

Small Interfering RNA Targeting T-cell Ig Mucin-3 Decreases Allergic Airway Inflammation and Hyperresponsiveness

Xiao-Xia Lu,^{1,4} Karen S. McCoy,² Jia-Li Xu,¹ Wei-Kun Hu,³ and He-Bin Chen¹

Abstract—Since CD4⁺ T cells play a pivotal role in the development of airway inflammation and hyperresponsiveness, targeting activated CD4⁺ T cell subsets and increasing the cells with regulatory function would be a logical therapeutic approach. We showed that this outcome can be achieved by local therapy with Tim-3, which is a negative regulator of CD4⁺ T cells. Tim-3 expression was up-regulated by ovalbumin (OVA) induction. Attenuating Tim-3 expression by RNA interference suppressed allergen-induced immune responses. Intranasal application of Tim-3 shRNA diminished airway inflammation and hyperresponsiveness. Multiple mechanisms were involved in the inhibitory effects, including regulation the imbalance of Th1/Th17 and increasing Treg cell expression. Our results indicate that the Tim-3 pathway is highly involved in the regulation of asthma. Targeting Tim-3 by siRNA may hold therapeutic potential in preventing the development of allergic asthma.

KEY WORDS: asthma; Tim-3; CD4⁺ T cell; siRNA.

INTRODUCTION

Asthma is a complex syndrome characterized by airway inflammation, a variable degree of airflow obstruction airway remodeling and airway hyperresponsiveness (AHR) [1]. The pulmonary infiltrates of asthmatic patients consist of eosinophils, mast cells, and T lymphocytes, which orchestrate allergen-specific immune responses. Among these, CD4⁺ T cells play critical roles in mediating asthmatic airway inflammation [2, 3].

Activation of CD4⁺ T cells produces antigen-specific T helper (Th) 1, Th2, Th17, and T regulatory (Treg) cells [4, 5]. Previous studies have reported that Th2 cells and imbalance of Th1/Th2 expression play central roles in the pathogenesis of asthma [6, 7]. However, the Th1/Th2 imbalance does not fully explain the aetiology of asthma. Increasing evidences showed that Th17 cells may also play a crucial role in the pathogenesis of allergic disorders. Interleukin-17 (IL-17) mRNA and/or proteins are observed in the lungs, sputum, broncho-alveolar lavage (BAL) fluids or sera in asthma, and IL-17 expression is correlated with the severity of asthma [8–10]. Furthermore, recent studies using murine asthma models have shown that IL-17 receptor-deficient (IL-17R^{-/-}) mice exhibit reduced recruitment of eosinophils (EOS) and neutrophils into the airways [8]. In addition, Harrington *et al.* [11, 12] reported that Th17 cell differentiation was inhibited by exogenous interferon- γ (IFN- γ), and IFN- γ can negatively regulate Th17 cell development. In addition to T effector cells, Treg cells deregulation results in autoimmune damage and is the major regulator of allergic airway inflammation [13, 14]. As different CD4⁺ T cell subsets are actively involved in the pathogenesis of asthma, understanding mechanisms of their function and

¹ Department of pulmonary Medicine, Wuhan Women and Children Health Care Center, Wuhan, China

² Department of Pediatrics, Division of Pulmonary Medicine, Nationwide Children's Hospital and Ohio State University School of Medicine and Public Health, Columbus, OH, USA

³ Department of Ophthalmology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

⁴ To whom correspondence should be addressed at Department of pulmonary Medicine, Wuhan Women and Children Health Care Center, Wuhan, China. E-mail: lusi74@163.com

ABBREVIATIONS: Tim-3, T-cell Ig and mucin domain-containing molecule-3; AHR, Airway hyperresponsiveness; BALF, Broncho-alveolar lavage fluid; RT-PCR, Real-time polymerase chain reaction; FCM, Flow cytometer; ELISA, Enzyme linked immunosorbent assay; IL, Interleukin; siRNA, Small interfering RNA

regulation would shed light on the pathogenesis of asthma.

T cell Ig- and mucin domain-containing molecule (Tim)-3 belongs to the Tim family, that map to a region of chromosome 11 termed T cell and airway phenotype regulator (Tapr), which has been shown to play a key role as a human susceptibility gene for asthma, allergy and autoimmunity. Tim-3 was initially identified on Th1 differentiated cells. Tim-3 interaction with ligand negatively regulates Th1 immune response [15, 16]. Current studies indicate that Tim-3 is also present on Th17 cells, and is involved in Th17 and Treg cell regulation [17–21]. Although it is clear that Tim-3 is actively involved in the negative regulation of CD4⁺ T cells and has been identified as an important target for the treatment of inflammation, atopic and autoimmune diseases [22–24], its role in the pathogenesis of asthma remains elusive. Therefore, we employed Tim-3 small interfering RNA (siRNA) to detect the effect of Tim-3 silence on development of ovalbumin (OVA)-induced airway pathology and AHR in mice.

