ORIGINAL ARTICLE

Intranasal Delivery of *T-bet* Modulates the Profile of Helper T Cell Immune Responses in Experimental Asthma

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Abstract

Background: Allergic asthma is caused by aberrant helper T (T_H) type 2 immune responses in susceptible individuals, characterized by airway hyperresponsiveness, chronic airway inflammation, and mucus hypersecretion. Its prevalence continues to increase, but optimal treatment remains a challenge. The transcription factor T-bet is a master regulator of T_H1 lineage commitment and strongly promotes interferon γ expression during T_H1 cell differentiation.
Objective: The aim of this study was to explore the role of intranasal delivery of *T-bet* on the differentiation of T_H cell subsets and airway inflammation in the ovalbumin (OVA)-induced mouse model of allergic airway inflammation.
Methods: BALB/c mice were sensitized by intraperitoneal injection of OVA and challenged with nebulized OVA. Four days before the inhalation challenge, the sensitized mice were subjected to intranasal delivery of a recombinant adeno-associated virus vector carrying murine *T-bet* gene (AAV-T-bet). Expression of the transcription factors *T-bet, GATA3*, and *Foxp3* was then assayed in the lungs, and airway histology was analyzed along with other inflammatory parameters, such as eosinophils and cytokines in bronchoalveolar lavage (BAL) fluid, and total and OVA-specific immunoglobulin (Ig) E in serum.
Results: Intranasal administration of AAV-T-bet efficiently balanced the T_H1/T_H2 transcription factor and cytokine profile and significantly decreased the number of eosinophils in BAL fluid. It also resulted in a reduction of peribronchial inflammation scores and serum IgE levels in OVA-sensitized and challenged mice during the effector phase.
Conclusions: Our data show that intranasal delivery of *T-bet* can promote a T_H1 immune response, restore a balanced Th immune response, and inhibit airway inflammation during the challenge phase in a mouse model of allergic airway inflammation.

Key words: Allergy. Transcription factor. Modulation. Immune response. T_H1/T_H2.

Resumen

Antecedentes: Las respuestas inmunitarias del linfocito T cooperador (T_C) de tipo 2 anómalas provocan asma alérgica en individuos susceptibles. Ésta se caracteriza por una hiperreactividad e inflamación crónica de las vías respiratorias e hipersecreción de moco. Su prevalencia va en aumento y hallar el tratamiento óptimo sigue siendo un reto. El factor de transcripción T-bet es el regulador principal del linaje T_C1 y estimula de modo notable la expresión del interferón γ durante la diferenciación celular de T_C1.
Objetivo: El objetivo de este estudio fue investigar el papel de la administración intranasal del *T-bet* sobre la diferenciación de las subclases de células T_C y la inflamación de las vías respiratorias, en un modelo murino de inflamación alérgica de las vías respiratorias inducido por ovoalbúmina (OVO).
Métodos: Se sensibilizaron ratones BALB/c mediante inyección intraperitoneal de OVO y se provocaron con OVO nebulizada. Cuatro días antes de la prueba de provocación por inhalación los ratones sensibilizados se sometieron a administración intranasal de un vector viral recombinante adenoasociado portador del gen *T-bet* murino (AAV-T-bet). La expresión de los factores de transcripción *T-bet, GATA3* y *Foxp3* fue analizada en pulmón y se analizó la histología de las vías respiratorias, junto con otros parámetros inflamatorios como los eosinófilos y las citocinas en el líquido del lavado broncoalveolar (LBA) y la inmunoglobulina (Ig) E sérica total y específica a OVO.
Resultados: La administración intranasal de AAV-T-bet equilibró de manera eficiente los factores de transcripción T_C1/T_C2 y el perfil de citocinas y provocó una disminución significativa del número de eosinófilos en el líquido del LBA. También provocó una reducción de la inflamación peribronquial y de las concentraciones de IgE séricas en ratones OVO-sensibilizados y provocados, durante la fase efectora.
Conclusiones: Nuestros datos muestran que la administración intranasal de *T-bet* puede fomentar una respuesta inmunitaria Th1, reestablecer una respuesta inmunitaria TC equilibrada e inhibir la inflamación de las vías respiratorias durante la fase de provocación en un modelo murino de inflamación alérgica de las vías respiratorias.

Palabras clave: Asma. Alergia. Factor de transcripción. Modulación. Respuesta inmunitaria T_C1/T_C2.

Introduction

Asthma is a chronic inflammatory disease characterized by airway hyperresponsiveness, mucus overproduction, and infiltration of inflammatory cells in the airway [1]. Type 2 helper T (T_H) cells (T_H2) are considered to play a critical role in the pathogenesis of asthma by producing a variety of cytokines such as interleukin (IL)-4, IL-5, IL-9, and IL-13 [2]. These cytokines promote immunoglobulin (Ig) isotype switching to IgE, mediate infiltration of inflammatory cells (such as eosinophils, mast cells, T lymphocytes, and neutrophils) in the airways, and lead to the production of a wide range of inflammatory mediators [3]. In addition, a reduced T_H1 immune response is also characteristic of allergic asthma.

