

## The effects of anti-Fas ribozyme on T lymphocyte apoptosis in mice model with chronic obstructive pulmonary disease

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### ABSTRACT

**Objective(s):** In this study, we aimed to investigate the effects of anti-Fas ribozyme on the apoptosis of T lymphocytes (T cells) in mice model with chronic obstructive pulmonary disease (COPD).

**Materials and Methods:** Male 6-week-old C57BL/6 mice were used to establish the COPD model by exposure to cigarette smoke. The COPD mice were sacrificed for spleen dissection and T cell isolation. T cells were randomly divided into four groups (n=10 per group). Group A was used as the control. B, C, and D groups were transfected with empty lentivirus, anti-Fas ribozyme, and an anti-Fas ribozyme mutant, respectively. The expression of Fas mRNA and protein in the T cells were evaluated using qPCR and Western blot, respectively. Flow cytometry was used to evaluate the apoptosis of CD<sup>4+</sup> T cells and calculate the ratio of CD<sup>4+</sup> to CD<sup>8+</sup> T cells (CD<sup>4+</sup>/CD<sup>8+</sup>).

**Results:** Anti-Fas ribozyme significantly inhibited the expression of Fas in the T cells of COPD mice. In addition, the number of apoptotic CD<sup>4+</sup> T cells and CD<sup>4+</sup>/CD<sup>8+</sup> of the C and D groups were significantly lower and higher than those of group A, respectively ( $P<0.05$ ). The apoptotic CD<sup>4+</sup> T cells and CD<sup>4+</sup>/CD<sup>8+</sup> of the C group were significantly lower and higher than those of group D, respectively ( $P<0.05$ ).

**Conclusion:** Anti-Fas ribozyme significantly inhibited the expression of Fas, increased CD<sup>4+</sup>/CD<sup>8+</sup>, and inhibited the apoptosis of T cells in COPD mice.

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### Introduction

Chronic obstructive pulmonary disease (COPD) is a chronic respiratory disease characterized by persistent airflow limitation (1, 2). Abnormal inflammatory responses to polluted air or harmful particles are associated with its development, which necessitates long-term treatment (3). The incidence of COPD is increasing, however its pathogenesis is still not fully understood. Airway inflammation, protease-antiprotease imbalance, oxidation-antioxidation imbalance, over secretion of airway mucus, small airway obstruction, persistent inflammation of small pulmonary vasculature, non-bacterial infections, and family genetic inheritance, have all been reported to be involved in the development of COPD (4). Of these, persistent airway inflammation is the most important factor contributing to COPD pathogenesis (5). As a chronic airway inflammatory disease, COPD is characterized by the accumulation of inflammatory cells in large and small airways, lung tissues, and pulmonary blood vessels, which secrete a number of cytokines in order to activate the inflammatory response, resulting in respiratory damage (6). T cells are important inflammatory cells and are activated by

macrophages by means of antigens presentation. Mature T cells can be divided into two subsets according to surface CD molecules, CD<sup>4+</sup> and CD<sup>8+</sup>. CD<sup>4+</sup> T cells are helper T cells and induce antibody production by B cells, as well as hypersensitive reactions by macrophages. CD<sup>8+</sup> T cells are cytotoxic cells, which recognize antigen peptides presented by class I major histocompatibility complex (MHC) (7-9). T cells can secrete a variety of inflammatory cytokines to activate airway epithelial cells and recruited more inflammatory cells, including neutrophils, macrophages, and eosinophils, to lung tissue (10-12). Finkelstein, *et al.* found this activation and subsequent airway infiltration of lymphocytes is one of the most important histological features of COPD (13). Therefore, T cells play an important role in COPD pathogenesis.