MATERIALS AND METHODS

Animals

Six- to eight-week old female BALB/c mice were fed with OVA-free diet under pathogen-free conditions in the Animal Experimental Institute of Tongji Medical College. All experimental protocols were approved by the Animal Care Committee of the Wuhan Women and Children Health Care Center Committees on Animal Experimentation (Wuhan, China).

Isolation and Culture of Peripheral Blood Mononuclear Cells

Allergen sensitization. Mice were exposed to OVA as previously described [25]. In brief, mice were systemically sensitized by an intraperitoneal injection of OVA (Grade VI, 100 µg/ml; Sigma-Aldrich), or sham-sensitized with PBS, adsorbed to adjuvant (10 mg/ml, Al(OH)₃; Pierce) in a total volume of 200 µl on day 0 and day 7.

Allergen challenge. From days 14 to 21, all mice were challenged by inhalation 1 % (weight/volume) OVA for 30 min in an exposure chamber (50×30×25 cm) once per day; control mice were exposed to aerosolized PBS. All mice were sacrificed 24 h after the final challenge, and blood samples were collected from the heart under sterile conditions.

Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples by Ficoll (Dakewei Biotech Co., China) density gradient centrifugation. Trypan blue exclusion was used to count viable cells. Cell concentration was adjusted to 2×10^6 /ml in RPMI 1640 media containing 10 % fetal calf serum.

Construction of Tim-3 shRNA Expressing Plasmids

As described by Elbashir *et al.* [26], three siRNA fragments targeting mice Tim-3 gene were designed respectively as follows: ss1: 5'-CACCG GTTGG TAAGA ATGCC TATCT-3'; ss2: 5'-CACCG CTTCT CCAAG AACCC TAACC-3'; ss3: 5'-CACCG GTGTC TTAAT CCTTA AATGG-3'. Sense and antisense sequences of short hairpin RNA were designed and synthesized by Shanghai Genepharma Biotechnology Company (China), containing *Bbs*I and *Bam*HI restriction sites at 5' and 3' ends. These fragments were annealed and cloned into pGP-U6/GFP/Neo/RNAi expression vector (Genepharma Biotechnology, Shanghai, China) to construct Tim-3 shRNA plasmids. All sequences were confirmed by sequencing analysis. GFP-specific shNC was used as control containing the targeting nucleotide site (GTTCTCCGAACGTGTCACGT). BLAST research was performed to ensure the specificity of Tim-3 shRNAs.

shRNA Transfection

PBMCs were digested and seeded in 6-well plate at a density of 5×10^4 cells per well. After 24 h, pGPU6/GFP/Neo-Tim-3 and pGPU6/GFP/Neo-shNC plasmids were transfected into PBMC using Lipofectamine 2000 (Invitrogen, California, USA) according to the manufacturer's instruction. The ratio of plasmid (µl) to Lipofectamine 2000 (µl) was used as 7.5:5. Cells were incubated at 37 °C in a 5 % CO₂ incubator for 48 h and stained with SYBR Green I. Positive transfectants were calculated and harvested by green fluorescence. Transfection efficiency was calculated by the ratio of transfected cells in one hundred of PBMC.

Real-Time PCR of Tim-3

Total RNA was isolated by TRIzol reagent (Invitrogen) according to the manufacturer's protocol. First-strand cDNA was synthesized using RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, USA). PCR was amplified by 7900HT Fast Real-Time PCR System (Applied Biosystems, USA) using the SYBR green PCR

master mix (Applied Biosystems, USA). Melting curve analyses following amplification were performed to ensure product specificity. The mRNA expression levels of Tim-3 were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The $\Delta\Delta C_t$ method was used to calculate fold changes of Tim-3 mRNA. Primer sequences for Tim-3 and GAPDH are listed in Table 1.

Western Blot

Cells were collected and washed with PBS, and cell lysate were harvested. Proteins were separated by 10 % SDS-PAGE gel electrophoresis, transferred onto nitrocellulose membranes, and incubated with Tim-3 antibodies (dilution 1/400). HRP-conjugated rabbit anti-mouse antibody was used as secondary antibody (dilution 1/4,000). Actin antibody was used as internal control.

