

Liver-specific allergen gene transfer by adeno-associated virus suppresses allergic airway inflammation in mice

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Short title: Liver OVA expression by AAV lowers allergic asthma

Abstract

Allergic airway inflammation driven by T helper 2 (Th2)-type immunity is characterized by airway hyperresponsiveness, eosinophilic infiltration, and elevated IgE production. Various novel strategies for managing asthma have been explored, such as DNA vaccines, T-cell peptides, and allergen-specific immunotherapy. A principal goal of most immunotherapeutic approaches is active and long-term allergen-specific tolerance. Liver-specific gene transfer using adeno-associated virus (AAV) has been shown to favorably induce tolerogenic responses to therapeutic products in various experimental models. AAV8 has strong liver tropism and induces immune tolerance in mice. The present study aimed to determine whether hepatocyte-specific allergen expression by pseudotyped AAV2/8 alleviates asthmatic symptoms in ovalbumin (OVA)-sensitized mice. Mice were intravenously injected with AAV2/8 vector carrying membrane-bound OVA transgene under transcriptional control of a hepatocyte-specific alpha 1 antitrypsin promoter (AAV2/8-OVA) and then sensitized with OVA. AAV2/8-OVA specifically transduced the OVA transgene in the liver. Airway hyperresponsiveness, eosinophilia, mucus hyper-secretion, and Th2 cytokines were significantly suppressed in both the lungs and secondary lymphoid organs of asthmatic mice infected with AAV2/8-OVA. Significant reduction of OVA-specific antibodies was detected in the bronchoalveolar lavage fluid from AAV2/8-OVA-treated mice. Moreover, AAV2/8-OVA treatment prominently promoted the expression of Foxp3, IL-10, and TGF- β in the liver. Enhanced Foxp3 expression was also detected in the lungs of asthmatic mice after AAV2/8-OVA treatment. Taken together, these results suggest that the induction of immune tolerance by hepatic AAV gene transfer may be beneficial for modulating allergic asthma.

Keywords: AAV, allergy, asthma, gene therapy, liver

Introduction

Allergic asthma is a pulmonary inflammatory disease characterized by airflow obstruction, persistent inflammation, and airway hyperresponsiveness (AHR) to common environmental aero-allergens.¹ Asthmatic patients exhibit elevated allergen-specific IgE, mucus hypersecretion, and eosinophilia in the lungs.² CD4⁺ T helper 2 (Th2) cells play a major role in the induction and maintenance of airway inflammation.³ Th2 cytokines, especially interleukin (IL)-4, IL-5, and IL-13, and other inflammatory mediators secreted by eosinophils and mast cells are primarily thought to orchestrate these pathological features of asthma.³ Most therapeutic strategies for asthma, such as β 2-adrenergic receptor agonists, anti-IgE blockers, antagonists of cytokines and mediators, as well as corticosteroids, only provide temporary alleviation by suppressing inflammatory responses and have certain side effects.⁴ Therefore, the development of more effective, safer, and long-lasting therapeutic or preventive approaches is pivotal for asthma.

The liver preferentially facilitates local or systemic immune tolerance via unique antigen-presenting cells (APCs) and the cytokine milieu.⁵ The advantages of sustained transgene expression and the tolerogenic immunity of the liver are widely utilized and investigated for the treatment of hepatitis, hemophilia, and inherited metabolic disorders by gene therapy.⁶ T cell activation in the liver promotes tolerant immune responses by inducing anergy, clonal deletion, or regulatory T cell (Treg) generation.⁷⁻⁹ Accumulated evidence indicates that *in vivo* hepatocyte-specific gene transfer contributes to the induction of immune tolerance and has therapeutic potential in mice.⁶ Some reports have proposed that antigen-specific Foxp3⁺ Tregs are generated following hepatic gene transfer, which prevents or suppresses the development of experimental autoimmune encephalomyelitis (EAE).^{7,10} Lüth et al. confirmed that specific expression of neural

autoantigen in hepatocytes, instead of other tissues, protects against neuron inflammation in EAE.¹⁰ CD4⁺CD25⁺Foxp3⁺ Tregs mainly engage in negative control of autoimmune responses and allergic diseases, such as asthma.¹¹ However, whether hepatocyte-specific allergen expression modulates allergic airway inflammation has not yet been reported.

Adeno-associated virus (AAV), a single-stranded DNA virus with low immunogenicity, consists of numerous serotypes that target various tissues and establish long-term transgene expression.¹² AAV1, AAV5, AAV8, and AAV9 efficiently infect muscle, retina, liver, and lung tissue, respectively.¹³ Recombinant AAV (rAAV) vectors derived from *rep* of AAV2 and *cap* of different serotypes would enhance gene transduction efficacy, change tissue tropism, and evade pre-existing neutralizing antibodies.¹³ Gene therapy with rAAV vectors has been employed for studies in animal models and clinical trials. Several reports have suggested that rAAV vectors carrying immunomodulatory transgenes, such as T-bet¹⁴ and Clara cell 10-kDa protein (CC10),¹⁵ or antisense sequences against Th2 cytokines¹⁶ and chemokines¹⁷ attenuate allergen-induced inflammation in asthmatic models. Because of the potential tolerogenic immune responses induced by hepatocyte-specific gene expression, we investigated whether Th2 cell-driven inflammation in allergic asthma is modulated by rAAV carrying an allergen transgene targeting hepatocytes. As the AAV8 serotype had been shown to superiorly transduce liver tissue and lead to immune tolerance in mice,^{18,19} we applied ovalbumin (OVA) as a model antigen and examined the immunomodulatory effects of AAV2/8 vector carrying a liver-specific membrane-bound OVA transgene (AAV2/8-OVA). Significant suppression of asthmatic symptoms was achieved in OVA-sensitized mice treated with AAV2/8-OVA. These results suggest that hepatocyte-specific allergen gene expression may be considered a novel approach for

alleviating allergic asthma.

Materials and Methods

Construction and preparation of pseudotyped AAV2/8 vector

A recombinant AAV (rAAV) construct was created with the coding sequence of chicken OVA or enhanced green fluorescence protein (EGFP) as the vector control. The full-length OVA DNA fragment (386 amino acids) was derived from pAc-neo-OVA (gift of Dr. MJ Bevan).²⁰ The OVA₃₈₆ fragment was fused with 178 COOH terminal amino acids of B7-1 domain including the transmembrane domain by PCR to generate membrane-bound OVA. Next, the OVA/B7-1-fused fragment was subcloned into pAAV vector containing the inverted terminal repeats (ITRs) of AAV serotype 2 at both ends. The expression cassette comprised a hepatocyte-specific alpha 1 antitrypsin (hAAT) promoter, a 9-amino-acid influenza virus HA tag, and a polyadenylation signal sequence.

Recombinant AAV2/8 vectors were generated using a tri-plasmid system as described previously.¹⁷ Briefly, the pAAV/hAAT plasmid with the OVA or EGFP transgene was co-transfected into HEK 293T cells with an adenovirus helper plasmid and a packaging chimeric plasmid encoding the AAV2 rep and AAV8 cap genes. Recombinant single strand AAV vectors were purified by cesium chloride sedimentation. Genome copies (GCs) of AAV2/8-OVA or AAV2/8-EGFP were determined by quantitative PCR (qPCR) using SYBR Green Master mix (Roche Diagnostics GmbH, Mannheim, Germany).

Animals and OVA-sensitization protocol

Three-week-old female BALB/c mice were purchased from National Laboratory Animal Center (Taipei, Taiwan, ROC) and acclimatized for 1 week. Four-week-old mice were administered 1×10^{11} GCs per mouse in 200 μ l of normal saline via intravenous

injection. Four weeks after virus treatment and before the sensitization protocol, blood samples were collected under anesthesia from the retro-orbital venous plexus using heparinized capillary tubes. All mice (8 weeks old) were sensitized by intra-peritoneal injection of 50 µg OVA (grade V, Sigma-Aldrich Inc., MO, USA) mixed with 0.8 mg aluminum hydroxide (Pierce Biotechnology Inc., Rockford, IL, USA) on days 56, 57, 58, and 70 after virus administration. These mice were challenged by inhaling 2% aerosolized OVA for 20 minutes using an atomizer (Pulmo-aide, DeVilbiss Health Care, Somerset, PA, USA) on days 70, 73, 77, 80, and 84. AHR was analyzed on day 85, and mice were sacrificed 24 hours later. The care and handling of mice in all experimental procedures was approved by the Institutional Animal Care Committee of Chang Gung University and complied with the guides of National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals.

Assessment of AHR and cell composition in bronchoalveolar lavage fluid (BALF)

AHR was assessed on day 85, 24 hours after the last challenge, using methacholine (Mch; Sigma). Mice were exposed to normal saline or increasing concentrations of Mch (10, 20, 30, and 40 mg/ml) for 3 minutes and then underwent whole body plethysmography while conscious. The enhanced pause (Penh) value was measured for 3 minutes and pulmonary airway obstruction analyzed by BioSystem XA software (Buxco Electronics, Inc., Wilmington, NC, USA). As described in detail in our previous reports, the sensitized mice had significantly enhanced Penh values compared to the normal healthy control mice.^{21,22}

Following the measurement of AHR, the tracheas of mice from different groups were cannulated and the lungs lavaged three times with 1 ml phosphate-buffer saline

(PBS) supplemented with 0.1 mM EDTA. Cells in the BALF were collected and counted using trypan blue dye exclusion. For differential cell counts, BALF cells were centrifuged (Shandon Cytospin 4; Thermo Scientific, Pittsburg, PA, USA) and stained with Liu staining solution. Different cell types were distinguished under light microscopy by counting at least 200 cells based on their morphological profile.

Lung histology

On day 86, non-lavaged lung tissues were removed and fixed with 4% formaldehyde for 24 hours at 4°C for histological examination. After dehydration, the tissues were embedded in paraffin and sliced into 4 µm sections. The tissue sections were subjected to hematoxylin and eosin (H&E) staining and periodic acid-Schiff (PAS) (Sigma) staining. Inflammatory cell infiltration and goblet cell hyperplasia were examined by automated MetaMorph microscopy and image analysis software (Molecular Devices).

Measurement of OVA-specific IgE

The titers of OVA-specific IgE antibodies in the BALF and serum were determined by ELISA. Briefly, the plates (Costar, Corning, NY, USA) were coated with 10 µg/ml OVA at 37°C for 1 hour. The OVA-coated plates were incubated with diluted BALF or serum samples at 37°C for 1 hour. Next, biotinylated rat anti-mouse IgE monoclonal antibodies (BD PharMingen, San Diego, CA, USA) and streptavidin-conjugated horseradish peroxidase (HRP) (BD PharMingen) were sequentially added to the plates. Finally, the plates were incubated with substrate solution (TMB, BD Biosciences) for 20 minutes and the reaction stopped with 2N H₂SO₄. Absorbance was measured on an ELISA reader at 450 nm (SpectraMax M2, Molecular Devices, CA, USA).