Fas, which is also known as Apo-1 or CD95, is an important factor involved in apoptosis (14). It is widely expressed in various cells, but particularly on the surface of immune cells. Increased Fas expression has been observed on activated T cells and binding of the anti-Fas antibody has been associated with Fas-induced apoptosis (15). Therefore, Fas has been nicknamed the "death receptor". Under normal

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conditions, Fas and Fas ligand (FasL) production can be induced in most tissues, which not only has a cytotoxic effect on Fas-targeted cells, but also clears over-activated immune cells, thereby down-regulating the immune response and maintaining host immune homeostasis (16). Recent studies have shown that down-regulation of Fas on activated T cells inhibited apoptosis and improved the anti-tumor ability of the host (17, 18). Numerous methods have been established to reduce Fas expression in cells, including ribozymes, antisense RNA, small RNA interference, anti-Fas antibodies, ligands, and soluble receptors (19, 20). Of these methods, ribozymes exhibit the most significant activities on gene regulation and have therefore been widely used in gene therapy. Ribozyme is an RNA molecule with endonuclease activity, which specifically cleaves its target RNA, in order to inhibit gene expression (21). Currently, six types of ribozymes have been identified and characterized, including group I intron (21), group II intron (22), RNase P (23), hammerhead (24), hairpin (25), and axotactic ribozymes. Structurally, all types can be classified into two categories: hammerhead and hairpin ribozymes. Hammerhead ribozymes are widely used in the field of gene therapy because of their simple structure, small molecular size, easy artificial design, and low cutting site restriction. Animal models are used to study COPD, to investigate the basic mechanisms of COPD, mice have been proven to be the best choice as animal models of COPD (26, 27).

In the present study, we designed anti-Fas ribozymes based on their homology and secondary structure. The anti-Fas ribozymes were transfected into T cells obtained from the COPD mice model, induced by cigarette smoke exposure, and study the effects of anti-Fas ribozyme on Fas expression in T cells and Fas-mediated T cells apoptosis.

## Materials and Methods

### Ethical statement

The study was approved by the ethic committee of Zunyi Medical University. All the animal procedures were strictly adhered to the guidelines for the care and use of laboratory animals of Zunyi Medical University.

### Mice model of COPD

Sixty male specific-pathogen-free (SPF) C57BL/6 mice (6 weeks of age, average weight of  $22.1 \pm 1.7$  g) were used to establish the COPD model. Mice were provided by the Experimental Animal Center, Zhuhai Campus of Zunyi Medical College (Guizhou, China) and raised at 24-26 °C with 60%-70% humidity for two weeks of adaptive feeding. The COPD model was established by exposure to cigarette smoke, according to previous studies (28, 29). Briefly, the control mice were placed in the same container without cigarette

smoke exposure. The smoking container (72 cm×44 cm×52 cm) had upper and lower compartments, which were separated by a clapboard with six holes (1.5 cm of diameter). Cigarettes were burned in the lower compartment to produce smoke, which diffused through the holes to the upper compartment, where the mice were situated. Six of Yangcheng brand cigarettes were used for smoke exposure for 30-40 min. Each cigarette contained 1 mg nicotine, 11 mg tar, and 13 mg carbon monoxide. After six cigarettes had been smoked, the container was left open for 10 min to allow the dissipation of the remaining smoke. The mice then experienced secondhand smoking for an additional 6 cigarettes. Cigarette smoking exposure was conducted for 12 weeks, six days per week, and twice per day. A total of 50 mice were used for the COPD model, but seven died during the model establishment. Finally, 40 mice were randomly selected for the follow-up experiments and 3 were selected for the preliminary experiment.

### Evaluation of the COPD mice model

The COPD mice model was evaluated based on function and histopathological changes observed in the airways and lungs. After intraperitoneal injection of 10% chloral hydrate (3 ml/kg body weight), the skin in front of trachea was shaved and disinfected for tracheotomy and intubation. Mouse pulmonary function tests, including peak expiratory flow (PEF), airway resistance (RI), and dynamic compliance (C<sub>dyn</sub>), were conducted according to the BUXCO small animal pulmonary function system (Buxco, USA). Animals were sacrificed to dissect lungs and airways, which were fixed in 10% formaldehyde for 24 hr. Lung tissues were dehydrated, embedded in dip wax, sectioned, stained with hematoxylin and eosin (HE), and examined under light microscopy.