T_H cell differentiation is regulated at various levels, such as the interaction of peptide antigens with the T cell receptor, cytokine signaling, actions of costimulatory molecules, and induction of the transcription factors GATA binding protein 3 (GATA3) and T-bet (T-box expressed in T cells). Among them, GATA3 and T-bet are critical for differentiation of naive T cells into T_H2 and T_H1 cells, respectively [4,5]. Studies have shown that T-bet, a member of the T-box family of transcription factors, is a master regulator of T_H1 lineage commitment [5]. T-bet is induced by STAT-1-mediated signals and strongly promotes IFN-γ and IL-12Rß2 expression during T_H1 cell differentiation while suppressing T_H2 differentiation [6,7]. GATA3 is a zinc finger protein that is preferentially expressed during the course of T_H2 differentiation in response to IL-4 signals [8,9]. Moreover, T-bet-deficient mice have hypersensitive airways and develop asthma spontaneously [10]. Thus, a balance between GATA3 and T-bet is believed to control T_H1/T_H2 polarization.

It is well known that T_H2 cells and their cytokines play a key role in the immunopathogenesis of allergic asthma. Previous studies have shown that T-bet has a specific regulatory role in T_H1 cells. Retroviral expression of T-bet in developing and developed T_H2 cells not only induces high-level production of IFN-γ, but also suppresses the expression of IL-4 and IL-5 in vitro [5]. Recently, we have cloned the cDNA of *T-bet* from Balb/c mice and constructed its corresponding recombinant adeno-associated viral vector (AAV-T-bet). In the present study, we explored whether intranasal delivery of AAV-T-bet to the airway mucosa in a murine model of allergic airway inflammation can regulate T_H1/T_H2 lineage development in vivo, prevent aberrant T_H1/T_H2 polarization, and subsequently relieve airway inflammation.

Materials and Methods

Reagents

A recombinant adeno-associated viral vector carrying the murine *T-bet* gene (AAV-T-bet) was constructed previously in our laboratory [11]. The AAV-T-bet vector was not checked for the presence of endotoxin, but the AAV-T-bet packing plasmids were purified with the EndoFree Plasmid Mega Kit (Qiagen, Valencia, California, USA). Ovalbumin (OVA, Grade V), aluminium hydroxide, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St Louis, Missouri, USA). Trizol reagents and First Strand cDNA Synthesis Kit for reverse-transcriptase polymerase chain reaction (RT-PCR) were purchased from Invitrogen Life Technologies (Carlsbad, California, USA) and QuantiTect SYBR Green PCR Kit for quantitative real-time PCR was from Qiagen. Biotinylated anti-mouse IgE antibodies and 3,3',5,5'-tetramethylbenzidine were obtained from Bethyl Laboratories Inc (Montgomery, Texas, USA). Mouse OVA-specific IgE enzyme-linked immunosorbent assay (ELISA) kit was from AbD Serotec (Oxford, UK). Mouse IL-4, IL-5, and IFN-γ ELISA kits were from Jingmei Biotech Inc (Shenzhen, China). All other chemicals or reagents were of the highest quality available.

Animals

Female Balb/c mice (6 weeks old), weighing 18 ± 2 g, were purchased from the laboratory animal center of Yangzhou University (Yangzhou, China). Mice were housed in plastic cages with sterilized wood-chip bedding, bred in animal rooms kept at a temperature of 23°C ± 2°C and a relative humidity of 55% ± 10% with a 12-hour light–dark cycle. They had free access to tap water and normal diet. Mice were separated into 4 groups (8 to 10 mice in each group): saline control, OVA control, AAV-treated control, and AAV-T-bet-treated. The saline control group was a sham control in which saline substituted OVA during sensitization and challenge. The OVA control was the model of allergic airway inflammation in which mice were sensitized and challenged with OVA. In the AAV-treated control group, the empty vector served as a control for gene delivery in the model of experimental asthma. In the AAV-T-bet-treated group, the AAV-T-bet vector was administered intranasally for gene delivery studies in the model of OVA-induced experimental asthma. Animal experiments were approved by the animal handling safety and ethics committee of Jiangsu University.

Induction of a Mouse Model of Allergic Airway Inflammation

The mouse model of allergic airway inflammation was established as described by Lommatzsch et al [12] with slight modifications. Briefly, the OVA control, AAV-treated control, and AAV-T-bet-treated groups were sensitized by intraperitoneal injection of 50 μg OVA protein and 2 mg aluminum hydroxide gel in saline. A second sensitization was given 10 days after the initial sensitization. On day 22 after initial sensitization, mice were placed in a Plexiglas chamber linked to an ultrasonic nebulizer and challenged with aerosolized OVA solution (10 mg/mL in saline) for 30 minutes. The provocation was performed once a day for 5 consecutive days and then the mice were assessed for allergic inflammation of the lungs 24 hours after the last aerosol exposure. Mice in the AAV-treated control group and the AAV-T-bet-treated group were treated intranasally with 1×10^{10} vector genome (vg) per mL AAV or AAV-T-bet vectors, respectively, in 15 μL PBS, from 3 to 4 days before the inhalation challenge. The applied AAV dose of *T-bet* differed from that used in the related study of Lassance et al [13]. Mice in the saline control group received intraperitoneal mock sensitization with saline and were challenged with an aerosol of saline without OVA (Figure 1).