Cell culture and cytokine assays

Spleens and mediastinal lymph nodes (mLNs) were removed and single cell suspensions prepared. Cells (5×10^6 cells/ml) were cultured in RPMI 1640 medium (Gibco, Life Technologies, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Gibco) with 100 μ g/ml OVA. Culture supernatants were harvested on day 6 and the levels of interleukin (IL)-4, IL-13 (DuoSet ELISA kit, R&D systems), and IL-5 (BD OptEIA set, BD Pharmingen) determined by ELISA according to the manufacturer's instructions.

RNA isolation and qPCR

Total RNA was extracted from homogenized lung and liver tissues using TRIzol reagent (Invitrogen, Life Technologies) and treated with DNase I (Fermentas, Thermo Scientific). The cDNA were generated using random primers and M-MLV reverse transcriptase (Invitrogen). The sequences of specific primers for qPCR of CD4 and CCL22 are listed in Supplementary Table S1. Other qPCR primers for IL-4, IL-5, IL-10, IL-12p35, IL-12p40, IL-13, IFN- γ , CCL11, CCL17, CCL24, Foxp3, TGF- β , β -actin, gob5, and Muc5ac were published previously.^{23,24} The cDNA samples were mixed with SYBR Green/ROX qPCR Master Mix (Fermentas) and amplified in a CFX Connect real-time PCR detection system (Bio-Rad). The thermal cycling conditions were 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. The relative expression of each gene was calculated by normalizing the levels to the expression of housekeeping gene β -actin. The expression of Foxp3 in CD4⁺ cells was calculated and normalized to CD4 expression. The fold change was relative to the untreated group.

Statistical analysis

Results are presented as mean $\pm$ SEM. Significance was assessed using a two-tailed *P*-value calculated by the Mann-Whitney *U* test. *P*<0.05 was considered significant. All graphs were generated and statistical analyses performed using GraphPad Prism 5.0 software.

Results

Liver-specific OVA expression by AAV2/8 vector virus suppressed AHR and OVA-specific IgE in OVA-sensitized mice

We constructed DNA encoding the membrane-bound OVA sequence using the rAAV2/8 vector with hepatocyte-specific alpha 1 antitrypsin promoter (Figure 1a) to achieve hepatocyte-specific OVA expression. The expression of OVA on the cell surface was confirmed in pAAV/hAAT-OVA-transfected Huh7 cells (Supplementary Figure S1a). Furthermore, mice infected with AAV2/8-OVA exhibited significant OVA transgene expression in the liver but not in the lung (Supplementary Figure S1b). To examine whether hepatic OVA expression alleviates the development of airway inflammation *in vivo*, mice were infected with AAV2/8-OVA or AAV2/8-EGFP control viruses and then sensitized with OVA (Figure 1b). After the final challenge, AHR was assessed in response to increasing concentrations of inhaled methacholine (Mch). When mice were stimulated with 40 mg/ml Mch, the enhanced pause (Penh) value was significantly lower in AAV2/8-OVA-treated mice than the non-treatment and AAV2/8-EGFP control groups (Figure 1c) and was comparable to healthy normal controls in our previous studies.^{21,22} Decreased Penh values were also measured in the AAV2/8-OVA vs. AAV2/8-EGFP control groups when mice were stimulated with 30 mg/ml Mch.

A significant reduction in OVA-specific IgG1, IgG2a (Supplementary Figure S2), and IgE (Figure 1d) was detected in the BALF from OVA-sensitized mice treated with AAV2/8-OVA compared to the non-treatment and AAV2/8-EGFP control groups. The data indicate that administration of AAV2/8-OVA attenuated AHR and inhibited OVA-specific antibody production in the lungs of OVA-sensitized mice.

AAV2/8-OVA significantly decreased cell infiltration and mucus hypersecretion in the lungs of OVA-sensitized mice

Because the infiltration of eosinophils and lymphocytes into lung tissue is one of the main characteristics of asthmatic patients, we analyzed the inflammatory cell composition of BALF from OVA-sensitized mice. A markedly decreased percentage of eosinophils was found in mice treated with AAV2/8-OVA compared to the non-treatment and AAV2/8-EGFP control groups (Figure 2a). The absolute numbers of total leukocytes, eosinophils, and lymphocytes were significantly lower in the BALF from AAV2/8-OVA-treated mice than the two control groups (Figure 2b-d). Therefore, AAV2/8-OVA reduced the infiltration of inflammatory cells into the lungs of OVA-sensitized mice.

We further analyzed whether AAV2/8-OVA can suppress cell infiltration and mucus secretion in the airways based on histological examination. Massive inflammatory cells infiltrated peribronchial and perivascular sites in the lungs of untreated and AAV2/8-EGFP control mice, whereas the number of inflammatory cells was much lower in AAV2/8-OVA-treated mice (Figure 3a). Quantitative analysis demonstrated a significant reduction in inflammatory cells in the lungs of AAV2/8-OVA-treated mice compared to the non-treatment and AAV2/8-EGFP control groups (Figure 3c). Similarly, decreased goblet cell hyperplasia and mucus production were observed in the airways of AAV2/8-OVA-treated mice compared to the two control groups (Figure 3b and d). To confirm this result, *gob5*, a molecule associated with mucin gene regulation and goblet cell hyperplasia, and *Muc5ac* expression were analyzed by qPCR (Figure 3e and f). Mice treated with AAV2/8-OVA expressed lower levels of *gob5* and *Muc5ac* than mice in the non-treatment and AAV2/8-EGFP control groups. Thus, AAV2/8-OVA administration was

able to attenuate the accumulation of inflammatory cells and decrease mucus production in the lungs of asthmatic mice.

Significant reduction of Th2 cytokine and chemokine gene expression in the lungs of AAV2/8-OVA-treated mice

Due to the substantial suppressive effect of AAV2/8-OVA on lung inflammation, we analyzed the expression levels of cytokine and chemokine genes in the lungs. OVA-sensitized mice treated with AAV2/8-OVA expressed significantly lower levels of Th2 cytokines compared to untreated and AAV2/8-EGFP control mice (Figure 4a). However, the RNA expression of Th1-related cytokines, such as IFN- γ , IL-12p35, and IL-12p40, was unchanged (Figure 4a). To determine whether the decreased number of lymphocytes in the lungs is attributed to the abolishment of Th2 cell recruitment, we measured CCL17 and CCL22, which selectively induce the chemotaxis of CD4⁺ Th2 cells into inflamed tissues in allergic diseases. The lungs of untreated and AAV2/8-EGFP control mice expressed large amounts of CCL17 and CCL22 compared to mice receiving AAV2/8-OVA (Figure 4b). The expression of the main chemokines for eosinophil recruitment (CCL11 and CCL24) was also reduced in AAV2/8-OVA-treated mice (Figure 4b). These results indicate that reduced chemokine production in response to AAV2/8-OVA treatment may prevent inflammatory cells from migrating into the lungs and alleviate the pathological features of OVA-sensitized mice.

AAV2/8-OVA repressed regional and systemic Th2-driven immune responses in the lungs of OVA-sensitized mice

To determine whether AAV2/8-OVA treatment suppressed local Th2 cell-derived

cytokines, culture supernatants of OVA-stimulated lymphocytes from mediastinal lymph nodes (mLNs) were examined. OVA-stimulated lymphocytes from mice receiving AAV2/8-OVA secreted less IL-4, IL-5, and IL-13 than lymphocytes from untreated and AAV2/8-EGFP control mice (Figure 5a). Similarly, OVA-stimulated splenocytes from AAV2/8-OVA-treated mice expressed significantly lower levels of Th2 cytokines compared to untreated and AAV2/8-EGFP control mice (Figure 5b). No significant differences were observed in IFN- γ secretion by OVA-stimulated lymphocytes from mLNs and spleens among the different groups (data not shown). Thus, AAV2/8-OVA inhibited Th2 cell-mediated immune responses and ameliorated the pathogenesis of allergic asthma.

We also examined whether AAV2/8-OVA administration inhibited antibody production against OVA in serum. Before encountering allergen, serum OVA-specific antibodies were hardly detected in all mice (data not shown), even in mice infected with AAV2/8-OVA. Significantly lower levels of serum OVA-specific IgG1 and IgG2a were detected in OVA-sensitized mice treated with AAV2/8-OVA than untreated or AAV2/8-EGFP control mice (Supplementary Figure S3a and S3b). The ratio of OVA-specific IgG1 to IgG2a was also significantly reduced (Supplementary Figure S3d). However, hepatic OVA expression did not seem to affect serum OVA-specific IgE in asthmatic mice (Supplementary Figure S3c). These data suggest that the administration of AAV2/8-OVA suppressed the production of OVA-specific antibodies in OVA-sensitized mice.

Higher Foxp3 expression was detected in liver and lung tissues from

AAV2/8-OVA-treated mice

To explore the possible mechanisms involved in modulating the hypo-immune responses to allergen, we examined the proportions and activation phenotypes of various lymphocyte subpopulations in the liver, spleen, liver-draining lymph nodes, and mLNs. The frequencies of NK, NKT, CD3⁺, CD4⁺, and CD8⁺ T cells were similar in all groups (Supplementary Figure S4a). The activation profiles of CD4⁺ and CD8⁺ T cells were comparable among the different groups (Supplementary Figure S4b). Hepatocyte-restricted transgene expression was previously reported to promote immune tolerance by inducing Foxp3 expression in CD4⁺CD25⁺ Treg cells.^{7,10} Thus, we investigated whether Foxp3 expression by CD4⁺ T cells from asthmatic mice was affected by AAV2/8-OVA treatment. The frequency of CD4⁺CD25⁺Foxp3⁺ T cells did not differ significantly in AAV2/8-OVA-treated mice and control mice (data not shown). The mean fluorescence intensity (MFI) of Foxp3 in liver-draining lymph node-isolated CD4⁺ T cells was elevated by AAV2/8-OVA treatment compared to the non-treatment group (Supplementary Figure S5). We further applied qPCR, a more sensitive method, to detect the Foxp3 RNA expression levels in CD4⁺ cells. The CD4⁺ cells from mice treated with AAV2/8-OVA expressed significantly increased levels of Foxp3 in the liver and lungs (Figure 6a and b). Higher TGF- β and IL-10 RNA levels were also observed in the livers of mice treated with AAV2/8-OVA compared to control groups (Figure 6c and e). In the lungs, IL-10 expression was greatly decreased in mice treated with AAV2/8-OVA compared to control groups (Figure 6f), but the level of TGF- β was not different between the groups (Figure 6d). These results imply that liver-specific allergen expression may create a tolerogenic microenvironment through enhanced amounts of TGF- β and IL-10 in the liver, promoting Foxp3 expression in CD4⁺ T cells.

Discussion

Allergic asthma has become a global public health issue that has been considerable burden on health services and incurs expensive social costs. Despite a growing number of pharmaceutical and immunotherapy strategies for asthma, the development of an active, effective, and long-term immunomodulatory approach has been difficult. Here, our results demonstrate for the first time that specific expression of allergen in hepatocytes by rAAV-mediated gene transfer induces tolerogenic immunity, which alleviates allergic airway inflammation and inhibits Th2 cell-driven immune responses. Pseudotyped AAV2/8 vector with hepatocyte-restricted promoter specifically transduced the allergen gene in the liver. OVA-specific Th2 immune responses and antibody production were significantly suppressed in asthmatic mice receiving AAV2/8-OVA. These results suggest that ectopic allergen expression in hepatocytes resulted in local and systemic hypo-immune responses to allergen re-challenge. Consequently, the application of the liver tolerance effect may be a novel strategy for therapeutic modulation and intervention in allergic asthma.