Flow Cytometry

Direct immunofluorescence was performed on PBMC as previously described [27]. PBMC were washed three times with ice cold PBS. The following antibodies were used for flow cytometry: anti-mouse IL-17-APC (eBiosciences, USA); anti-mouse CD4-FITC (eBiosciences); anti-mouse CD25-APC (eBiosciences) and anti-mouse foxp3-PE (eBiosciences). All samples were analyzed for the level of cell surface marker by flow cytometer (FACS-440; Becton Dickinson, USA). Flow cytometric data were collected in a logarithmic mode on light scatter-gated live cells using CellQuest software (Becton Dickinson).

Cytokine Measurement

Levels of IL-4, IL-10, IL-17, IFN- γ and TGF- β were quantified in the supernatants of PBMC by ELISA according to the manufacturer's protocol (Dakewe Biotech Company Limited, China).

Detecting Suppression Efficiency of ShRNA *In Vivo*

Mice were sensitized and challenged as described previously [25]. Thirty minutes before airway challenge, animals were anesthetized with pentobarbital sodium and intranasally treated with 50 μ g of Tim-3 shRNA (OVA/Tim-3shRNA group) or the same amount of GFP-shNC (PBS/GFP-shNC or OVA/GFP-shNC group). To determine appropriate dose of Tim-3 shRNA, we intranasally administered different amounts of Tim-3 shRNA and found that 50 μ g of Tim-3shRNA as the optimal dose (data not shown).

Airway Hyperresponsiveness Measurement

AHR was assessed 24 h after the final aerosol challenge by calculating respiratory system resistances using various concentrations of methacholine (MCh), as described previously [28]. Briefly, Mice were anesthetized with 60 mg/kg 1 % pentobarbital sodium (Sigma), tracheostomized and mechanically ventilated at a rate of 90 breaths/min, a tidal volume of 5 ml/kg and a positive end-expiratory pressure of 3–4 cm H₂O with a computer controlled small animal ventilator (AniRes 2003 Beijing Xin AoCheng Ltd., China). Once stabilized, aerosols containing varying concentrations of MCh (0–270 g/kg) were delivered to the inspiratory line for 3 min, and data were presented as RL of three independent experiments.

Broncho-alveolar Lavage

BAL was performed via a tracheal tube with Hank's balanced salt solution (3 $\times$ 1 ml). The total cell number was counted using a hemocytometer. The supernatant was immediately stored at –80 °C for IL-17 and IL-10 measurement by ELISA. Differential cell counts were stained with Wright–Giemsa.

Histology

After BAL, the left lobe of each individual lung was fixed with 10 % neutralized buffered formalin.

Table 1. Primer Sequences of Real-Time PCR

	Sequence (5' to 3')	TA (°C)	Cycles
Tim-3	Forward: TTGGAGTGGGAGTCTCTGCT	59	40
	Reverse: ATCCTGACTGCTCCTGCATT		
GAPDH	Forward: AACTTTGGCATTGTGGAAGG	59	40
	Reverse: GGATGCAGGGATGATGTTCT		

TA annealing temperature, GAPDH glyceraldehyde-3-phosphate dehydrogenase

Sections (5 mm thick) were prepared, subjected to H&E staining and examined by light microscopy. Intensity of inflammatory cell infiltrate (0–4+ scale) was evaluated [29].

Statistical Analysis

Graph generation and statistical analysis were performed by using Prism 5.00 software (GraphPad). Values for all measurements are expressed as mean $\pm$ SEM. Pairs of treatment groups were compared using Mann–Whitney *U*-test. A *P* value <0.05 was considered statistically significant.

RESULTS

Tim-3 Expression is Up-regulated on PBMCs After OVA Induction

To explore Tim-3 roles affecting the outcome of OVA-induced inflammation, mice were sensitized and challenged with OVA, and Tim-3 protein and mRNA expressions were measured by real-time PCR and Western. Elevations of Tim-3 on PBMCs were detected as previous described [30, 31]. Tim-3 mRNA expression in OVA-sensitized mice increased 8.0 ± 2.4 -fold, compared to normal control mice (Fig. 1a). Similar to mRNA, Tim-3 protein levels in OVA-sensitized mice also increased from 1.11 % to 6.58 %, in comparison to normal controls ($P < 0.05$) (Fig. 1b).