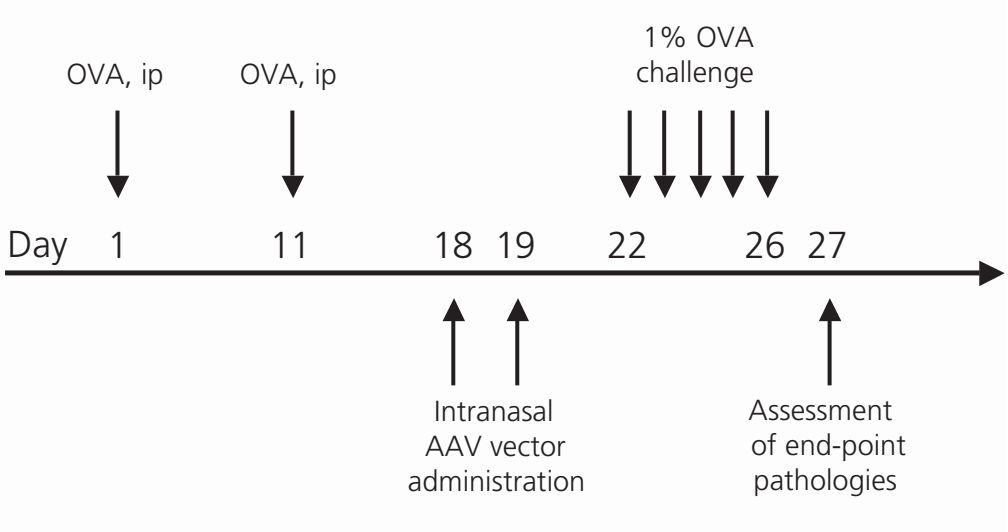

Figure 1. Schematic diagram of the experimental protocol. Mice were sensitized on day 1 and 11 by intraperitoneal (ip) injection of 50 μg of ovalbumin (OVA) emulsified in 2 mg aluminum. On day 22, 23, 24, 25, and 26 after the primary sensitization, the mice were challenged with an aerosol of 1% OVA solution or saline (control) for 30 minutes every day using an ultrasonic nebulizer. Adeno-associated virus vector (AAV) was administered either as empty vector or containing *T-bet* (AAV-T-bet) intranasally on days 18 and 19.

Bronchoalveolar Lavage

Under ether anesthesia, mice were bled and sacrificed on day 27. Bronchoalveolar lavage (BAL) fluid was collected by washing the trachea twice with 1.0 mL of pyrogen-free PBS containing 0.05 mM ethylene diamine tetraacetic acid. The BAL fluid was centrifuged at 300 *g* for 5 minutes at 4°C, and the cells were separated from the fluid. The first lavage fluid was stored at –20°C until it was analyzed. The cell pellets from the BAL fluid were resuspended in 0.5 mL PBS, and the total number of cells was determined using a hemocytometer. Differential cell counts were determined with cytospin preparations followed by Giemsa staining.

Enzyme-Linked Immunosorbent Assay for Cytokines and IgE

The presence of IL-4, IL-5, and IFN-γ in BAL fluid was assessed by ELISA. Blood samples were collected from experimental animals by cardiac puncture after ether anesthesia and the sera were stored at –80°C prior to analysis. Total IgE and OVA-specific IgE in serum from each animal were also analyzed according to the manufacturer's instructions. Cytokine, total IgE, and OVA-specific IgE concentrations were calculated by comparison with cytokine and IgE standards of known concentration. The assay sensitivities for cytokines were 15-2000 pg/mL (IL-4 and IL-5 system) and 15-2000 pg/mL (IFN-γ system). The sensitivity for total IgE was 0-1000 ng/mL and for OVA-specific IgE it was 8.0–800 ng/mL. The experiments were performed in triplicate.

Respiratory Tract Histology

Lungs were harvested on day 27. The right lung was preserved in liquid nitrogen for total RNA extraction while the left lung was fixed in 10% phosphate-buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin (H&E), Giemsa (for identification of eosinophils), and periodic acid Schiff (PAS, for identification of mucus). The histopathological changes were graded according to a semiquantitative scoring system [14] as mild (score 1-2), moderate (score 3), or severe (score 4) by 2 researchers without prior knowledge of the treatment group. Briefly, this score described the following categories: 0, no inflammation; 1, few inflammatory cells; 2, a ring of inflammatory cells 1 cell layer deep; 3, a ring of inflammatory cells 2–4 cells deep; and 4, a ring of inflammatory cells >4 cells deep. Mucus production was assessed by determining the percentage of goblet cells that stained strongly. Lung sections from each group of mice were counted with at least 1000 epithelial cells analyzed per section. The results were calculated as the percentage of positive cells from each group of mice. Data are presented as median (range). All experiments were performed in triplicate.

Reverse-Transcriptase and Real-Time Polymerase Chain Reaction for T-bet, GATA3, *and* Foxp3

Total RNA was extracted from lung tissue. Reverse transcription was carried out with 1 μg of total RNA using an oligo (dT)12-18 primer, and real-time quantitative PCR was performed for *T-bet*, *GATA3*, and *Foxp3* on the Roter-Gene 2000 Sequence detector using the QuantiTect SYBR Green PCR Kit according to the manufacturer's protocol. Standard curves were generated using plasmids (pGEM T-easy, Promega) containing either *T-bet*, *GATA3*, or *Foxp3* inserts. The primer sequences were as follows: *ß2 microglobulin (ß2MG)*, sense 5'-TCACTGACCGGCCTGTATGCTATC3', antisense 5'-GTGAGGCGGGTGGAACTGTGT-3'; *T-bet*, sense 5'-TCCCATTCCTGTCCTTCA-3', antisense 5'-GCTGCCTTCTGCCTTTC-3'; *GATA3*,

sense 5'-ACCACGGGAGCCAGGTATG-3', antisense 5'-CGGAGGGTAAACGGACAGAG-3'; *Foxp3*, sense 5'-GCTCCCGGCCTGGTCTGCTC-3', antisense 5'-AGGTGGCGGGG TGGTTTCTGA-3'. The primers were constructed to generate fragment of 372 bp for *T-bet*, 170 bp for *GATA3*, 252 bp for *Foxp3*, and 123 bp for *ß2MG*. Each PCR amplification used the following conditions: 10 minutes at 94°C, followed by a total of 35 or 40 cycles of 15 seconds at 94°C and 1 minute at 60°C. The melting point analysis was carried out by heating the amplicon from 65°C to 95°C, allowing the characteristic melting point to be found for each product. The length of the product was confirmed by electrophoresis. Gene expression was quantified relative to the expression of *ß2MG*. Experiments were performed in triplicate.