Antigen-specific tolerance has been demonstrated by transgene expression in hematopoietic stem cells, immature APCs, and tolerogenic tissues, such as the liver and thymus.⁵ Gene transfer-mediated immune tolerance has been applied in the treatment of hereditary protein deficiencies²⁵ and autoimmune diseases¹⁰ in animal models and clinical trials.²⁶ In a murine model of hemophilia B, hepatocyte-restricted factor IX expression sustained therapeutic protein levels and evaded unwanted immune responses.²⁷ In addition, hepatocyte-specific gene transfer of α -galactosidase and acid sphingomyelinase has been shown to trigger tolerogenic immunity and improve pathological features of Fabry and Nieman-Pick disease.^{18,28} In an animal model of human multiple sclerosis,

neural autoantigen-expressing hepatocytes prevented and repressed CD4⁺ T cell-driven neuroinflammation.¹⁰ Some potential mechanisms of immune tolerance by hepatic gene transfer have been proposed that involve the elimination and unresponsiveness of effector T cells or the induction of Tregs.^{7,8,10} The development of transgene-specific CD4⁺CD25⁺Foxp3⁺ Tregs has also been reported following hepatic gene transfer.^{7,10} For allergic disease, the current study demonstrates that allergen-expressing hepatocytes effectively protect against asthma development.

How lung inflammation was alleviated by allergen expression in the liver is currently not clear. The phenomenon of “liver tolerance” has been shown to be local and systemic tolerance of self and foreign antigens presented by non-parenchymal cells or expressed in hepatocytes. Several non-parenchymal cells are involved in the tolerogenic properties of the liver, including dendritic cells,²⁹ liver sinusoidal endothelial cells (LSECs),³⁰ Kupffer cells,³¹ and hepatic stellate cells.³² These unique cells regulate tolerant responses via the production of anti-inflammatory cytokines, such as IL-10, TGF- β , or programmed cell death ligand-1.³³ Furthermore, hepatocytes directly contact circulating T cells through the specialized fenestrations of LSECs. The immunological functions of hepatocytes are facilitated by the polarized expression of MHC and ICAM-1 molecules towards the sinusoidal lumen.³⁴ A previous study suggested that T cells activated by hepatocytes die prematurely, leading to immune tolerance.³⁵ Thus, it is likely that antigen presented by hepatocytes or other cell populations is able to modulate dysregulated immune responses.

The induction of CD4⁺CD25⁺ Tregs in tolerance to the transgene product or autoantigen has been reported following hepatic *in vivo* gene transfer.^{7,10} Based on the aberrant number of Tregs and Foxp3 mRNA levels in patients allergic to house dust mites

(HDMs),^{36,37} the increased Foxp3 gene expression in the liver and lungs of AAV2/8-OVA-treated mice suggested the possibility of the development of Tregs. Liver-generated CD4⁺Foxp3⁺ cells are recruited to or circulate in the inflamed lung tissue to suppress Th2 cell-driven inflammation. Thus, the increased Foxp3⁺ gene expression levels in the liver and lungs (Figures 6a and b) suggest that hepatic gene transfer-induced CD4⁺Foxp3⁺ Tregs partially contributed to the tolerogenic effect. Similar to oral tolerance, the reduction of AHR, lung inflammation, and Th2 cytokines is associated with an increased proportion of airway CD4⁺Foxp3⁺ Tregs in asthmatic mice.³⁸ IL-10 and TGF- β are well documented to be crucially involved in the maintenance of immune tolerance to transgene products and peripheral Treg conversion.^{10,39} In the allergen milieu of the liver, significantly increased expression of TGF- β and IL-10 was detected in OVA-sensitized mice treated with AAV2/8-OVA (Figure 6). Allergen stimulation combined with immunosuppressive cytokines in the liver probably facilitated Foxp3 acquisition of CD4⁺ cells with both cytokines. Nevertheless, IL-10 expression was significantly decreased following AAV2/8-OVA treatment in the lungs. Although the reduction in IL-10 may be attributed to reduced lymphocyte recruitment into the lungs, whether IL-10 directly suppresses lung inflammation or indirectly targets allergen-mediated responses through enhanced Foxp3 expression in the lung tissues is not clear. Nevertheless, we cannot formally prove which mechanism is really involved in the suppression of lung inflammation in asthmatic mice, though it is clear that the alleviated allergic responses occurred when the allergen gene was expressed in the liver.

Various factors, including dose, nature, and expressed form of the antigen, exposure route, delivery system, and mouse strain determine the development of immune activation or tolerance. The location where antigen encounters antigen-presenting cells

(APCs) is also important.^{40,41} We tested whether different subcellular antigen localizations will influence immune tolerance by hepatic gene transfer. Although both membrane-anchored and secreted antigens induce antigen-specific antibodies,^{42,43} the activation of CD4⁺ T cells was greater in mice receiving membrane-anchored antigen than those receiving secreted antigen.⁴² Other studies have indicated that mice immunized with soluble antigens encoded by DNA-based vaccine provoke a strong IgG1 response and greater levels of IL-4, but this does not occur with the membrane-bound antigen.^{44,45} Our results indicate that mice receiving secreted OVA (treated with AAV2/8-sOVA) had higher eosinophilia and Th2 cytokine RNA expression in the lungs than mice treated with vector carrying membrane-anchored OVA (Supplementary Figure S6). Highly efficient immune tolerance was also induced by the transmembrane form of neural autoantigen in hepatocytes in autoimmune neuroinflammation.¹⁰ On the other hand, low doses of antigen have been demonstrated to efficiently induce and expand Tregs compared to high doses of antigen.^{46,47} Lower levels of OVA transgene expression were observed in the livers of mice receiving vector carrying membrane-anchored OVA compared to those receiving secreted OVA (data not shown). Thus, we speculate that membrane-bound OVA provides an environment for less effective activation of Th2 cells and less antigen exposure promotes Treg function compared to secreted OVA.

AAV is a favorable vector for gene transfer due to its non-pathogenic nature, widespread infectivity, and stable transgene expression.¹² Over the past few decades, pseudotyped AAV vectors have been developed extensively and exerted therapeutic efficacy in disease models. A growing number of successful gene therapies have also been reported in clinical trials of AAV for inherited disorders and acquired diseases.²⁶ Our previous studies successfully applied an AAV2/9 vector carrying CC10 to efficiently

target lung epithelial cells and restore CC10 levels, alleviating lung inflammation in OVA-sensitized mice.¹⁵ In addition, AHR, eosinophilia, and airway remodeling were significantly mitigated in HDM allergen-sensitized mice with AAV2/9-delivered CCL11 small hairpin RNA.¹⁷ Compared to overall immune suppressive chemokine-modulatory gene sequences, antigen-specific immune tolerance may have fewer side effects. In addition, the liver is a more tolerogenic organ and evidence has demonstrated that hepatic gene transfer using AAV vector may promote transgene-specific systemic immune tolerance.^{7,48,49} The AAV8 serotype preferentially targets the liver to support sustained transgene products and induce active tolerogenic responses.^{50,51} AAV2/8 vector carrying therapeutic transgenes predominantly assists tolerant responses to transgenes in mice with lysosomal storage disorders.^{18,28} The livers of patients with hemophilia B have been transduced successfully by AAV2/8-Factor IX vectors with no lasting toxicity and transiently achieving therapeutic levels.⁵² We established liver-restricted allergen expression with AAV2/8 vectors, which resulted in immune hypo-responsiveness to OVA-induced antibody production and Th2 cytokine secretion in OVA-sensitized mice. Thus, AAV2/8 vector combined with a hepatocyte-specific promoter was a beneficial vehicle for hepatic gene transfer to suppress hypersensitive lung responses to allergen. Similar strategies may be applied for other allergic diseases that do not have a localized target, such as atopic dermatitis. Furthermore, liver-mediated immune tolerance may be beneficial for systemic or organ-specific autoimmune diseases.

In conclusion, ectopic expression of antigen in the liver by AAV-directed gene transfer has been applied gradually to induce tolerant responses against transgene products. For the first time, our study indicates that hepatocyte-restricted allergen expression by pseudotyped AAV2/8 vector effectively suppressed asthmatic pathogenesis

in allergen-sensitized mice. AAV2/8 vector carrying the OVA transgene up-regulated the expression of immunosuppressive cytokines, which may facilitate Foxp3 acquisition of CD4⁺ cells in the liver. Therefore, targeting allergen expression to the liver serves as a novel approach to induce active tolerance for the treatment of allergic disease.

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Author Disclosure

None of the authors has any financial conflict of interest related to this manuscript.

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Figure legends

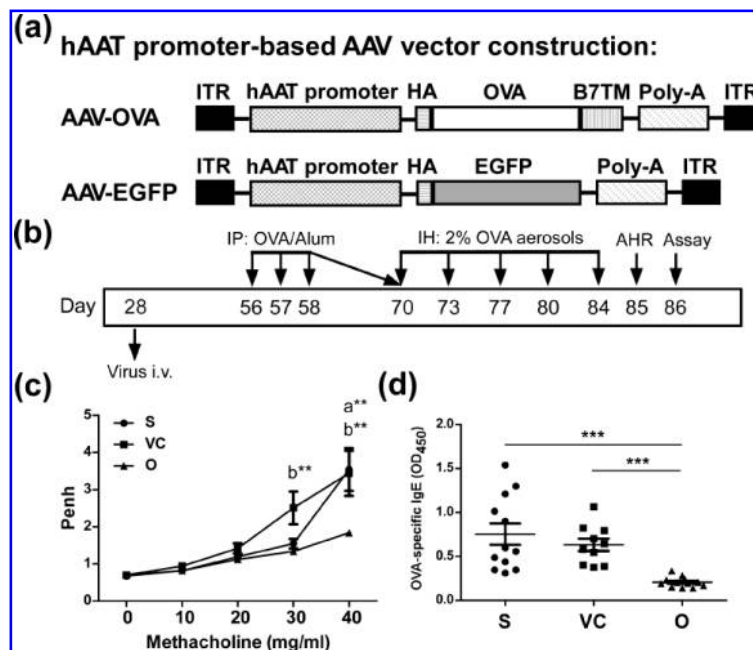


Figure 1. AAV2/8-OVA ameliorated airway hyper-responsiveness (AHR) and inhibited OVA-specific antibody secretion in OVA-sensitized mice. **(a)** Schematic diagrams of plasmids encoding OVA and EGFP transgenes, which are composed of the hepatocyte-specific alpha 1 antitrypsin (hAAT) promoter, a 9-amino-acid influenza virus HA tag, and a polyadenylation sequence. The membrane-bound OVA expression cassette was constructed by fusing the murine B7-1 transmembrane domain (B7TM) with a cytosolic tail. ITR, inverted terminal repeat of AAV. **(b)** Experimental procedure for rAAV2/8 vector treatment and OVA sensitization. Mice were treated with AAV2/8 vector and sensitized with OVA. IV, intravenous; IP, intraperitoneal; IH, inhalation. **(c)** Methacholine-induced AHR. OVA-sensitized mice were analyzed in response to increasing concentrations of inhaled methacholine on day 85, after the last OVA challenge. Data were represented as enhanced pause (Penh) values (mean $\pm$ SEM; n=10-12 per group). Bronchoalveolar lavage fluid (BALF) samples were collected at the

end of the experiment. **(d)** The levels of OVA-specific IgE (1:2 dilution) were determined by ELISA. Data are presented as mean $\pm$ SEM (n=10-12 per group). S, sensitized control; VC, AAV2/8-EGFP vector control; O, AAV2/8-OVA vector. ** $P<0.01$ vs. S in (a) or vs. VC in (b), *** $P<0.001$.

