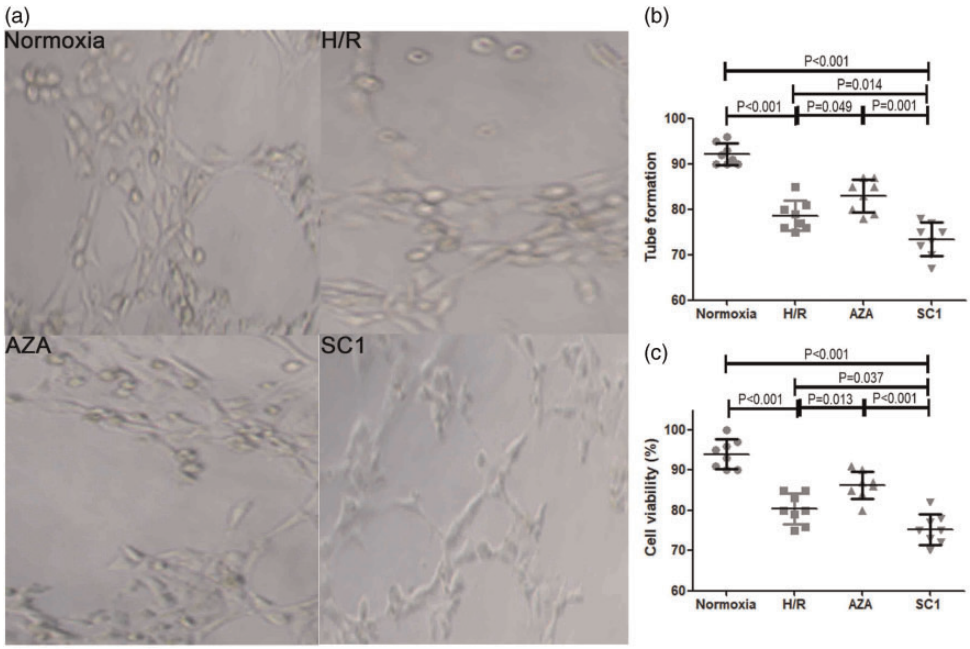

Figure 6. Inhibition of DNA methylation aggravates tube formation capacity and viability of the endothelium after reoxygenation. After reoxygenation, the tube formation capacity of the endothelium (a) was reduced and improved by AZA. However, SC1 deteriorated tube formation. The viability and tube formation capacity scores are shown (b, c) (●, normoxia group; ■, H/R group; ▲, AZA group; ▼, SC1 group) H/R, hypoxemia and reoxygenation; LIRI, lung ischemia–reperfusion injury; AZA, azathioprine; SC1, pluripotin.

During LIRI, the oxidative stress response activates NF-kB signaling in the endothelium,[48,49] leading to the release of chemokines from the endothelium after reoxygenation.[50,51] The chemokines ICAM-1 and MCP-1 further recruit inflammatory cells into the injured tissue, resulting in local inflammation. In this study, we found that the inhibition of methylation by AZA significantly reduced inflammatory factors in lung tissue, as well as the chemokines ICAM-1 and MCP-1, from the endothelium, but promoted the anti-inflammatory factor IL-10. This effect may be due to the inhibition of NF-κB. During cellular sectioning, the activity and phosphorylation of NF-κB were upregulated by H/R, and the upregulation of NF-κB was inhibited by AZA and exacerbated by SC1. These results indicated that DNA methylation aggravated the activation of NF-κB, and that inhibition of DNA methylation inhibited the activity and phosphorylation of NF-κB.[52,53]

Limitations

Although we found that AZA inhibited DNA methylation and reduced LIRI, we did not investigate the exact mechanism of DNA methylation in LIRI. DNMT and TET are important modulators in epigenetic regulation. In our future research, we will further investigate the role and mechanism of DNMT and TET in LIRI. Additionally, in this study, we did not observe an effect of AZA on inflammatory cells, such as macrophages and T cells, during LIRI. Finally, the results of this study were preliminary