Statistical Analysis

Total and differential cell counts in BAL fluid displayed a non-normal distribution, and parameters were expressed as median values (range). Therefore, differences in cell counts between the 4 groups were assessed by Kruskal-Wallis test. The concentrations of IFN-γ, IL-4, and IL-5 in BAL fluid, and those of total and OVA-specific serum IgE had a log-normal distribution, and data were shown as geometric mean (SEM). Expression of *T-bet*, *GATA3*, and *Foxp3* were normally distributed, and values were expressed as means (SEM). Differences among all groups were assessed by analysis of variance (ANOVA). $P < .05$ was considered statistically significant. Relationships between IFN-γ, IL-4, *T-bet* and *GATA3* in BAL fluid were examined by calculation of Pearson correlation coefficients.

Results

Effects of T-bet *Gene Delivery In Vivo on the Cell Numbers in BAL Fluid*

Compared with the saline control group, the total number of cells in BAL fluid was increased in the OVA control and AAV-treated control groups ($P < .05$), but it was dramatically reduced in the AAV-T-bet group ($P < .05$, Table 1). Also, the counts of macrophages, lymphocytes, and eosinophils were significantly increased in OVA and AAV-treated control mice when compared with the saline control group ($P < .05$), while the counts of those cells were significantly decreased in the mice treated with AAV-T-bet compared with the OVA control group ($P < .05$, Table 1).

Effects of T-bet *on Pulmonary Inflammation*

Lung tissue was collected 24 hours after the last challenge. Inflammatory cells were analyzed by H&E staining (Figure 2A-D). OVA challenge induced marked infiltration of inflammatory cells into the peribronchial and perivascular tissue as compared with the saline control group. Most infiltrated inflammatory cells were macrophages, lymphocytes, and eosinophils, as detected by Giemsa staining (data not shown). Inflammation was abated in the AAV-T-bet group when compared with that of the AAV-treated control or OVA control groups ($P < .05$, Figure 2I), and was close to the saline control ($P > .05$). Allergen-induced mucus secretion was detected by PAS staining (Figure 2E-H). The percentage of PAS-positive mucus-containing epithelial cells was higher in the OVA control group than the saline control group. The percentage of mucus-secreting cells decreased significantly after intranasal delivery of AAV-T-bet when compared with the OVA control or AAV-treated control groups, but it was higher than in the saline control group $P < .05$, Figure 2J).

Effects of T-bet *on Cytokines, Total IgE and OVA-Specific IgE*

Cytokine profiles in BAL fluid were analyzed by ELISA 24 hours after the last OVA inhalation. The levels of IL-4 in BAL fluid were elevated in the OVA control group (257.08 [1.51] pg/mL) compared to the saline control group (56.86 [1.48] pg/mL, $P < .05$), and reduced in AAV-T-bet-treated mice (149.61 [1.41] pg/mL) when compared with the OVA control or AAV control mice (290.86 [1.45] pg/mL, $P < .05$), but were higher than in the saline control group. In addition, IL-5 levels in BAL fluid were similar to those of IL-4, which were higher in OVA control (189.91 [1.55] pg/mL) and AAV control mice (158.45 [1.70] pg/mL) than in the saline control group (29.56 [1.58] pg/mL), and decreased

Table 1.Total and Differential Cell Counts in BAL Fluid 24 Hours After the Last Allergen Challenge[a]

Group	No	Total Cells	Macrophages	Lymphocytes	Eosinophils	Neutrophils
Saline control	10	0.24 (0.15-0.39)	0.22 (0.13-0.36)	0.02 (0.01-0.03)	0.(0-0)	0 (0-0)
OVA control	10	0.63 (0.33-0.98)[b]	0.39 (0.26-0.56)[b]	0.13 (0.02-0.18)[b]	0.15 (0.05-0.34)[b]	0.01 (0-0.04)
AAV control	10	0.64 (0.32-0.91)[b]	0.36 (0.20-0.48)[b]	0.09 (0.04-0.24)[b]	0.16 (0.08-0.22)[b]	0.02 (0.01-0.03)
AAV-T-bet	8	0.34 (0.22-0.52)[c,d]	0.28 (0.18-0.40)[c]	0.02 (0.00-0.07)[c]	0.02 (0.01-0.02)[c,d]	0.01 (0-0.02)

Abbreviations: AAV, adeno-associated viral vector; BAL, bronchoalveolar lavage; OVA, ovalbumin.
[a] Data are shown as the median (range) number of cells ($\times 10^6$) per mL in 3 comparable experiments.
[b] $P < .05$ compared with saline control group
[c] $P < .05$ compared with OVA control group
[d] $P < .05$ compared with AAV-treated control group.