Tim-3 shRNA Attenuates Tim-3 Expression on PBMCs

Three Tim-3 shRNA plasmids were constructed. Prior to Tim-3 shRNA application in mice, we first verified efficiently inhibition of Tim-3 expression by shRNA in PBMCs. We transduced PBMCs with Tim-3 shRNA (ss1, 2, 3) to suppress Tim-3 expression. Transfections without shRNA or with GFP-specific shNC were served as controls. pGP-NC or pGPsh-Tim-3 transfectants expressed green fluorescent protein. Thus observation of strong green luminescence indicated successful transfection (Fig. 2a). Tim-3 mRNA and protein expressions on PBMCs were measured by RT-PCR and Western blot, respectively. As shown in Fig. 2b and c, all three shRNA successfully attenuated Tim-3 expression on PBMCs, while ss2 exhibited strongest suppression. Expression levels between the control

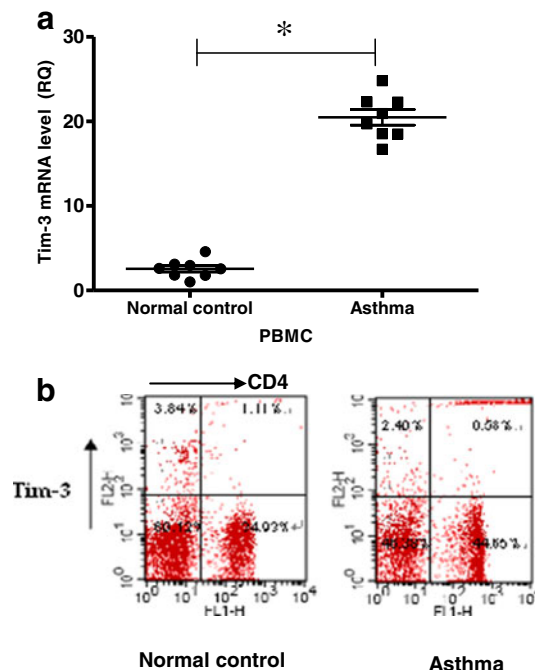


Fig. 1. Tim-3 expression in OVA-induced PBMCs. **a** As measured by RT-PCR, Tim-3 mRNA expressions were increased in PBMCs from asthmatic mice compared with normal controls. **b** Tim-3 expression in CD4⁺ T cells as assessed by flow cytometry ($n=8$). Percentages of Tim-3 + CD4⁺ cells were increased from 1.11 % in control group to 6.58 % in the Asthma group. Experiments were repeated at least three times.

group and the GFP-NC group did not show significant difference ($P > 0.05$).

Application of Tim-3 shRNA *In Vivo* Reduces Tim-3 Expression and Suppresses Eosinophilic Airway Inflammation in Murine Asthmatic Models

Since Tim-3 shRNA suppresses Tim-3 expression *in vitro*, we subsequently applied Tim-3 shRNA in animal models. shRNAs were administered on days 15–21 before airway challenge. On day 22, BAL was performed and lungs were removed. As shown in Fig. 3a and b, repeated intranasal administration of Tim-3 shRNA significantly reduced Tim-3 expression in PBMCs, and decreased numbers of eosinophils and lymphocytes in BALFs, in comparison with GFP-specific shNC. A histological examination also showed that eosinophilic accumulation in the airways was largely decreased in Tim-3 shRNA treated mice that were repeatedly administered Tim-3 shRNA, compared with those that were administered GFP-specific shNC controls (Fig. 3c,d).