Figure. 2. AAV2/8-OVA reduced the infiltration of eosinophils and lymphocytes in the BALF from OVA-sensitized mice. Lung tissues were lavaged with PBS and **(a)** the percentages of different cells and absolute numbers of **(b)** total leukocytes, **(c)** eosinophils, and **(d)** lymphocytes were assessed by light microscopy using Liu staining. Data are presented as mean $\pm$ SEM (n=10-12 per group). Eo, eosinophils; Ne, neutrophils; Ly, lymphocytes; Mo, monocytes. S, sensitized control; VC, AAV2/8-EGFP vector control; O, AAV2/8-OVA vector. * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

Figure. 3. Lung histopathology of OVA-sensitized mice treated with AAV2/8-OVA. Lung tissues were harvested and fixed in formaldehyde at the end of the sensitization protocol. Paraffin-embedded lung tissues were sliced and subjected to **(a)** hematoxylin and eosin (H&E) and **(b)** periodic acid-Schiff (PAS) staining. Representative sections are shown for each group. Scale bar = 200 μ m. **(c)** Inflammatory cell infiltration and **(d)** goblet cell hyperplasia were quantified by MetaMorph and presented as percentages (mean $\pm$ SEM). Quantitative results in untreated mice are defined as 100%. The RNA expression levels of **(e)** gob5 and **(f)** Muc5ac were analyzed by quantitative PCR (n=9 per group) and normalized to β -actin. The fold change was contrasted to untreated mice and presented as a percentage (mean $\pm$ SEM). S, sensitized control; VC, AAV2/8-EGFP vector control; O, AAV2/8-OVA vector. * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

Figure. 4. Expression of Th2-related cytokines and eotaxin chemokines was suppressed in the lungs of OVA-sensitized mice treated with AAV2/8-OVA. Total RNA was extracted from homogenized lung tissues and the RNA expression levels of **(a)** Th2 or Th1 cytokines and **(b)** chemokines detected by quantitative PCR (n=9 per group). The relative quantity of each RNA was normalized to β -actin and the fold change determined relative to the untreated group. Data are presented as mean $\pm$ SEM. S, sensitized control; VC, AAV2/8-EGFP vector control; O, AAV2/8-OVA vector. * P <0.05; ** P <0.01; *** P <0.001.

Figure. 5. Inhibited Th2 cytokine secretion by OVA-stimulated lymphocytes from the mediastinal lymph nodes (mLNs) and spleens of OVA-sensitized mice treated with AAV2/8-OVA. Lymphocytes from **(a)** mLNs (n=4-6 samples in each group, each sample was pooled cells from 2-3 mice) and **(b)** spleens (n=10-12 per group) were collected and re-stimulated with 100 μ g/ml OVA for 6 days. Culture supernatants were measured for IL-4, IL-5, and IL-13 using ELISA. Data are presented as mean $\pm$ SEM. S, sensitized control; VC, AAV2/8-EGFP vector control; O, AAV2/8-OVA vector. * P <0.05; ** P <0.01; *** P <0.001.

Figure. 6. AAV2/8-OVA significantly enhanced Foxp3 expression in CD4⁺ cells from the livers and lungs of OVA-sensitized mice. The livers (n=6-8 per group) and lungs (n=10 per group) were homogenized for RNA extraction at the end of the sensitization protocol. Expression levels of **(a, b)** Foxp3, **(c, d)** TGF- β , and **(e, f)** IL-10 mRNA were determined by quantitative PCR. The relative quantity of each mRNA was normalized to β -actin. Foxp3 expression levels in CD4⁺ cells were calculated as the normalized levels of Foxp3

expression divided by the normalized levels of CD4 expression. The fold change was relative to the untreated group. Data are presented as mean $\pm$ SEM. S, sensitized control; VC, AAV2/8-EGFP vector control; O, AAV2/8-OVA vector. * $P < 0.05$; ** $P < 0.01$.

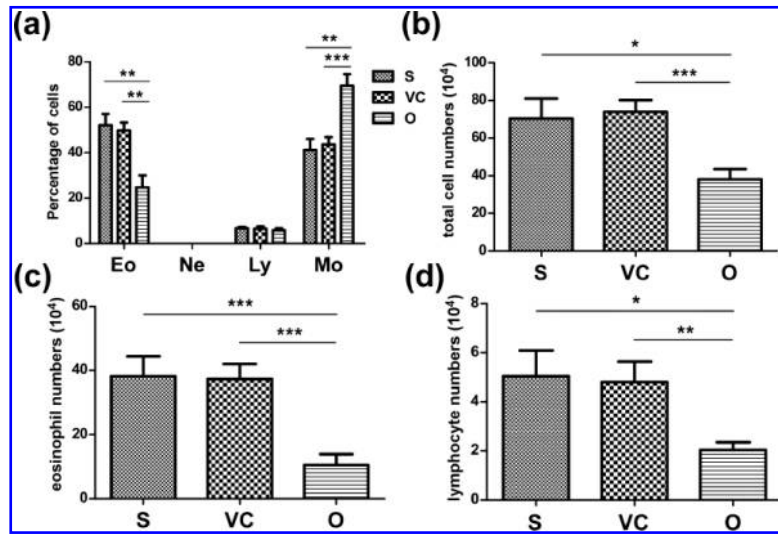


Fig. 2. AAV2/8-OVA virus reduced the infiltration of eosinophils and lymphocytes in BALF from OVA-sensitized mice.

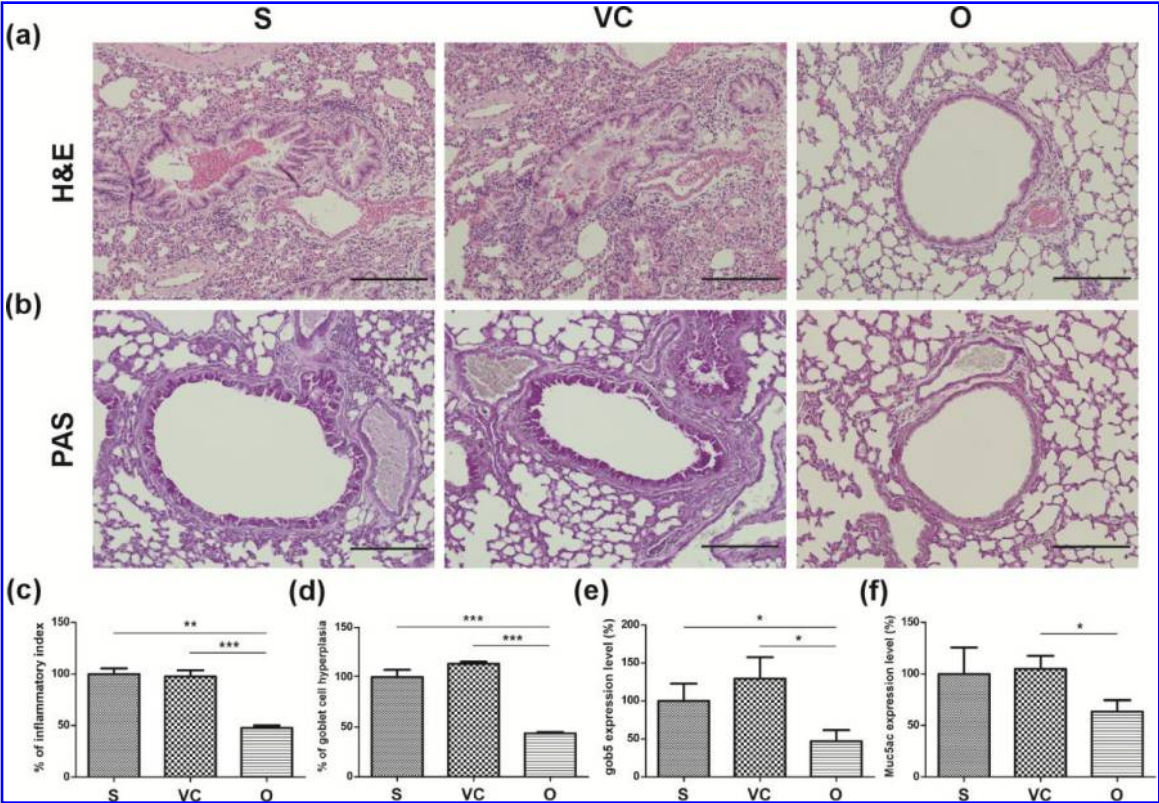


Fig. 3. Lung histopathology of OVA-sensitized mice receiving AAV2/8-OVA viruses.