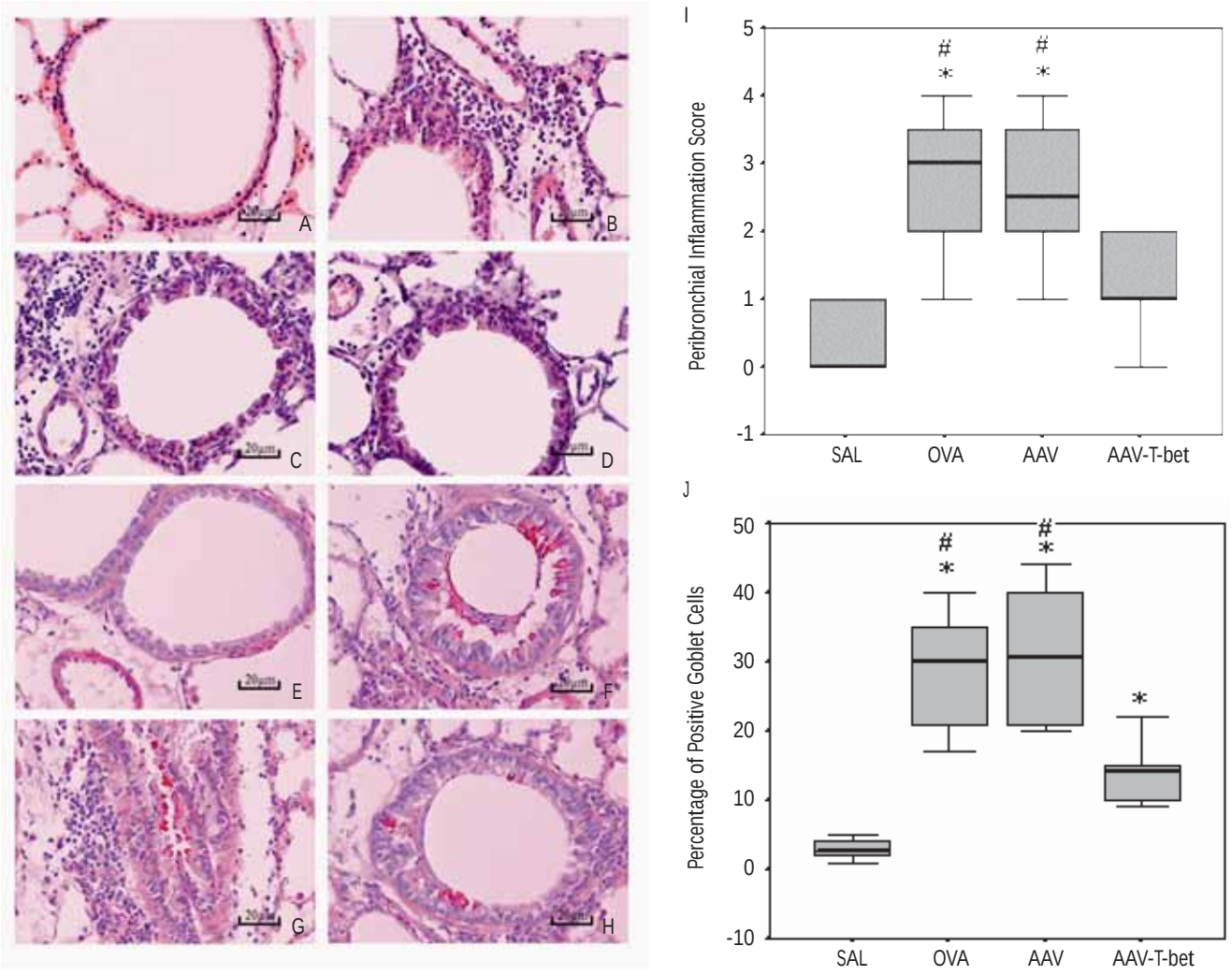

Figure 2. Effects of *T-bet* gene delivery on allergen-induced pulmonary inflammation and quantification of histological inflammation. The figure shows photomicrographs of lung tissue from the 4 groups of mice (8-10 mice in each group). Balb/c mice exposed to saline (A, E), ovalbumin (OVA) alone (B, F), or OVA plus treatment with adeno-associated virus vector (AAV), without a gene insert (C, G) or containing the *T-bet* gene (D, H). Sections were stained with hematoxylin-eosin (A-D) or periodic acid Schiff (E-H). Original magnifications, × 400. I and J show boxplots of the peribronchial inflammation scores (I) and mucus score (percentage of positive goblet cells), respectively. Kruskal-Wallis test and Nemenyi test were used for comparisons among groups. * indicates $P < .05$ compared with the saline control group; #, $P < .05$ compared with the AAV-T-bet-treated group. Comparable results were obtained in 3 representative experiments

Table 2. Effects of ***T-bet*** Delivery on the Levels of Cytokines in BAL Fluid and IgE in Serum[a]

Group	No	IL-4, pg/mL	IL-5, pg-mL	IFN-γ, pg/mL	Total IgE, ng/mL	OVA-Specific IgE, ng/mL
Saline control	10	56.86 (1.48)	29.56 (1.58)	130.20 (1.58)	25.44 (1.38)	6.61 (1.70)
OVA control	10	257.08 (1.51)[b]	189.91 (1.55)[b]	54.67 (1.55)[b]	233.67 (1.45)[b]	34.95 (1.45)
AAV control	10	290.86 (1.45)[b]	158.45 (1.70)[b]	63.87 (1.45)[b]	218.90 (1.58)[b]	29.28 (1.55)[b]
AAV-T-bet	8	149.61 (1.41)[b,c,d]	64.54 (1.45)[c,d]	108.48 (1.38)[c,d]	109.63 (1.48)[c,d]	15.41 (1.58)[c,d]

Abbreviations: AAV, adeno-associated viral vector; BAL, bronchoalveolar lavage; IFN, interferon; IgE, immunoglobulin E; IL, interleukin; OVA, ovalbumin.