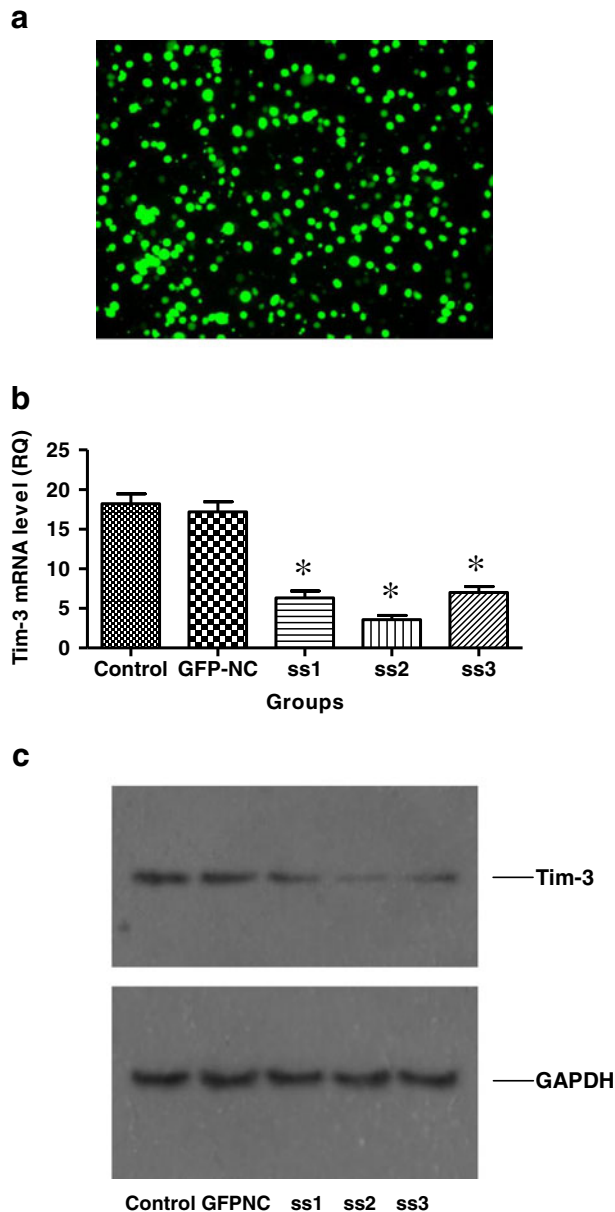


Fig. 2. Tim-3 shRNA suppresses Tim-3 expression *in vitro*. **a** Representative picture showing pGFP-NC or pGpsi-Tim-3 transfected PBMCs with green fluorescent protein. Transfecting Tim-3 shRNAs in PBMCs inhibited Tim-3 mRNA and protein expression, as assessed by RT-PCR (**b**) or Western blot (**c**). In three different shRNAs, ss2 exhibited the strongest inhibition effect. * $P < 0.01$ vs. GFP-NC, $\Delta P < 0.01$ vs. ss1 or ss3.

Intranasal Delivery of Tim-3 shRNA Suppressed AHR Development

We studied whether Tim-3 shRNA affects AHR development in murine asthmatic models. Airway

responses were assessed by changes in RL in response to increasing doses of aerosolized MCh. BALB/c mice that sensitized and challenged with OVA showed increase of lung resistance to MCh inhalation, compared to PBS-sensitized and challenged mice (Fig. 4).

Similarly, AHR was increased in OVA-sensitized and challenged mice receiving the GFP-shNC. However, RL levels in Tim-3 shRNA treatment group were similar as those of the PBS group, indicating that Tim-3 shRNA inhibited OVA-induced AHR formation.

Tim-3 shRNA Induces Th1, Foxp3+ Treg Production and Limits Th17 Generation *In Vitro*

To understand mechanisms underlying inhibitory effects of Tim-3 shRNA, we further investigated the effect of Tim-3 shRNA treatment on CD4⁺ T cell subsets expression on PBMCs. First, we measured the frequency of Th1 (CD4⁺ IFN- γ ⁺), Th2 (CD4⁺ IL-4⁺), Treg (CD4⁺ CD25⁺ FoxP3⁺) and Th17 (CD4⁺ IL-17⁺) cells by FCM. Sensitization and airway allergen challenges (OVA/GFP-shNC) resulted in increased Th2, Th17 cell productions and decreased Th1 and Treg cells production in PBMC compared with control animals (PBS/GFP-shNC). The intranasal application of Tim-3 shRNA to sensitized animals before and during airway allergen challenges (OVA/ss2) reduced Th17 cells (by 63 %), and increased Th1 (by 56 %) and Treg (by 58 %) production, in comparison to GFP control mice (OVA/GFP-shNC) (Fig. 5). The frequency of Th2 cells did not exhibit significant changes after Tim-3 shRNA treatment (data not shown). Subsequently, we assessed IFN- γ , IL-4, IL-10, IL-17, TGF- β protein levels in PBMCs culture supernatant by ELISA. We detected moderate to strong expression of IL-4, IL-17 and decreased levels of IFN- γ and IL-10 in OVA/GFP-shNC group, whereas IL-17 expression in PBMCs from OVA/ss2-treated mice were significantly reduced, and IFN- γ and IL-10 expression were significantly increased by Tim-3 shRNA treatment (Table 2). The level of IL-4 was unchanged by Tim-3 shRNA treatment and TGF- β did not alter in the presence or absence of Tim-3 shRNA after allergen challenge (data not shown). These results suggested that Tim-3 shRNA induced Th1 and Treg cell increase and Th17 cell decrease were involved in suppression of OVA-induced airway inflammation and AHR.