### Design of anti-Fas ribozyme

The anti-Fas ribozyme cleavage site on mouse Fas mRNA was targeted on the GUA codon (596-598). The sequences of the anti-Fas ribozyme, RZ596, were A5'TCTAGAGATATATAAACTGATGAGTCCGTCCTGAG-GACGACGAAACAAGTGGATCC-3' and B5'-GGATCCAC-TTGTTCGTCCTCACGGACTCATCAGTTTATATATCTCT-A GA-3'. An anti-Fas ribozyme mutant, in which the G nucleotide following the GUA cleavage site was replaced by an A, was also designed and produced (dRZ596). The sequences were cloned into the prokaryotic vector pBSKU6 between the *Xba* I and *Bam*H I cleavage sites and were confirmed by DNA sequencing. The vectors, U6-RZ596 and U6-dRZ596, inserted with RZ596 and dRZ596, respectively, were used as templates to amplify the U6 promoter and the fragment of anti-Fas ribozyme, which were inserted into the *Mlu* I cleavage site of the green GFP vector pEGFPC1 to produce pU6-RZ596 and pU6-dRZ596 vectors. The vectors were packaged into lentiviruses

for follow-up experiments and the virus titer was set to be  $1.5 \times 10^8$  TU/ml.

### **T cell isolation and cultivation**

Mice were sacrificed under anesthesia for spleen dissection and were soaked in alcohol for 10 min and fixed on a board. Under sterile conditions, the abdominal cavities were opened to dissect spleens. The dissected spleens were washed with PBS and adipose tissues were removed in a Petri dish. Spleens were then cut into 1-2 mm<sup>3</sup> pieces, mixed with PBS, and centrifugated at 1500 g for 20 min. After the supernatant was removed, trypsin at a measurement of 3 times the spleen volume was added to digest spleen at room temperature for 20-30 min with vibration. After centrifugation at 1500 g for 5 min, the cloudy materials were aspirated and RPMI1640 was added to the digested spleen tissue, which was again centrifuged at 1500 g for 5 min. The cell pellet was then resuspended in RPMI1640. After the addition of lymphocyte separation medium (3 ml), the solution was centrifugated at 1500 g for 20 min, the transparent supernatant was removed, and cells were resuspended in RPMI1640. To prepare the lymphocyte separation column, nylon wool was boiled in ultra-pure water six times (20 min each time), dried in an oven to the single fiber state, assembled in a 10 ml syringe (2/3 of the syringe volume), and sterilized with high temperature and pressure. The cell suspension was run through the lymphocyte separation column (1 drop/45 sec) to collect T cells, which were suspended in RPMI1640 with IL-2 (100 U/ml), 20% fetal bovine serum, and conA (2.5 µg/ml). Harvested T cells were cultured under standard conditions for 3-4 days and counted under microscopy. The concentration of T cells ( $1-1.5 \times 10^7$ ) was determined using flow cytometry.

### **Experimental grouping and T cell transfection**

T cells were randomly divided into four groups (n=10, A, B, C, and D). T cells from group A did not receive the anti-Fas ribozyme transfection, those from group B were treated with the empty vector, those from group C were transfected with anti-Fas ribozymes, and those from group D were transfected with the anti-Fas ribozyme mutant. T cells transfected with anti-Fas ribozyme ribozyme was performed according the previous study with minor modification (30). Briefly, the harvested T cells were cultured for 3-4 days and the cell concentration was set to be  $1 \times 10^6$ /ml before transfection. T cells (1 ml) from mice in each group were inoculated onto 12-well culture plates and cultured for 24 hr. Transfection reagent (0.5 ml), anti-Fas ribozyme (0.5 ml), and anti-Fas ribozyme mutant (0.5 ml), were added to the B, C, and D group cells, respectively (multiplicity of infection = 50) and cells were incubated in the dark for 8-12 hr. The cells were centrifuged at 1500 g for 10 min and the cell pellets were

re-suspended and transferred onto a 96-well plate for culture. The cells were examined under fluorescence microscopy to count transfected cells and calculate the transfection rate according to the following formula: rate of transfection (%) = number of transfected cells / total number of cells.