[a] Data are shown as geometric means (SEM) of 3 comparable experiments.

[b] $P < .05$ compared with saline control group

[c] $P < .05$ compared with OVA control group

[d] $P < .05$ compared with AAV-treated control group

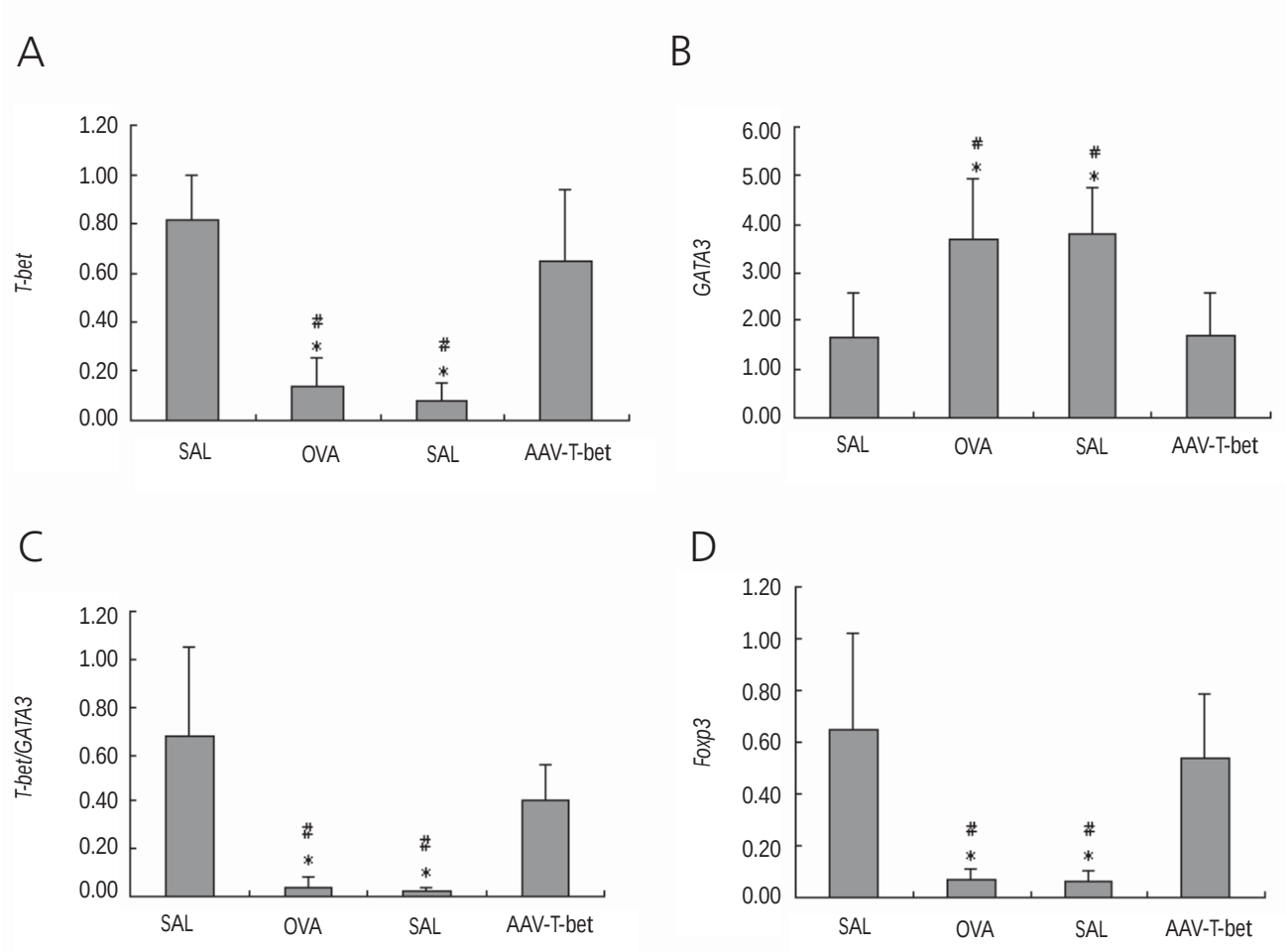

Figure 3. Effects of *T-bet* gene delivery on the expression of *T-bet* and *GATA-3*. The mRNA levels of transcription factors *T-bet* (A), *GATA-3* (B), *T-bet*/*GATA-3* (C), and *Foxp3* (D) in lung tissue were measured using real-time quantitative polymerase chain reaction at 24 hours after the last ovalbumin challenge in 4 groups of mice. Data are shown as the ratio of gene expression compared with *ß2 microglobulin*, except for (C), which shows the ratio of *T-bet* to *GATA-3* expression. Bars show the mean of 8-10 mice in each group; whiskers show SEM. SAL indicates saline control; OVA, ovalbumin control; AAV, adeno-associated virus vector; AAV-T-bet, adeno-associated virus vector containing the *T-bet* gene. Asterisks (*) indicate $P < .05$ compared with the saline control group; #, $P < .05$ compared with the AAV-T-bet-treated group. Comparable results were obtained in 3 separate experiments.