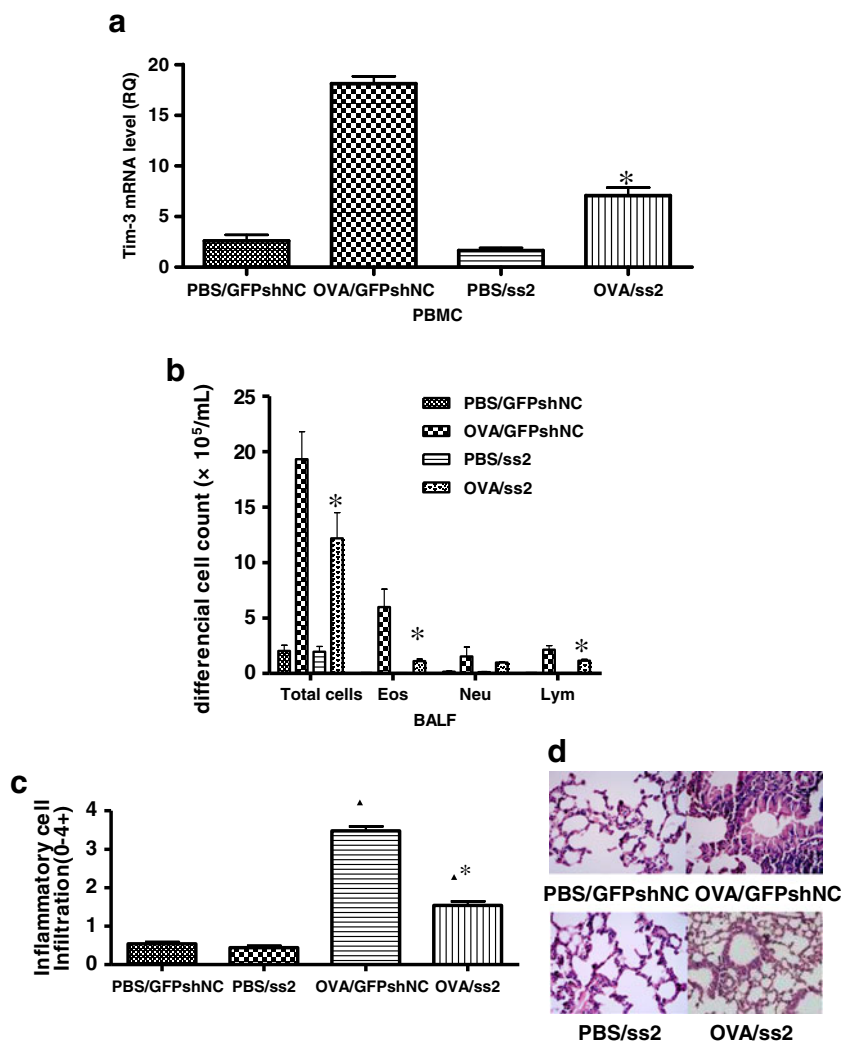


Fig. 3. Tim-3 shRNA inhibited airway inflammation *in vivo*. **a** Tim-3 mRNA levels in PBMCs among different groups. **b** Differential cell counts in BAL fluids from asthmatic mice. **c** intensity of inflammatory cell infiltrate (0–4+ scale); **d** lung histology (hematoxylin and eosin staining) of different groups. $\Delta P < 0.05$, compared to PBS/GFPshNC group; $*P < 0.05$, compared to OVA/GFPshNC group.

DISCUSSION

The pathogenesis of asthma is complicated, involving different cells, mediators and cytokines, all with their variety of signaling cascades and regulation pathways. Controlling chronic inflammation lesions represents a major therapeutic strategy for asthma. Since current studies indicate that these lesions are strongly suspected to result from the disorder of the activation and regulation of CD4⁺ T cell subset [32, 33], a logical therapeutic approach could be to suppress the function of activated T cell subsets and increase the representation of cells that express regulatory function. Tim-3, a

negative regulator of CD4⁺ T cells, has been identified as an important target for the treatment of inflammation, allergy and autoimmune diseases [16, 23]. In present study, we designed Tim-3 shRNA and employed a murine model of OVA-induced airway disease to study Tim-3 roles in the progression of asthma. Our data showed that silencing Tim-3 significantly reduced two cardinal features of bronchial asthma, airway inflammation and AHR, by affecting the polarization and function of different T cell subsets and regulation the imbalance of Th1/Th17.

Tim-3 belongs to a novel family of genes that map to a region of chromosome 11 termed Tapr, which

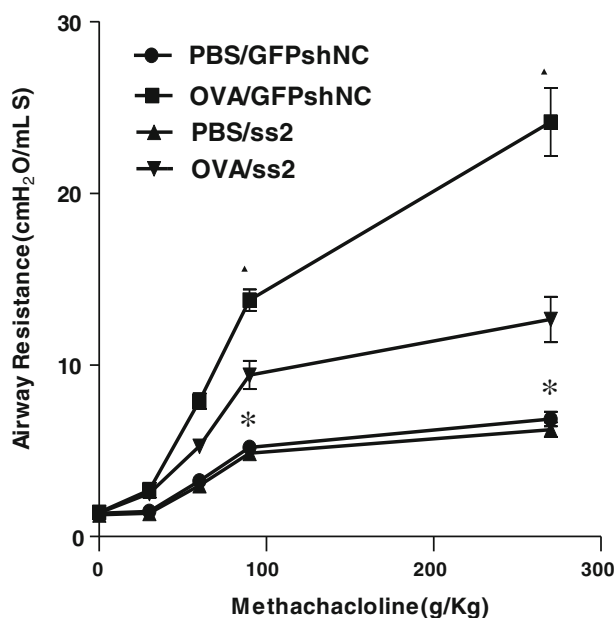


Fig. 4. Tim-3 shRNA inhibited OVA-induced AHR formation in BALB/c mice. Airway function was measured by invasive body plethysmography. Data were presented as mean $\pm$ SEM of the pulmonary resistance (*RL*) from three individual experiments. $\blacktriangle P < 0.05$, compared to PBS/GFP-shNC group; $*P < 0.05$, compared to OVA/GFP-shNC group.