### **Real-time quantitative PCR (qPCR)**

Total RNA was isolated from the T cells of each group using Trizol reagent and DNase I (20 µl) was added to the RNA to remove genomic DNA. Isolated RNA (2 µl) was separated in an agarose gel by electrophoresis and the OD<sub>260</sub>/OD<sub>280</sub> was determined. cDNA was synthesized using a TaKaRa reverse transcription kit (TaKaRa, Japan). The primers used for qPCR were Fas forward: 5'-GCTGCAGACATGCTGTGGATC-3', Fas reverse: 5'-TCACAGCCAGGAGAATCGCAG-3', internal control β-actin forward: 5'-ATCTTCAAACCTCCATGATG-3', and internal control β-actin reverse: 5'-ACCCCACTGAAAAAGATGA-3'. The qPCR reaction system contained 10 µl SYBR® Premix Ex Taq™ (Tli RNaseH Plus, Takara, Japan), 5 µl cDNA, 0.5 µl forward primer, 0.5 µl reverse primer, and 4 µl ddH<sub>2</sub>O. qPCR was conducted in a ViiA7 real-time PCR machine (Life Technologies, Carlsbad, CA) according to the following conditions: pre-denaturation at 95 °C for 30 sec, 45 cycles of 95 °C for 30 sec, and extension at 60 °C for 34 sec. The relative quantity of Fas mRNA was determined according to the 2<sup>-ΔΔC<sub>T</sub></sup> method.

### **Western blotting**

Total proteins were isolated from T cells and quantified using the BCA method. After being separated on 10% SDS-PAGE gel by electrophoresis, proteins were transferred to a nylon membrane and incubated with the first antibody (rabbit anti-rat PDGFR-α, 1:1000) overnight at room temperature for 10 min at 4 °C. The second day, the membrane was rinsed and incubated with rabbit anti-rat PDGF-C (1:1000) at room temperature for 10 min at 4 °C overnight. It was then incubated with the second antibody (1:5000) at room temperature for 90 min. The film was developed and the images were analyzed using Image J analysis software to calculate the relative expression of Fas protein.

### **Flow cytometry**

Forty-eight hr after the transfection, T cells were transferred into a centrifuge tube and suspended in PBS. After centrifugation at 1000 g for 5 min, Annexin V-PE (5 µl) and Annexin V-PE binding buffer (195 µl) were mixed with the cells and incubated at room temperature for 10 min. Cells were centrifuged again at 1000 g for 5 min, 7-AAD (5 µl) and Annexin V-PE binding buffer (190 µl) was added. Cells were then incubated in the dark in an ice bath before the flow cytometry assay. To determine the CD<sup>4+</sup>/CD<sup>8+</sup>, the T cells were centrifuged at 1000 g for 5 min. The cell pellet was suspended in PBS, mixed with FITC-labeled CD<sub>4</sub> antibody, and incubated in the dark for 30 min. Then, cells were mixed with PE-labeled CD<sub>8</sub> antibody

**Table 1.** Comparison of pulmonary functions between the control mice and COPD mice

Group	RI (cmH <sub>2</sub> O • mL <sup>-1</sup> • sec <sup>-1</sup> )	Cdyn (ml/cmH <sub>2</sub> O)	PEF (ml/sec)
Control	0.54±0.02	0.06±0.05	4.29±0.49
COPD	1.07±0.10*	0.03±0.01*	0.99±0.01*

Notes: RI: Airway resistance; Cdyn: Dynamic lung compliance; PEF: Peak expiratory flow. \* $P < 0.05$ , significantly difference compared with the control group

and incubated for 30 min. After being rinsed twice with PBS, cells were analyzed by flow cytometry.

### Statistical analyses

The data were analyzed using SPSS software 20.0. Quantitative data were presented as mean ± standard deviation. One-way ANOVA was used for statistical analyses and the LSD method was used to compare two groups.  $P$ -value  $< 0.05$  was considered statistically significant.