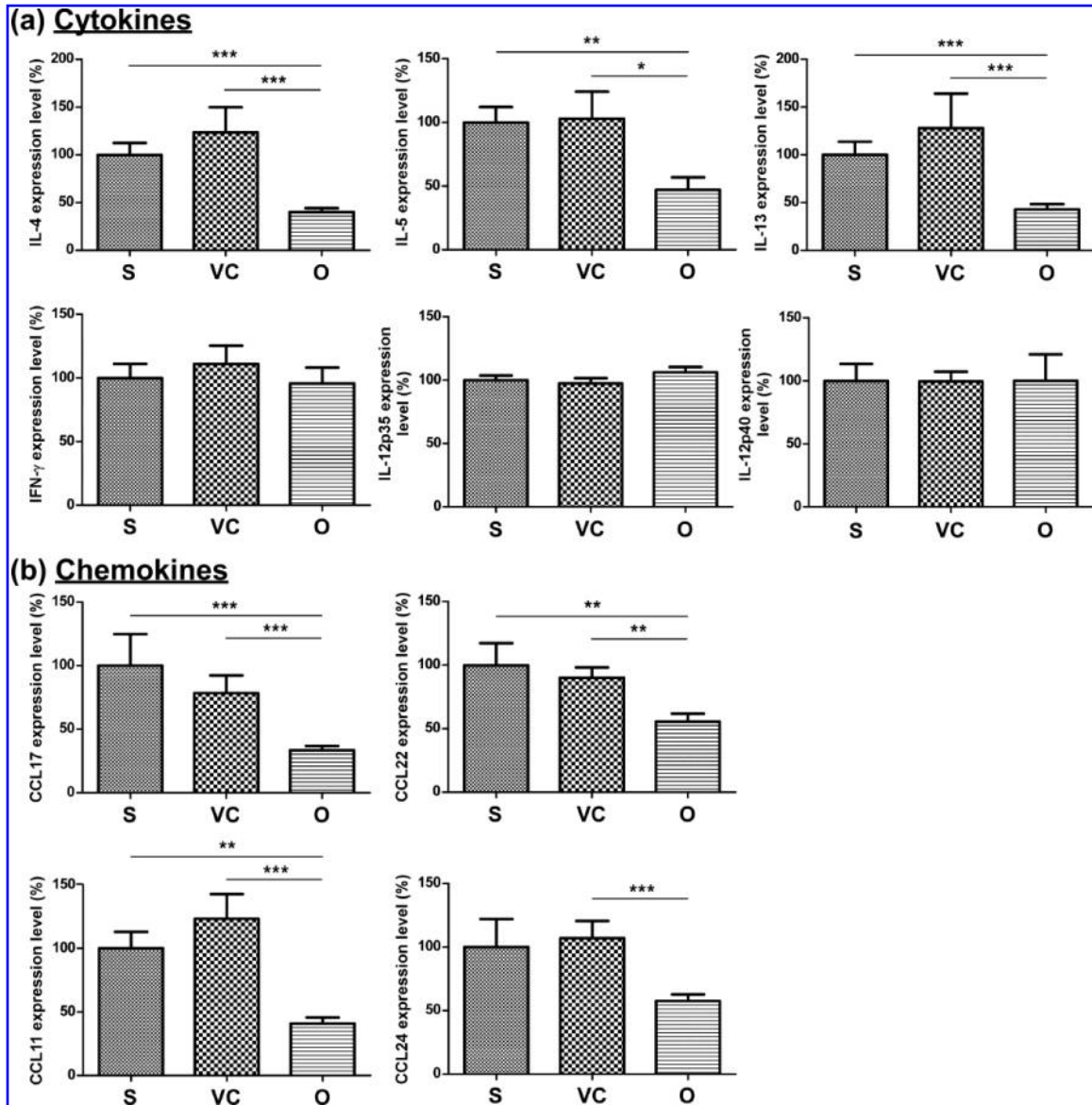


Fig. 4. Expression of Th2-related cytokines and eotaxin chemokines were suppressed in the lungs of OVA-sensitized mice with AAV2/8-OVA virus treatment.

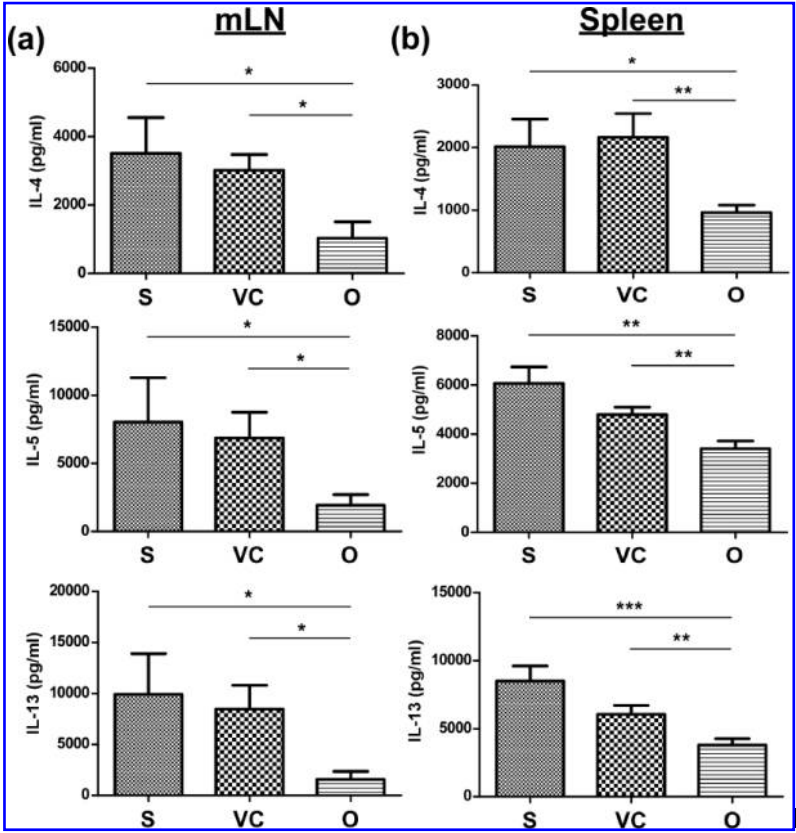


Fig. 5. Th2 cytokine secretion by OVA-stimulated lymphocytes from mLNs and spleens of OVA-sensitized mice with AAV2/8-OVA virus treatment was inhibited.

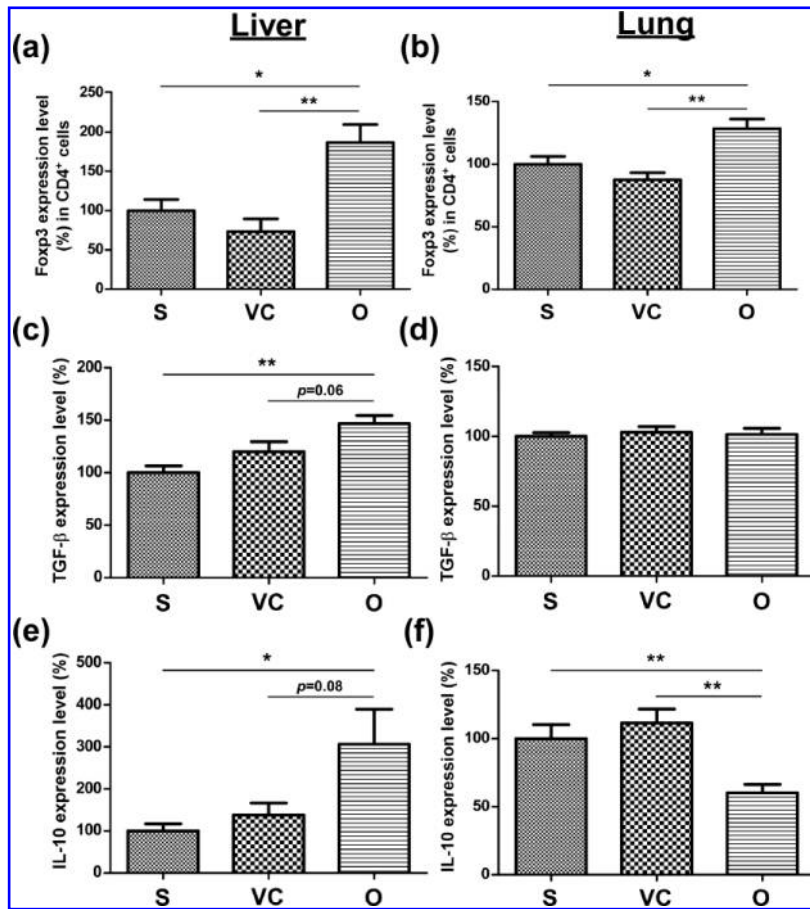


Fig. 6. AAV2/8-OVA virus significantly enhanced Foxp3 expression in CD4⁺ cells from liver and lung tissues of OVA-sensitized mice.

Supplementary Figures

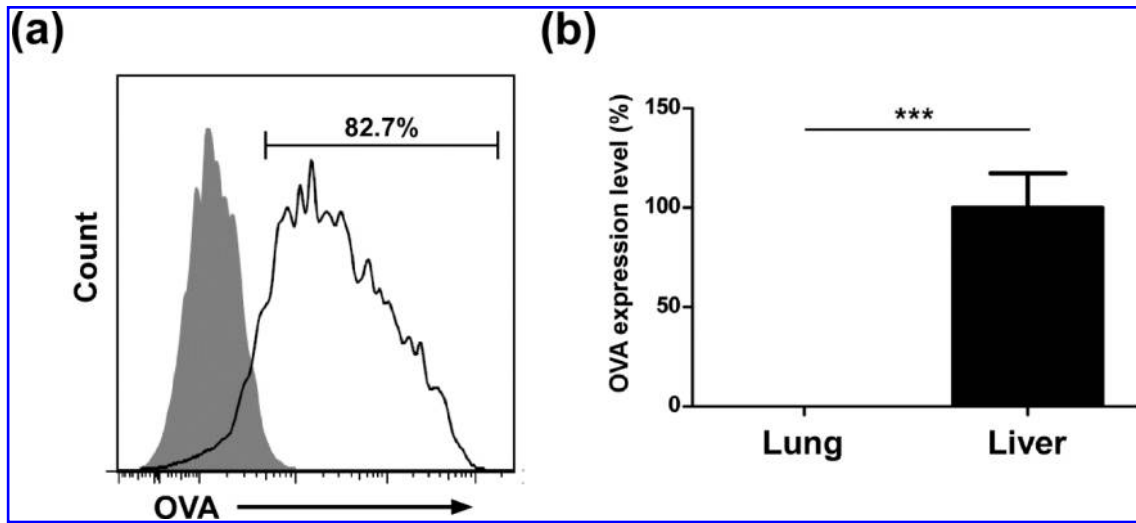


Figure S1. OVA transgene expression *in vitro* and *in vivo*. **(a)** Huh7 cells were transfected with pAAV/hAAT-EGFP (gray area) or pAAV/hAAT-OVA (black line) vectors. The expression of membrane-bound OVA was analyzed by flow cytometry using mouse anti-OVA mAb. **(b)** Total RNA was extracted from the livers or lungs of mice receiving recombinant AAV2/8-OVA and OVA transgene expression detected by quantitative PCR. The fold changes in the lungs were contrasted to those in the liver. Data are presented as mean $\pm$ SEM (n=10 per each tissue). *** P <0.001.

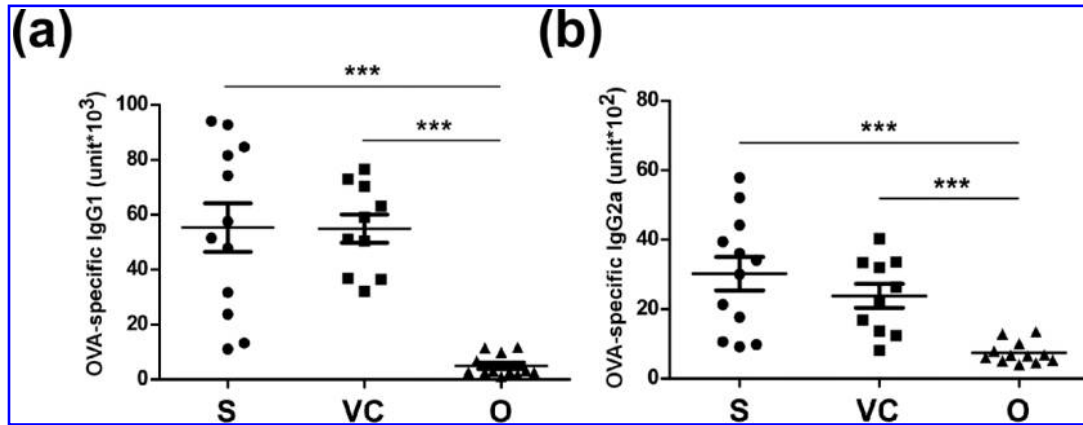


Figure. S2. The secretion of OVA-specific IgG1 and IgG2a was inhibited in the BALF of OVA-sensitized mice treated with AAV2/8-OVA. At the end of the sensitization protocol, the BALF supernatants were harvested and diluted (IgG1: 1:10,000; IgG2a: 1:500) to determine the levels of OVA-specific **(a)** IgG1 and **(b)** IgG2a by ELISA. Data are presented as mean $\pm$ SEM (n=10-12 per group). S, sensitized control; VC, AAV2/8-EGFP vector control; O, AAV2/8-OVA vector. *** P <0.001.