protects against airway hyperreactivity (AHR). In addition, polymorphisms of Tim-3 have been associated with atopy, suggesting that the Tim family may play functional roles in human allergic diseases [34, 35]. Previous reports have shown that activated CD4⁺ T cells may up-regulate Tim-3 expression in allergic and inflammatory disease, and neutralization of Tim-3 resulted in decreased allergic inflammation [30, 36]. Our studies investigating Tim-3 roles in OVA-induced inflammatory disease showed that Tim-3 shRNA resulted in the decreased AHR response, inflammatory cells infiltrated around airways and EOS numbers in BALF.

To provide a mechanistic link between dysregulated Tim-3 expression and CD4⁺ T cells, we assessed the effect of Tim-3 shRNA on PBMC CD4⁺ subset cells and related cytokines. We found Th2 cell response increased significantly *in vivo*, but no change observed after Tim-3 shRNA administration. Our finding suggested that Tim-3 is not involved in the suppression of Th2 cell proliferation in this setting.

Several studies [15, 16, 18] found that blockage of Tim-3 induced Th1 cell hyperproliferation, cytokine release and augmented Th1 responses. Recent studies

reported that Tim-3 is also involved in the regulation of Th17 cell function [18–20, 24, 37]. For example, Seki et al. [19] found that interaction of Tim-3 with its ligand galectin-9 suppresses the generation of Th17 cells and ameliorates collagen-induced arthritis (CIA), so we sought to further establish the effect of Tim-3 shRNA treatment on Th1 and Th17 cells expression in the lung. To this end, we found that the percentage of CD4 cells expressing IL-17 was significantly decreased by Tim-3 shRNA treatment compared with GFP-shNC. In contrast, Tim-3 shRNA treatment significantly increased IFN- γ production from CD4 cells. As Th1 and Th17 cells can cross-regulate each other, Harrington *et al.* [12] reported that Th17 cell differentiation was inhibited by exogenous IFN- γ . Furthermore, it has been reported that Th17 cell and related cytokines inhibit Th1 cell biology *in vivo* [17]. It is conceivable that Tim-3 shRNA treatment may suppress Th17-mediated, allergen-induced inflammation by inducing hyperproliferation of Th1 cells in the lung and increased IFN- γ expression.

In addition, a reciprocal induction of Th17 and Treg cells during differentiation has been reported [5, 38]. TGF- β 1 induces the differentiation of naive CD4⁺ T cells into CD4⁺CD25⁺Foxp3⁺ Treg cells, whereas TGF- β 1 in combination with IL-6 induces differentiation into Th17 but prevents differentiation into Treg cells. Thus, we also investigated whether Tim-3 shRNA affected the number of Treg cells in OVA-induced asthmatic mice, and found that attenuating Tim-3 by shRNA caused an increase in CD4⁺CD25⁺Foxp3⁺ *in vivo*. These findings indicate that Tim-3 is involved in the regulation of Foxp3⁺ Treg cells in OVA-induced asthma.

Targeting mRNA is a more efficient way of reducing gene expression at the protein level, compared with direct blocking of protein activity by monoclonal antibodies or inhibitor compounds, because multiple copies of protein (approximately 5,000 copies) are produced by each mRNA [39]. Therefore, pharmacological modulation of gene expression has been received increasing attention in allergic diseases. siRNAs were also reported to inhibit activation of effector cells and Th2 responses in models of allergic disease *in vitro* [40, 41]. Furthermore, concerning the choice between local and systemic delivery for allergic airway diseases, the local administration of siRNA by intranasal application according to a possible inhalation therapy for humans is strongly preferable. Previous studies [42, 43] also indicated the intra-airway administration of siRNAs that targeted gene suppressed allergic airway inflammation