## Results

### Activity and pulmonary function changes in mice model with COPD mice

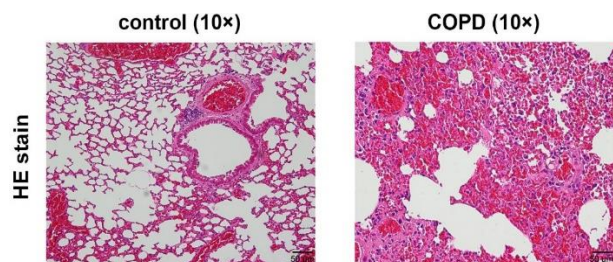
In the first several days of exposure to cigarette smoke, the mice became more active and irritable. In the last several days, they experienced shortness of breath and apathy. Compared with the control mice, COPD mice had lower body weights and their fur became yellow and lost its luster. Based on pulmonary function tests, the COPD mice exhibited lower dynamic lung compliance (Cdyn, ml/cmH<sub>2</sub>O) (0.06±0.05 vs. 0.03±0.01) and peak expiratory flow (PEF, ml/sec) (4.29±0.49 vs. 0.99±0.01), and higher airway resistance (RI, cmH<sub>2</sub>O • mL<sup>-1</sup> • s<sup>-1</sup>) (0.54±0.02 vs. 1.07±0.10) compared with the control mice (Table 1,  $P < 0.05$ ), suggesting irreversible airway obstruction and COPD in the exposed mice.

### Histopathological changes of lung tissues

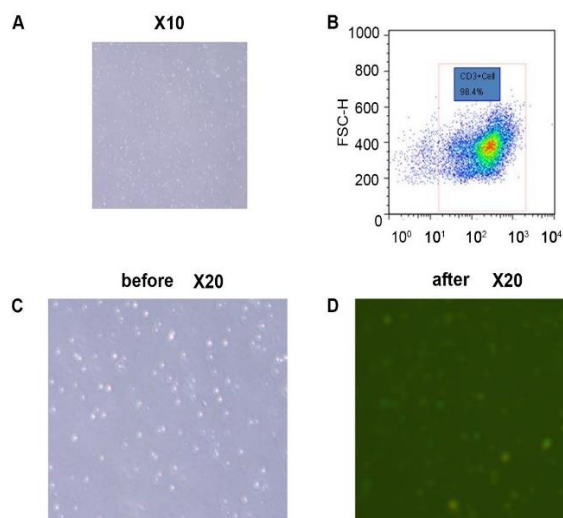
As shown in Figure 1, the histopathological of control mice lung tissues exhibited normal airway and vascular endothelial structures. However, we observed alveolar septal rupture and inflammatory cell infiltration in the lung tissues of COPD mice. In addition, the vascular endothelium was damaged with smooth muscle hyperplasia. We also observed disorder and loss of airway epithelia, increased goblet cells, and alveolar expansion and fusion to form cysts, symptoms that are characteristic of emphysema. These histopathological changes suggest that the COPD model was successfully established in mice.

### Efficient transfection of T cells by lentivirus-packaged anti-Fas ribozyme

Isolated T cells were counted under a microscope on a cell counting plate, and the purity of the isolated T cells was measured with flow cytometry. Figure 2A shows the T cells under a microscope, and Figure 2B shows their purity (98.4%). These results suggest that



**Figure 1.** Histopathological changes of lung tissues in control mice and COPD mice. Magnification: 10×; Scale bar=50 μm



**Figure 2.** (A) Isolated T cells examined under microscopy (×10); (B) the purity (98.4%) of T cells was measured by flow cytometry; (C) before the transfection, the T cells were examined under microscopy (magnification: ×20); (D) 72 hr after the transfection, the T cells were examined under microscopy (magnification: ×20)

lentivirus-packaged anti-Fas ribozyme can effectively transfect mouse T cells. The B, C, and D COPD groups received transfections of empty vector, anti-Fas ribozyme, and anti-Fas ribozyme mutant, respectively. 72 hr after the transfection, the T cells were examined under a microscope and the transfection rate was about 40% (Figure 2C and Figure 2D).

### Anti-Fas ribozyme significantly reduced Fas mRNA and protein levels in COPD mice T cells

As shown in Figure 3A and Table 2, no significant difference was found in Fas mRNA levels between groups A and B ( $P > 0.05$ ) based on qPCR assays. Compared with group A, the Fas mRNA levels in groups C and D were significantly lower ( $P < 0.05$ ), and the Fas mRNA level in group C was significantly lower than that of the D group ( $P < 0.05$ ). Western blotting showed that there was no significant difference in the Fas protein levels between groups A and B ( $P > 0.05$ ). Compared with group A, the Fas protein levels in groups C and D were significantly lower ( $P < 0.05$ ), and the Fas protein level in group C was significantly lower than that of group D ( $P < 0.05$ ) (Figure 3B).