treated mice (64.54 [1.45] pg/mL, $P < .05$). In contrast to T_H2 cytokines, IFN-γ levels in BAL fluid from OVA control mice (54.67 [1.55] pg/mL) were lower than in the saline control group (130.20 [1.58] pg/mL, $P < .05$), but were increased in AAV-T-bet-treated mice (108.48 [1.38] pg/mL) compared with OVA control or AAV control mice (63.87 [1.45] pg/mL, $P < .05$). Administration of AAV-T-bet decreased the levels of total IgE (109.63 [1.48] ng/mL) and OVA-specific IgE (15.41 [1.58] ng/mL) in serum, and the difference was statistically significant ($P < .05$, Table 2).

Effects of AAV-T-bet on Transcription Factor mRNA Expression

Real-time quantitative PCR was used to measure lung tissue mRNA expression of T_H-associated transcription factors 24 hours after the last OVA inhalation. In lung tissue, the ratio of *T-bet* to *ß2MG* mRNA was lower in OVA control (0.14 [0.11]) and AAV control (0.08 [0.07]) mice than in the saline control group (0.82 [0.18], $P < .05$). After treatment with AAV-T-bet, the *T-bet* mRNA levels increased significantly (0.65 [0.29], $P < .05$, Figure 3A). Also, higher mRNA levels of *GATA3* in the lung were detected in OVA control (3.70 [1.25]) and AAV control (3.79 [0.95]) mice than in the saline control group (1.63 [0.97], $P < .05$). However, *GATA3* mRNA levels decreased to near normal levels after intranasal delivery of AAV-T-bet (1.67 [0.93], $P < .05$, Figure 3B). The ratio of *T-bet*/*ß2MG* versus *GATA3*/*ß2MG* was similar to the results of *T-bet* mRNA levels for each group, ie the ratio was lower in OVA control (0.04 [0.04]) and AAV control mice (0.02 [0.02]) than in the saline control group (0.68 [0.37], $P < .05$), and recovered in AAV-T-bet-treated mice (0.40 [0.16], $P < .05$, Figure 3C). As for the expression of *T-bet*, mRNA levels of *Foxp3* were lower

in OVA control (0.07 [0.04]) and AAV control (0.06 [0.04]) groups than in saline control mice (0.65 [0.37], $P < .05$). But the levels increased significantly in the AAV-T-bet-treated group (0.54 [0.25], $P < .05$, Figure 3D).

Analysis of the Correlation Between Cytokine and Transcription Factor Expression

The correlations between expression of cytokines and transcription factors were examined by calculation of Pearson correlation coefficients. In the AAV-T-bet-treated group, the IFN-γ level in BAL fluid was positively correlated with mRNA expression of *T-bet* in lung tissue ($r = 0.76$, $P < .05$), and IL-4 concentration was also positively correlated with lung mRNA levels of *GATA3* ($r = 0.81$, $P < .05$). However, there were negative correlations between IFN-γ and IL-4 ($r = –0.79$, $P < .05$) and between *T-bet* and *GATA3* ($r = –0.77$, $P < .05$).

Discussion

The purpose of this study was to explore the effect of intranasal delivery of *T-bet* by AAV-T-bet on the differentiation of T_H cell subsets in a mouse model of allergic airway inflammation. We demonstrated that more inflammatory cells, including macrophages, lymphocytes, and eosinophils, infiltrated the lung in OVA control mice. The serum levels of total and OVA-specific IgE and airway mucus secretion were increased after allergen inhalation in sensitized mice, findings which were consistent with a T_H2-dominated transcription factor (GATA3) and cytokine (IL-4, IL-5) profile. Intranasal application of 1.5×10^8 vg AAV-T-bet twice before inhalation challenge abrogated the imbalance of T_H2 response in vivo including overproduction of the T_H2 cell signature cytokine IL-4 and the specific transcription factor *GATA3*, while the T_H1 signature cytokine IFN-γ and specific transcription factor *T-bet* showed opposite changes. At the same time, the expression of *Foxp3* mRNA was increased, and inflammatory cell infiltration of the airway and mucus gland hypersecretion were inhibited. In addition, repeated AAV-T-bet administration also significantly decreased serum levels of total and OVA-specific IgE.

The gene *TBX21* (GenBank accession No. NM-013351) encodes T-bet, which is a T_H1 cell-specific transcription factor and shows the ability to direct T_H2 cells to a T_H1 lineage [15]. It is now clear that IFN-γ and IL-4 play important roles in the differentiation of T_H1 and T_H2 cells by controlling the expression of *T-bet* and *GATA3* [5,16]. T-bet and GATA3 interact with each other, and their production is induced by IFN-γ and IL-4 through feedback loops [17]. Specifically, T-bet promotes a T_H1 response by inducing the production of IFN-γ and inhibiting IL-4 and IL-5 expression [5,10,18,19]. At the same time, GATA3 promotes a T_H2 response through 3 different mechanisms: induction of T_H2 cytokine production, selective growth of T_H2 cells, and inhibition of T-bet [20]. Moreover, *T-bet* knock-out mice manifest multiple pathological and inflammatory features of asthma, suggesting that the T-bet deficiency itself may induce allergic asthma in the absence of allergen [10]. With the administration of AAV-T-bet in the murine model of allergic airway inflammation in the present study, we discovered that the levels of IFN-γ and IL-4 in BAL fluid were positively correlated with mRNA expression of *T-bet* and *GATA3*, respectively, in the lung, and there was a negative correlation between IFN-γ and IL-4 and between *T-bet* and *GATA3*. These findings suggest that the secretion of IL-4 and IFN-γ was controlled by T-bet and GATA3, respectively. Our findings also indicate that intranasal delivery of AAV-T-bet before allergen challenge potently activated the expression of IFN-γ, increased T_H1 cell number and/or activity, decreased T_H2 cytokine production and corrected the T_H2-biased immune response.