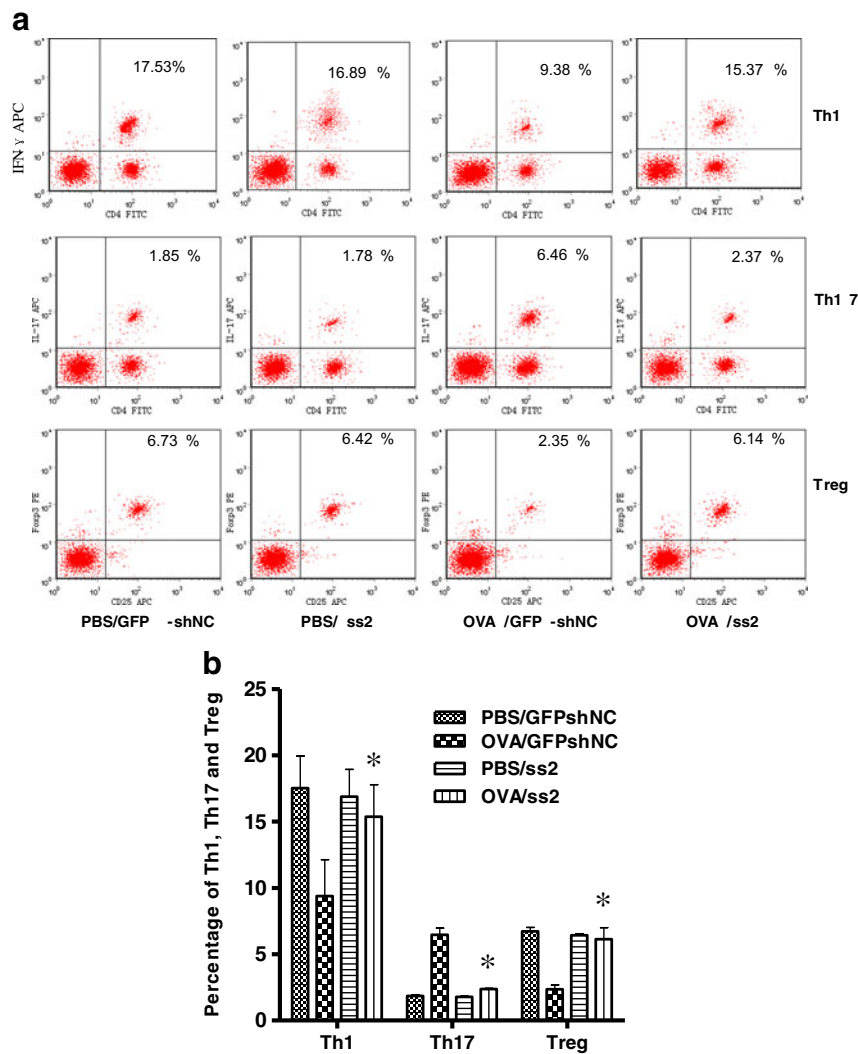


Fig. 5. Tim-3 shRNA reduced Th17 and induced Th1 and Treg cell production in PBMCs. Mice received OVA challenge and intranasal application of Tim-3 shRNA or control GFP-shNC, PBMCs were stained and assessed by flow cytometry. **a** Numbers of Th1+, Th17+ and Treg + cells relative to CD4+ blood cells. **b** Quantifications of Th1, Th17 or Treg cells. **P*<0.05, compared with OVA/GFP-shNC group.

and AHR in murine models of acute asthma. Therefore, in the current study, we constructed a short hairpin RNA of Tim-3 administered intranasally to investigate their

therapeutic effects in OVA-induced asthma. We showed that the down-regulation of Tim-3 using shRNA suppressed Th17 differentiation, elevated IFN-γ production,

Table 2. IL-4, IL-10, IL-17 and IFN-γ Expressions in PBMC Culture Supernatant

Cytokines (pg/ml) (n=8)	PBS/GFP-shNC	PBS/SS2	OVA/GFP-shNC	OVA/ss2
IL-4	44.3±11.5	45.2±12.1	69.3±10.5*	48.6±9.32
IL-10	120.5±24.8	123.5±22.6	45.4±12.6*	83.9±15.5*▲
IL-17	72.5±13.6	70.3±12.9	118.8±16.5*	73.6±12.5*▲
IFN-γ	88.0 ±17.6	87.1±16.4	40.4±15.4*	78.6±14.7*▲

Data are presented as mean ± SEM
 **P*<0.01 vs. PBS/GFP-shNC
 ▲*P*<0.01 vs. OVA/GFP-shNC

and inhibited asthmatic responses in mice. Because Tim-3 expression is increased in allergic diseases including asthma, targeting Tim-3 by shRNA or chemical compounds may hold therapeutic potential in preventing the development of allergic asthma.

ACKNOWLEDGMENTS

This work was supported by grant Wu Jieping Medical Fund 320.6750.1285, grant 2010CDB08804 from the Natural Science Foundation of Hubei Province, and grant WX11B10 from the Foundation of Wuhan Health Bureau.

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