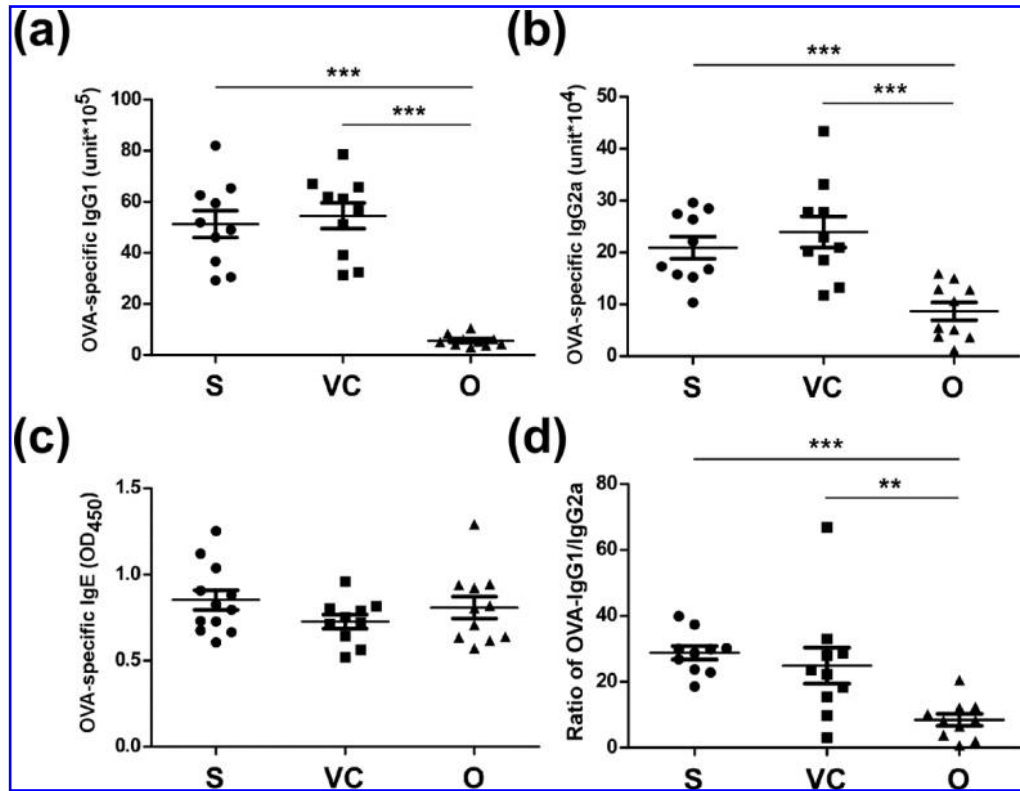


Figure. S3. Serum OVA-specific IgG1 and IgG2a levels were suppressed by AAV2/8-OVA treatment. Serum samples were collected and diluted (IgG1: 1:500,000; IgG2a: 1:10,000; IgE: 1:20). Levels of OVA-specific (a) IgG1, (b) IgG2a, and (c) IgE were determined by ELISA, and (d) the ratio of OVA-specific IgG1 to IgG2a was also determined. Data are presented as mean $\pm$ SEM (n=10 per group). S, sensitized control; VC, AAV2/8-EGFP vector control; O, AAV2/8-OVA vector. **P<0.01; ***P<0.001.

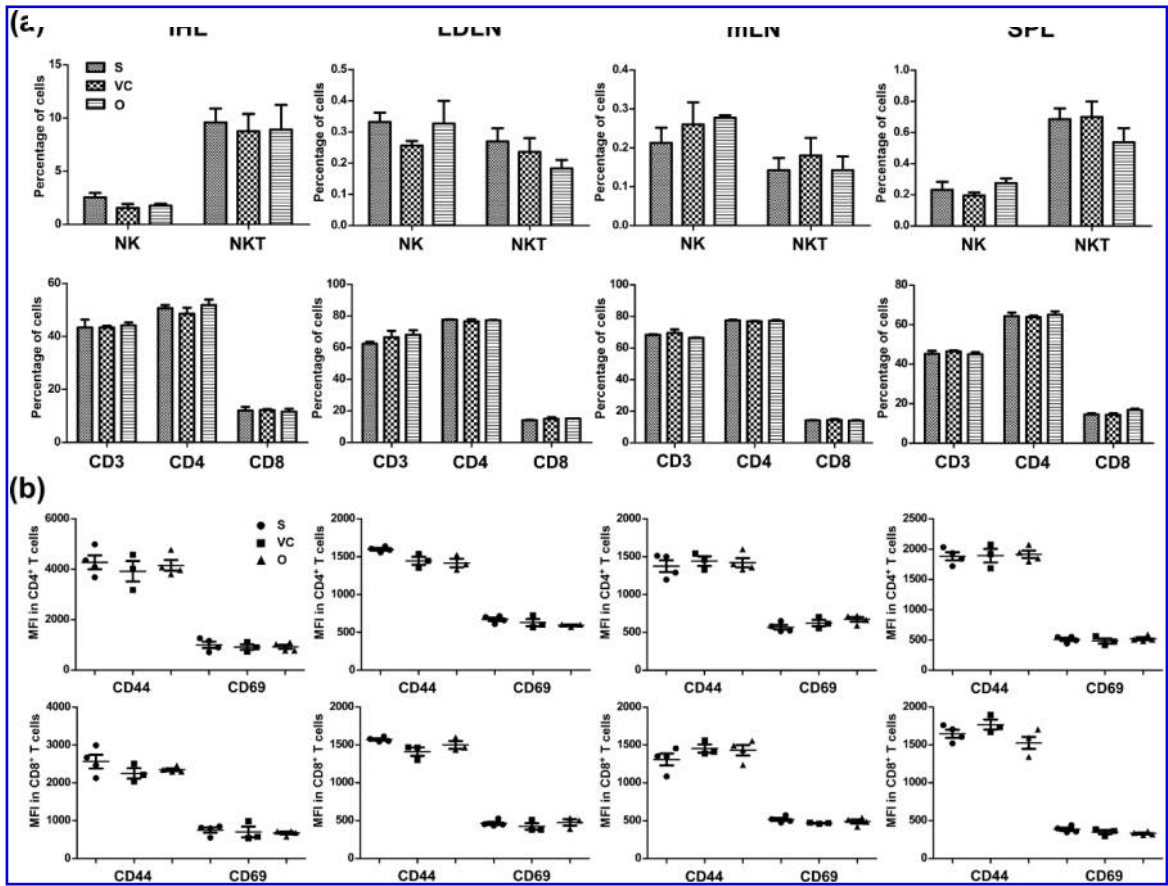


Figure. S4. AAV2/8-OVA did not influence the frequencies and activation profiles of various lymphocyte subpopulations in the livers, spleens, and draining lymph nodes of OVA-sensitized mice. At the end of the sensitization protocol, single cell suspensions from the liver, spleen, and liver-draining and mediastinal lymph nodes (LNs) were prepared as described in *Supplementary Materials and Methods*. **(a)** The frequency of different lymphocyte populations in the innate (NK and NKT cells) or adaptive (CD3, CD4, and CD8) immune system were evaluated by flow cytometry (n=4 per group). **(b)** For the activation profiles of CD4⁺ and CD8⁺ T cells, CD44 and CD69 expression was examined (n=4 per group). Data are presented as mean $\pm$ SEM. IHL, intrahepatic lymphocyte; LDLN, liver-draining LN; mLN, mediastinal LN; SPL, spleen lymphocyte; S, sensitized control; VC, AAV2/8-EGFP vector control; O, AAV2/8-OVA vector.

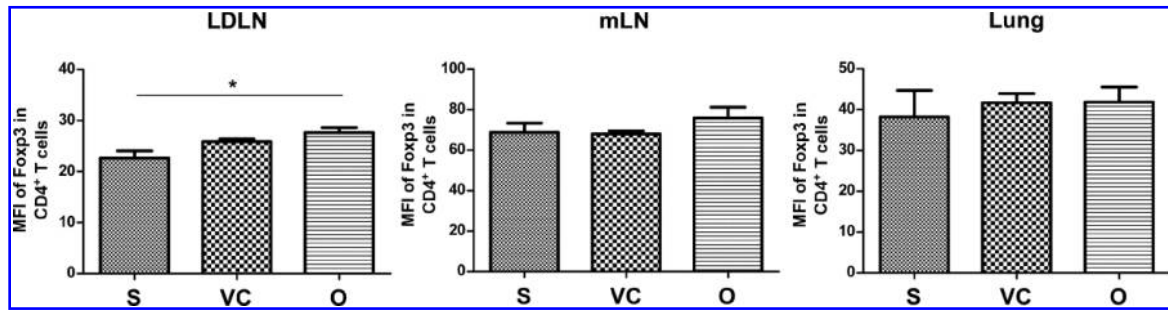


Figure. S5. Foxp3 intensity in CD4⁺ T cells from the lungs and draining lymph nodes of OVA-sensitized mice treated with AAV2/8-OVA. Foxp3 expression by CD4⁺ T cells from liver-draining lymph nodes (LDLNs), mediastinal lymph nodes (mLNs), and lungs was determined by flow cytometry for the CD4⁺ gated cell population (n=4 per group). Data are presented as mean $\pm$ SEM. MFI, mean fluorescent intensity; S, sensitized control; VC, AAV2/8-EGFP vector control; O, AAV2/8-OVA vector.

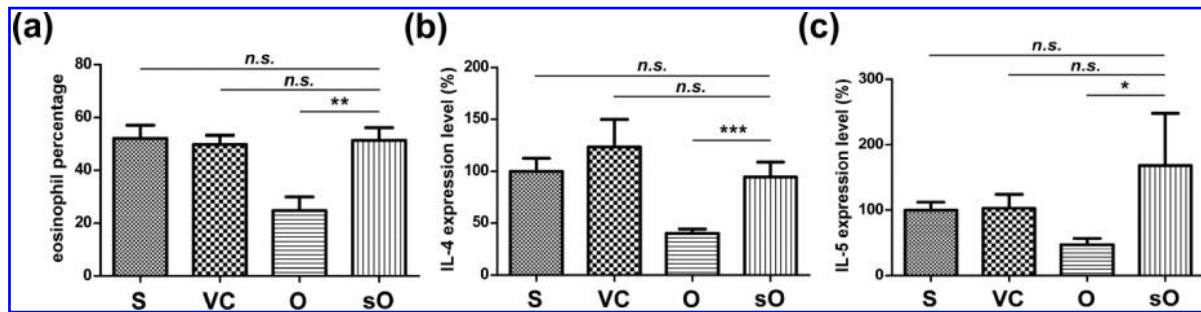


Figure. S6. Recombinant AAV vector carrying the secreted form of OVA did not reduce eosinophilic infiltration and Th2 cytokine expression in the lungs of OVA-sensitized mice.