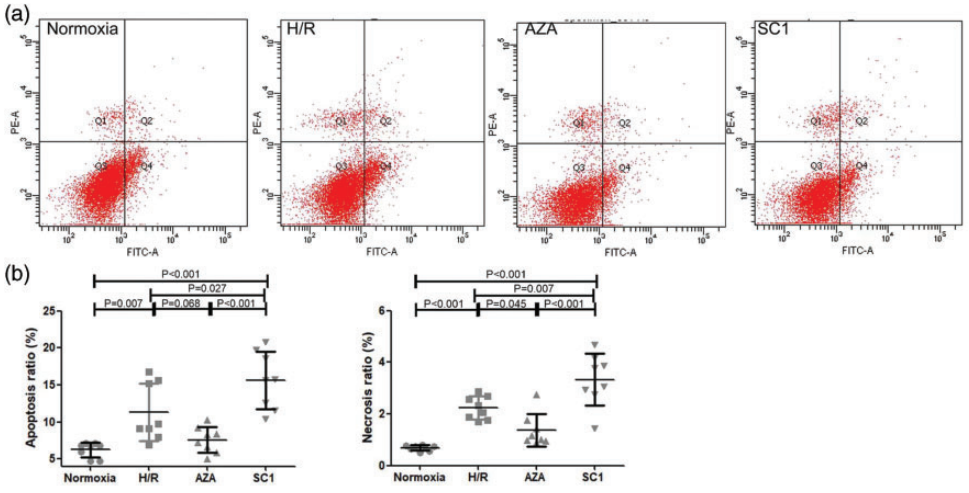

Figure 7. Inhibition of DNA methylation increases necrosis and apoptosis of the endothelium after reoxygenation. After reoxygenation, there was substantial endothelial necrosis (upper left quadrant) and apoptosis (right lower quadrant) (a). Necrosis and apoptosis of the endothelium were reduced by AZA, but worsened by SC1 compared with H/R. However, the difference in the apoptosis ratio between the H/R and SC1 groups was not significant (b and c).
FITC, fluorescein isothiocyanate; H/R, hypoxemia and reoxygenation; LIRI, lung ischemia–reperfusion injury; AZA, azathioprine; SC1, pluripotin.

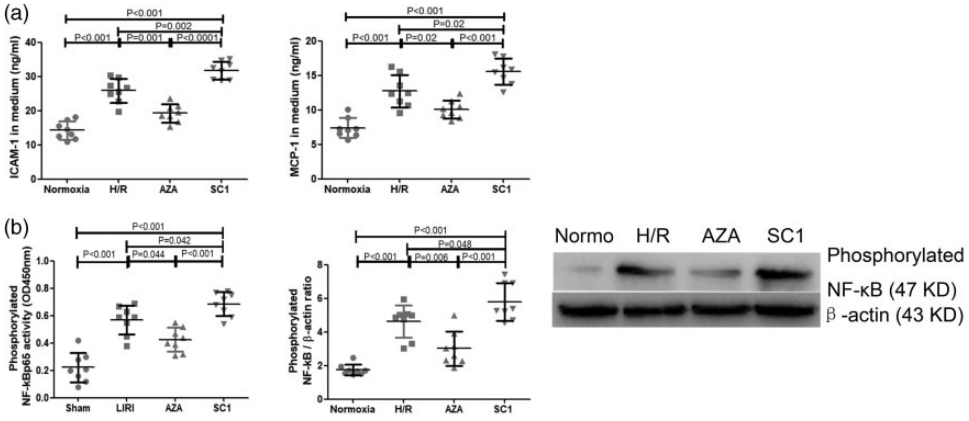

Figure 8. Effect of DNA methylation on endothelial inflammation. After 24 hours of reoxygenation, ICAM-1 and MCP-1 concentrations were significantly upregulated in the cellular medium. ICAM-1 and MCP-1 concentrations were decreased by AZA, but increased by SC1 (a). AZA also notably downregulated the activity and phosphorylation of NF-κB after reoxygenation (b) (●, normoxia group; ■, H/R group; ▲, AZA group; ▼, SC1 group).
ICAM-1, intercellular adhesion molecule-1; H/R, hypoxemia and reoxygenation; LIRI, lung ischemia–reperfusion injury; AZA, azathioprine; SC1, pluripotin; MCP-1, monocyte chemotactic protein-1; NF-κB, nuclear factor-κB.

and require further validation in future studies. In the next study, we will co-culture inflammatory cells and endothelial cells, and administer AZA and SC1 to detect their effect and mechanism of DNA methylation on inflammatory cells to further examine the possible mechanism of DNA methylation in LIRI.

Conclusion

This study shows that DNA methylation is involved in LIRI after lung transplantation. Our results suggest that the inhibition of DNA methylation can attenuate histological injury, improve pulmonary function, reduce endothelial apoptosis, and balance inflammation and the oxidative stress response.

Author Contributions

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Declaration of conflicting interests

The authors declare that there is no conflict of interest.

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Availability of data and materials

The datasets used and/or analyzed during the study are available from the corresponding author on reasonable request.

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