Although the exact biological mechanism underlying the regulatory role of T-bet remains to be elucidated, previous studies demonstrated that retroviral transfection of *T-bet* in primary T cells or developing T_H2 cells resulted in activation of IFN-γ production, whereas mice lacking *T-bet* failed to develop T_H1 cells and displayed a dramatic reduction of IFN-γ production by $CD4^+$ T cells [15]. Overexpression of T-bet by retroviral gene transduction in primary T cells is reported to be sufficient to induce IFN-γ production by chromatin remodeling at the IFN-γ gene locus and direct transactivation of the IFN-γ gene promoter in an IL-12-independent fashion [5,7]. Recent reports have shown that IFN-γ is acutely regulated by T-bet activity and is a direct gene target of T-bet [21], as well as demonstrating that the principal function of T-bet in developing T_H1 cells is to negatively regulate GATA3 expression rather than to positively regulate the expression of IFN-γ [22]. Additional studies showed that T-bet could be expressed in dendritic cells at levels comparable to those in T_H1 cells and that it was necessary for the optimal production of IFN-γ and optimal activation of antigen-specific T_H1 cells [23,24]. Hence, it is not inconceivable that intranasal administration of AAV-T-bet may deliver *T-bet* to T_H cells and dendritic cells of the airway and blood in our mouse model of allergic airway inflammation and eventually induce a T_H1 response.

Although treatment with AAV-T-bet restored the T_H1/T_H2 balance and suppressed airway inflammation, the signature cytokine of T_H1 cells, IFN-γ, has a stimulatory role in inflammation. To address why an enhanced T_H1 response did not aggravate airway inflammation and whether transfer of *T-bet* can induce an enhanced immune response, we measured the mRNA levels in lungs of *Foxp3*, which is a specific transcription factor in regulatory T (Treg) cells. It has been reported that Treg cells are a subpopulation of suppressor T cells, and they have recently gained attention since they appear to play a key role in the maintenance of immunological balance [25]. It was reported that allergen-specific Treg cells were thought to be present at a higher frequency in nonallergic individuals than in allergic patients and that this was responsible for downregulating immune responses at the level of the effector T cells, either through regulation of IL-2 production or through modulating the expression of IL-10, CTLA-4, or transforming growth factor ß [26,27]. Our experimental results showed that levels of *Foxp3* expression were lower in the model of allergic airway inflammation than in saline control mice. After administering AAV-T-bet, we found that levels of *Foxp3* were clearly increased. This suggests that gene transfer of *T-bet* induced the expression of *Foxp3* and the

activity of Treg cells, and that this prevented an excessive T_H1 immune response.

In initial studies of Foxp3, $CD4^+CD25^+$ T cells were usually considered the only cell subpopulation that expressed Foxp3. Recent studies have shown that Foxp3 is also expressed in T_H1-like cells or T_H2-like cells [28,29]. T_H1-like cell expressed both T-bet and Foxp3, while T_H2-like cell expressed both GATA3 and Foxp3. These cells function as both T_H1/T_H2 cells and are involved in immunosuppression, as well as playing a role in the maintenance of peripheral tolerance. Thus, further studies will be required to determine whether transcription-factor feedback loops induced differentiation of T_H1-like cells or the transfer of *T-bet* induced the differentiation of Treg cells while promoting T_H1 cell differentiation.

In the present study, we observed that administration of AAV-T-bet decreased bronchial eosinophilic inflammation and the levels of total and OVA-specific serum IgE in a model of allergic airway inflammation. We attributed those phenomena to lessened production of IL-5 and IL-4 in bronchi and lungs from mice treated with AAV-T-bet. Indeed, circulating IL-5 is one of the major stimuli for differentiation of eosinophils and migration of eosinophils toward the airways [30]. Previous studies have reported that eosinophil infiltration could cause exacerbations of asthma. One possible cause was that eosinophil-derived fibrogenic factors and growth factors amplified airway remodeling and associated mucus production relatively rapidly, and then accelerated disease progression [31]. Furthermore, IL-4, together with the related cytokine IL-13, is important for isotype switching of B lymphocytes to secrete IgE, the characteristic antibody that underlies atopy, and serum level of IgE is generally used as a marker for in vivo IL-4 activity. Therefore, we suggest that administration of AAV-T-bet reduced the production of IgE by suppressing immunoglobulin isotype class switching.

In conclusion, intranasal transfer of *T-bet* prevented a T_H2-biased immune response by restoring the T_H1/T_H2 balance during the challenge phase, resulting in the inhibition of airway inflammation. These findings suggest that T-bet may represent a target for the treatment of allergic airway inflammation. Taken together, our results indicate that the development of an immunomodulation strategy based on T-bet might shed light on more effective treatments for allergic airway inflammation.

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