(a) The percentage of eosinophils (n=10-12 in sensitized control (S), AAV2/8-EGFP vector control (VC), and AAV2/8-OVA vector with membrane OVA (O) groups; n=9 in AAV2/8-sOVA vector carrying secreted form of OVA (sO) group) and RNA expression levels of **(b)** IL-4 and **(c)** IL-5 (n=9 in S, VC, and O groups; n=6 in sO group) were examined as described in the *Materials and Methods*. The relative quantity of each RNA was normalized to β -actin, and the fold change was relative to the non-treatment group. Data are presented as mean $\pm$ SEM. n.s., no significance; * P <0.05; ** P <0.01; *** P <0.001.

Supplementary Table S1. Primer sequences for quantitative PCR

Gene		Sequence
CD4	forward	5'-AGGAAGTGAACCTGGTGGTG-3'
	reverse	5'-CTCCTGCTTCAGGGTCAGTC-3'
CCL22	forward	5'-AGGTCCCTATGGTGCCAATGT-3'
	reverse	5'-CGGCAGGATTTTGAGGTCCA-3'
OVA	forward	5'-CACAAGCAATGCCTTTCAGA-3'
	reverse	5'-GACTTCATCAGGCAACAGCA-3'

Supplementary Materials and Methods

RNA isolation and quantitative PCR

The extraction and reverse transcription of total RNA from the liver and lung was performed as described in the *Materials and Methods*. The complementary DNA were analyzed by quantitative PCR using specific primers (Supplementary Table S1) to detect OVA transgene expression.

Measurement of serum and BALF OVA-specific IgG1 and IgG2a antibodies

The levels of OVA-specific IgG1 and IgG2a antibodies in the BALF or serum were analyzed by ELISA. Briefly, the plates (Costar, Corning, NY, USA) were coated with 10 µg/ml OVA at 37°C for 1 hour and then incubated with diluted samples at 37°C for another hour. Next, the biotinylated rat anti-mouse IgG1 and IgG2a monoclonal antibodies (BD PharMingen, San Diego, CA, USA) and streptavidin-conjugated horseradish peroxidase (HRP) (BD PharMingen) were sequentially added to the plates. Finally, the plates were incubated with substrate solution (TMB, BD Biosciences) for 20 minutes and the reaction stopped with 2N H₂SO₄. Absorbance was measured using an ELISA reader at 450 nm (SpectraMax M2, Molecular Devices, CA, USA). The concentration of either OVA-specific IgG1 or IgG2a was defined in arbitrary units according to each of the standard curves based on the pooled serum obtained from multiple OVA-sensitized mice.

Cell isolation

Intrahepatic lymphocytes (IHLs) were prepared as described previously.¹ Briefly, the

liver was perfused immediately with digestion buffer (HBSS containing 0.01% collagenase IV, Sigma) through the hepatic portal vein after sacrifice. Following perfusion, the liver was minced into small pieces and incubated in digestion buffer at 37°C for 40 minutes. Cell suspensions were obtained from homogenized liver gently passed through a cell strainer (100 μ m, BD Bioscience). Parenchymal cells were separated from the IHLs by centrifugation at 50 x g for 5 minutes. The remaining cells were suspended in a 33% Percoll gradient solution (GE Healthcare, Uppsala, Sweden) to purify the hepatic lymphocytes by centrifugation at 754 x g for 18 minutes.

Similar procedures were used to isolate pulmonary lymphocytes. The lung tissue was perfused with PBS via the right ventricle, excised from the thoracic cavity, and minced into small pieces in digestion solution (RPMI 1640 with 1% penicillin/streptomycin, 20 mM HEPES, 200 U/ml collagenase, and 0.05% DNase I) prior to incubation at 37°C for 30 minutes on a rotary agitator (190 rpm). After incubation, the digested lung tissue was gently grinded using a cell strainer (40 μ m) to generate cell suspensions. Lung lymphocytes were prepared in a 47% Percoll gradient solution by centrifugation at 1500 rpm for 10 minutes at 4°C. Cells were isolated from the spleens and draining lymph nodes as described in the *Materials and Methods*.

Flow cytometry

To detect the expression of the OVA transgene, human hepatoma Huh7 cells were transiently transfected with pAAV/hAAT-OVA vectors using Lipofectamine 2000 (Invitrogen). After 24 hours, cells were harvested and then incubated with mouse anti-OVA monoclonal antibody (mAb) (OVA-14, Sigma) and Alex Fluor 488-conjugated goat anti-mouse IgG (Invitrogen). For cell population and activation analysis, isolated

single cells from various tissues were suspended in staining buffer (1% BSA in PBS) and pre-incubated with anti-mouse CD16/32 mAb (clone 93) for 20 minutes on ice to block non-specific binding of Abs to Fc receptors. Surface marker expression was assessed by flow cytometry using phycoerythrin (PE)-conjugated anti-CD3 ϵ (145-2C11), allophycocyanin (APC)-conjugated or fluorescein isothiocyanate (FITC)-conjugated anti-CD4 (GK1.5), PE-conjugated or APC-eFluor 780-conjugated anti-CD8 (53-6.7), PE-Cy7-conjugated anti-CD44 (IM7), FITC-conjugated anti-CD69 (H1.2F3), and brilliant violet 421-conjugated anti-NK1.1 (PK136). Staining reactions were performed at 4°C for 30 minutes in the dark. For intracellular Foxp3 staining, cells were pre-incubated with relevant mAbs against cell surface markers and washed. Next, the cell pellets were re-suspended in fixation/permeabilization buffer (Foxp3 Staining Buffer Set, eBioscience, San Diego, CA, USA) for 16 hours at 4°C in the dark. Finally, the cells were stained with APC-conjugated anti-Foxp3 or isotype control Ab. All mAbs were obtained from BioLegend or eBioscience. Stained cells were analyzed by FACSCalibur or CantoII flow cytometry and the data processed using CellQuest or DIVA software (BD Bioscience).

Construction and preparation of AAV2/8 vector carrying the secreted OVA gene fragment

The recombinant AAV (rAAV) construct with the coding sequence for OVA (designated as AAV2/8-sOVA) was similar to membrane-bound OVA as described in the *Materials and Methods*, but this vector did not contain the 178 COOH terminal amino acids of the B7-1 domain including the transmembrane domain. The procedure to generate the AAV2/8-sOVA vector and determine copy number was performed similar to other vectors as described in the *Materials and Methods*.

Supplementary references

1. Chang CM, Lo CH, Shih YM et al. Treatment of hepatocellular carcinoma with adeno-associated virus encoding interleukin-15 superagonist. Hum Gene Ther 2010;21:611-621.

Liver-specific allergen gene transfer by adeno-associated virus suppresses allergic airway inflammation in mice

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Short title: Liver OVA expression by AAV lowers allergic asthma

Abstract

Allergic airway inflammation driven by T helper 2 (Th2)-type immunity is characterized by airway hyperresponsiveness, eosinophilic infiltration, and elevated IgE production. Various novel strategies for managing asthma have been explored, such as DNA vaccines, T-cell peptides, and allergen-specific immunotherapy. A principal goal of most immunotherapeutic approaches is active and long-term allergen-specific tolerance. Liver-specific gene transfer using adeno-associated virus (AAV) has been shown to favorably induce tolerogenic responses to therapeutic products in various experimental models. AAV8 has strong liver tropism and induces immune tolerance in mice. The present study aimed to determine whether hepatocyte-specific allergen expression by pseudotyped AAV2/8 alleviates asthmatic symptoms in ovalbumin (OVA)-sensitized mice. Mice were intravenously injected with AAV2/8 vector carrying membrane-bound OVA transgene under transcriptional control of a hepatocyte-specific alpha 1 antitrypsin promoter (AAV2/8-OVA) and then sensitized with OVA. AAV2/8-OVA specifically transduced the OVA transgene in the liver. Airway hyperresponsiveness, eosinophilia, mucus hyper-secretion, and Th2 cytokines were significantly suppressed in both the lungs and secondary lymphoid organs of asthmatic mice infected with AAV2/8-OVA. Significant reduction of OVA-specific antibodies was detected in the bronchoalveolar lavage fluid from AAV2/8-OVA-treated mice. Moreover, AAV2/8-OVA treatment prominently promoted the expression of Foxp3, IL-10, and TGF- β in the liver. Enhanced Foxp3 expression was also detected in the lungs of asthmatic mice after AAV2/8-OVA treatment. Taken together, these results suggest that the induction of immune tolerance by hepatic AAV gene transfer may be beneficial for modulating allergic asthma.

Keywords: AAV, allergy, asthma, gene therapy, liver

Introduction

Allergic asthma is a pulmonary inflammatory disease characterized by airflow obstruction, persistent inflammation, and airway hyperresponsiveness (AHR) to common environmental aero-allergens.¹ Asthmatic patients exhibit elevated allergen-specific IgE, mucus hypersecretion, and eosinophilia in the lungs.² CD4⁺ T helper 2 (Th2) cells play a major role in the induction and maintenance of airway inflammation.³ Th2 cytokines, especially interleukin (IL)-4, IL-5, and IL-13, and other inflammatory mediators secreted by eosinophils and mast cells are primarily thought to orchestrate these pathological features of asthma.³ Most therapeutic strategies for asthma, such as β 2-adrenergic receptor agonists, anti-IgE blockers, antagonists of cytokines and mediators, as well as corticosteroids, only provide temporary alleviation by suppressing inflammatory responses and have certain side effects.⁴ Therefore, the development of more effective, safer, and long-lasting therapeutic or preventive approaches is pivotal for asthma.

The liver preferentially facilitates local or systemic immune tolerance via unique antigen-presenting cells (APCs) and the cytokine milieu.⁵ The advantages of sustained transgene expression and the tolerogenic immunity of the liver are widely utilized and investigated for the treatment of hepatitis, hemophilia, and inherited metabolic disorders by gene therapy.⁶ T cell activation in the liver promotes tolerant immune responses by inducing anergy, clonal deletion, or regulatory T cell (Treg) generation.⁷⁻⁹ Accumulated evidence indicates that *in vivo* hepatocyte-specific gene transfer contributes to the induction of immune tolerance and has therapeutic potential in mice.⁶ Some reports have proposed that antigen-specific Foxp3⁺ Tregs are generated following hepatic gene transfer, which prevents or suppresses the development of experimental autoimmune encephalomyelitis (EAE).^{7,10} Lüth et al. confirmed that specific expression of neural

autoantigen in hepatocytes, instead of other tissues, protects against neuron inflammation in EAE.¹⁰ CD4⁺CD25⁺Foxp3⁺ Tregs mainly engage in negative control of autoimmune responses and allergic diseases, such as asthma.¹¹ However, whether hepatocyte-specific allergen expression modulates allergic airway inflammation has not yet been reported.