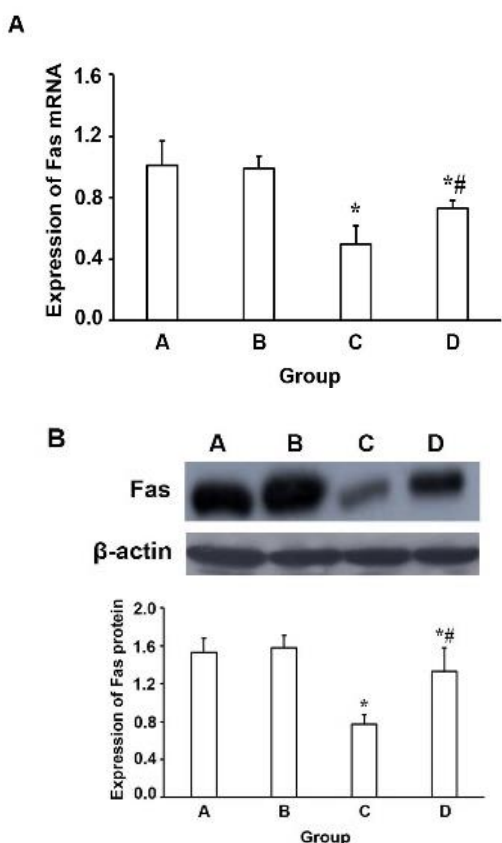
**Table 2.** Comparison of the Fas mRNA level in T cells between four groups

Group	Relative level of Fas mRNA
A (COPD mice)	1.01±0.16
B (Empty vector transfection)	0.99±0.08
C (Anti-Fas ribozyme transfection)	0.50±0.12*
D (Anti-Fas ribozyme mutant transfection)	0.73±0.05*#

Notes: \**P*<0.05, significantly difference when compared with group A; #*P*<0.05, significantly difference when compared with group C

**Anti-Fas ribozyme significantly increased the CD4<sup>+</sup>T/CD8<sup>+</sup> T cell ratio in COPD mice**

We determined the apoptosis of CD4<sup>+</sup> T cells using flow cytometry and calculated CD4<sup>+</sup>/CD8<sup>+</sup>. As shown in Figure 4 and Table 3, no significant differences were found between groups A and B in the apoptosis of CD4<sup>+</sup> T cells or CD4<sup>+</sup>/CD8<sup>+</sup> (*P*>0.05). Compared with group A, the C and D groups exhibited significantly lower CD4<sup>+</sup> T cell apoptosis rates and significantly higher CD4<sup>+</sup>/CD8<sup>+</sup> (*P*<0.05). In addition, group C exhibited a significantly lower CD4<sup>+</sup> T cell apoptosis rate and a significantly higher CD4<sup>+</sup>/CD8<sup>+</sup> than group D (*P*<0.05).

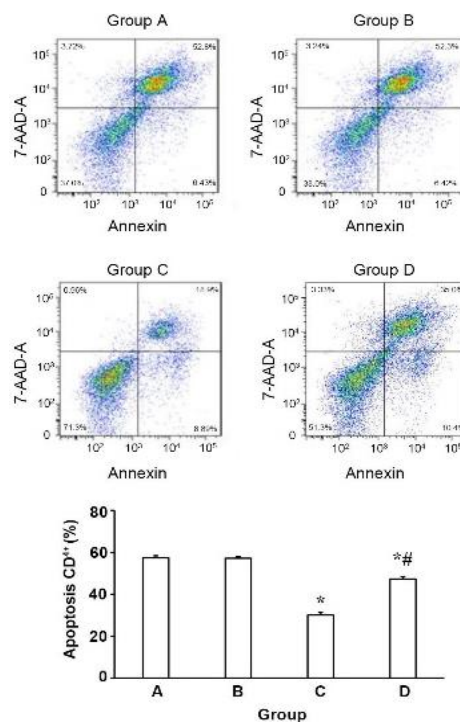


**Figure 3.** (A) The expression of Fas mRNA in each group were detected by real-time quantitative PCR; (B) The expression of Fas protein in each group were detected by western blotting and bar chart of the quantity was observed in western blotting. \**P*<0.05 vs. group A and B; #*P*<0.05 vs. group C

**Table 3.** The ratio of CD4<sup>+</sup> to CD8<sup>+</sup> T cells (CD4<sup>+</sup>/CD8<sup>+</sup>) in four groups

Group	CD4 <sup>+</sup> /CD8 <sup>+</sup>
A (COPD mice)	0.52±0.04
B (Empty vector transfection)	0.55±0.07
C (Anti-Fas ribozyme transfection)	0.94±0.07*
D (Anti-Fas ribozyme mutant transfection)	0.65±0.07*#

Notes: \**P*<0.05, significantly difference when compared with group A; #*P*<0.05, significantly difference when compared with group C



**Figure 4.** Flow cytometry for the apoptosis of CD4<sup>+</sup> T cells in each group. \**P*<0.05 vs. group A and B; #*P*<0.05 vs. group C

**Discussion**

In the present study, we successfully established a mouse COPD model by exposure to passive smoke, which is a widely used method in COPD research with animal models, due to the resulting persistent inflammation in the airway and lung tissues (31, 32). Based on pulmonary function tests and histopathological examination of lung tissues, the COPD model was successfully established, and was then used to investigate the effects of ribozymes on the inhibition of Fas expression in T cells from COPD mice. In addition, we designed an anti-Fas ribozyme based analysis of Fas protein homology and secondary structures, and transfected anti-Fas ribozymes into T cells isolated from COPD mice using the lentiviral transfection method, in order to investigate its effect on T cell apoptosis. Our results showed no significant effects of the transfection of empty vectors on either T cell Fas expression or apoptosis in COPD mice. In addition, the CD4<sup>+</sup>/CD8<sup>+</sup> was not affected by the transfection of empty vectors. However, anti-Fas

ribozymes reduced the levels of Fas mRNA and protein, inhibited T cell apoptosis, and increased the CD<sup>4+</sup>/CD<sup>8+</sup> isolated from COPD mice, suggesting that anti-Fas ribozymes may be useful for COPD treatment. Our results also suggest that Fas is involved in the apoptosis of CD<sup>4+</sup> T cells and that reducing Fas expression can inhibit the Fas/FasL-mediated apoptosis pathway. In addition, anti-Fas ribozymes can inhibit T cell apoptosis and inflammation, which is important for the treatment of COPD. We also found that anti-Fas ribozyme was more effective than the ribozyme mutant for inhibiting Fas expression. While both anti-Fas ribozyme and its mutant specifically bound with target mRNA, the anti-Fas ribozyme mutant exhibited significantly lower cleavage efficacy than anti-Fas ribozyme, due to the mutation of the third nucleotide on the cleavage site, suggesting that a slight change in the catalytic center leads to a significant reduction of anti-Fas ribosome activity. Anti-Fas ribozyme can potentially be used for the treatment of COPD because it not only binds specifically with, but also efficiently degrades Fas mRNA.

T cells are one of the most important immune cells in the body (33). Normally, the proliferation and apoptosis of immune cells play essential roles in host defense and surveillance. Previous studies have reported that T cell infiltration into the airway and lung parenchymal tissue are closely associated with the development of COPD (33). Our preliminary results showed a reduced number of CD<sup>4+</sup> T cells due to apoptosis in COPD patients of both stable and acute exacerbation stages, leading to the inhibition of various T cell functions, such as lymphokine production, inducing antibody production by B cells, and activating other lymphocytes (34). Therefore, consistent cellular immune dysfunction may cause repeated infections in COPD patients. In addition, inhibited CD<sup>8+</sup> T cell apoptosis led to a reduced CD<sup>4+</sup>/CD<sup>8+</sup>, which disrupts the balance between the proliferation and apoptosis of immune cells, delays healing of chronic inflammation, and causes a poorer COPD prognosis (35). Increased Fas expression was associated with the apoptosis of CD<sup>4+</sup> cells, suggesting that Fas activation played an essential role in the apoptosis of CD<sup>4+</sup> T cells.

### Conclusion

Taken together, anti-Fas ribozyme can effectively inhibit Fas expression in CD<sup>4+</sup> T cells in COPD mice, reducing the number of CD<sup>4+</sup> T cells, increasing CD<sup>4+</sup>/CD<sup>8+</sup>, and improving inflammatory responses in COPD.

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