Adeno-associated virus (AAV), a single-stranded DNA virus with low immunogenicity, consists of numerous serotypes that target various tissues and establish long-term transgene expression.¹² AAV1, AAV5, AAV8, and AAV9 efficiently infect muscle, retina, liver, and lung tissue, respectively.¹³ Recombinant AAV (rAAV) vectors derived from *rep* of AAV2 and *cap* of different serotypes would enhance gene transduction efficacy, change tissue tropism, and evade pre-existing neutralizing antibodies.¹³ Gene therapy with rAAV vectors has been employed for studies in animal models and clinical trials. Several reports have suggested that rAAV vectors carrying immunomodulatory transgenes, such as T-bet¹⁴ and Clara cell 10-kDa protein (CC10),¹⁵ or antisense sequences against Th2 cytokines¹⁶ and chemokines¹⁷ attenuate allergen-induced inflammation in asthmatic models. Because of the potential tolerogenic immune responses induced by hepatocyte-specific gene expression, we investigated whether Th2 cell-driven inflammation in allergic asthma is modulated by rAAV carrying an allergen transgene targeting hepatocytes. As the AAV8 serotype had been shown to superiorly transduce liver tissue and lead to immune tolerance in mice,^{18,19} we applied ovalbumin (OVA) as a model antigen and examined the immunomodulatory effects of AAV2/8 vector carrying a liver-specific membrane-bound OVA transgene (AAV2/8-OVA). Significant suppression of asthmatic symptoms was achieved in OVA-sensitized mice treated with AAV2/8-OVA. These results suggest that hepatocyte-specific allergen gene expression may be considered a novel approach for

alleviating allergic asthma.

Materials and Methods

Construction and preparation of pseudotyped AAV2/8 vector

A recombinant AAV (rAAV) construct was created with the coding sequence of chicken OVA or enhanced green fluorescence protein (EGFP) as the vector control. The full-length OVA DNA fragment (386 amino acids) was derived from pAc-neo-OVA (gift of Dr. MJ Bevan).²⁰ The OVA₃₈₆ fragment was fused with 178 COOH terminal amino acids of B7-1 domain including the transmembrane domain by PCR to generate membrane-bound OVA. Next, the OVA/B7-1-fused fragment was subcloned into pAAV vector containing the inverted terminal repeats (ITRs) of AAV serotype 2 at both ends. The expression cassette comprised a hepatocyte-specific alpha 1 antitrypsin (hAAT) promoter, a 9-amino-acid influenza virus HA tag, and a polyadenylation signal sequence.

Recombinant AAV2/8 vectors were generated using a tri-plasmid system as described previously.¹⁷ Briefly, the pAAV/hAAT plasmid with the OVA or EGFP transgene was co-transfected into HEK 293T cells with an adenovirus helper plasmid and a packaging chimeric plasmid encoding the AAV2 rep and AAV8 cap genes. Recombinant single strand AAV vectors were purified by cesium chloride sedimentation. Genome copies (GCs) of AAV2/8-OVA or AAV2/8-EGFP were determined by quantitative PCR (qPCR) using SYBR Green Master mix (Roche Diagnostics GmbH, Mannheim, Germany).

Animals and OVA-sensitization protocol

Three-week-old female BALB/c mice were purchased from National Laboratory Animal Center (Taipei, Taiwan, ROC) and acclimatized for 1 week. Four-week-old mice were administered 1×10^{11} GCs per mouse in 200 μ l of normal saline via intravenous

injection. Four weeks after virus treatment and before the sensitization protocol, blood samples were collected under anesthesia from the retro-orbital venous plexus using heparinized capillary tubes. All mice (8 weeks old) were sensitized by intra-peritoneal injection of 50 µg OVA (grade V, Sigma-Aldrich Inc., MO, USA) mixed with 0.8 mg aluminum hydroxide (Pierce Biotechnology Inc., Rockford, IL, USA) on days 56, 57, 58, and 70 after virus administration. These mice were challenged by inhaling 2% aerosolized OVA for 20 minutes using an atomizer (Pulmo-aide, DeVilbiss Health Care, Somerset, PA, USA) on days 70, 73, 77, 80, and 84. AHR was analyzed on day 85, and mice were sacrificed 24 hours later. The care and handling of mice in all experimental procedures was approved by the Institutional Animal Care Committee of Chang Gung University and complied with the guides of National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals.

Assessment of AHR and cell composition in bronchoalveolar lavage fluid (BALF)

AHR was assessed on day 85, 24 hours after the last challenge, using methacholine (Mch; Sigma). Mice were exposed to normal saline or increasing concentrations of Mch (10, 20, 30, and 40 mg/ml) for 3 minutes and then underwent whole body plethysmography while conscious. The enhanced pause (Penh) value was measured for 3 minutes and pulmonary airway obstruction analyzed by BioSystem XA software (Buxco Electronics, Inc., Wilmington, NC, USA). As described in detail in our previous reports, the sensitized mice had significantly enhanced Penh values compared to the normal healthy control mice.^{21,22}

Following the measurement of AHR, the tracheas of mice from different groups were cannulated and the lungs lavaged three times with 1 ml phosphate-buffer saline

(PBS) supplemented with 0.1 mM EDTA. Cells in the BALF were collected and counted using trypan blue dye exclusion. For differential cell counts, BALF cells were centrifuged (Shandon Cytospin 4; Thermo Scientific, Pittsburg, PA, USA) and stained with Liu staining solution. Different cell types were distinguished under light microscopy by counting at least 200 cells based on their morphological profile.

Lung histology

On day 86, non-lavaged lung tissues were removed and fixed with 4% formaldehyde for 24 hours at 4°C for histological examination. After dehydration, the tissues were embedded in paraffin and sliced into 4 µm sections. The tissue sections were subjected to hematoxylin and eosin (H&E) staining and periodic acid-Schiff (PAS) (Sigma) staining. Inflammatory cell infiltration and goblet cell hyperplasia were examined by automated MetaMorph microscopy and image analysis software (Molecular Devices).

Measurement of OVA-specific IgE

The titers of OVA-specific IgE antibodies in the BALF and serum were determined by ELISA. Briefly, the plates (Costar, Corning, NY, USA) were coated with 10 µg/ml OVA at 37°C for 1 hour. The OVA-coated plates were incubated with diluted BALF or serum samples at 37°C for 1 hour. Next, biotinylated rat anti-mouse IgE monoclonal antibodies (BD PharMingen, San Diego, CA, USA) and streptavidin-conjugated horseradish peroxidase (HRP) (BD PharMingen) were sequentially added to the plates. Finally, the plates were incubated with substrate solution (TMB, BD Biosciences) for 20 minutes and the reaction stopped with 2N H₂SO₄. Absorbance was measured on an ELISA reader at 450 nm (SpectraMax M2, Molecular Devices, CA, USA).

Cell culture and cytokine assays

Spleens and mediastinal lymph nodes (mLNs) were removed and single cell suspensions prepared. Cells (5×10^6 cells/ml) were cultured in RPMI 1640 medium (Gibco, Life Technologies, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Gibco) with 100 μ g/ml OVA. Culture supernatants were harvested on day 6 and the levels of interleukin (IL)-4, IL-13 (DuoSet ELISA kit, R&D systems), and IL-5 (BD OptEIA set, BD Pharmingen) determined by ELISA according to the manufacturer's instructions.

RNA isolation and qPCR

Total RNA was extracted from homogenized lung and liver tissues using TRIzol reagent (Invitrogen, Life Technologies) and treated with DNase I (Fermentas, Thermo Scientific). The cDNA were generated using random primers and M-MLV reverse transcriptase (Invitrogen). The sequences of specific primers for qPCR of CD4 and CCL22 are listed in Supplementary Table S1. Other qPCR primers for IL-4, IL-5, IL-10, IL-12p35, IL-12p40, IL-13, IFN- γ , CCL11, CCL17, CCL24, Foxp3, TGF- β , β -actin, gob5, and Muc5ac were published previously.^{23,24} The cDNA samples were mixed with SYBR Green/ROX qPCR Master Mix (Fermentas) and amplified in a CFX Connect real-time PCR detection system (Bio-Rad). The thermal cycling conditions were 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. The relative expression of each gene was calculated by normalizing the levels to the expression of housekeeping gene β -actin. The expression of Foxp3 in CD4⁺ cells was calculated and normalized to CD4 expression. The fold change was relative to the untreated group.

Statistical analysis

Results are presented as mean $\pm$ SEM. Significance was assessed using a two-tailed *P*-value calculated by the Mann-Whitney *U* test. *P*<0.05 was considered significant. All graphs were generated and statistical analyses performed using GraphPad Prism 5.0 software.

Results

Liver-specific OVA expression by AAV2/8 vector virus suppressed AHR and OVA-specific IgE in OVA-sensitized mice

We constructed DNA encoding the membrane-bound OVA sequence using the rAAV2/8 vector with hepatocyte-specific alpha 1 antitrypsin promoter (Figure 1a) to achieve hepatocyte-specific OVA expression. The expression of OVA on the cell surface was confirmed in pAAV/hAAT-OVA-transfected Huh7 cells (Supplementary Figure S1a). Furthermore, mice infected with AAV2/8-OVA exhibited significant OVA transgene expression in the liver but not in the lung (Supplementary Figure S1b). To examine whether hepatic OVA expression alleviates the development of airway inflammation *in vivo*, mice were infected with AAV2/8-OVA or AAV2/8-EGFP control viruses and then sensitized with OVA (Figure 1b). After the final challenge, AHR was assessed in response to increasing concentrations of inhaled methacholine (Mch). When mice were stimulated with 40 mg/ml Mch, the enhanced pause (Penh) value was significantly lower in AAV2/8-OVA-treated mice than the non-treatment and AAV2/8-EGFP control groups (Figure 1c) and was comparable to healthy normal controls in our previous studies.^{21,22} Decreased Penh values were also measured in the AAV2/8-OVA vs. AAV2/8-EGFP control groups when mice were stimulated with 30 mg/ml Mch.

A significant reduction in OVA-specific IgG1, IgG2a (Supplementary Figure S2), and IgE (Figure 1d) was detected in the BALF from OVA-sensitized mice treated with AAV2/8-OVA compared to the non-treatment and AAV2/8-EGFP control groups. The data indicate that administration of AAV2/8-OVA attenuated AHR and inhibited OVA-specific antibody production in the lungs of OVA-sensitized mice.

AAV2/8-OVA significantly decreased cell infiltration and mucus hypersecretion in the lungs of OVA-sensitized mice

Because the infiltration of eosinophils and lymphocytes into lung tissue is one of the main characteristics of asthmatic patients, we analyzed the inflammatory cell composition of BALF from OVA-sensitized mice. A markedly decreased percentage of eosinophils was found in mice treated with AAV2/8-OVA compared to the non-treatment and AAV2/8-EGFP control groups (Figure 2a). The absolute numbers of total leukocytes, eosinophils, and lymphocytes were significantly lower in the BALF from AAV2/8-OVA-treated mice than the two control groups (Figure 2b-d). Therefore, AAV2/8-OVA reduced the infiltration of inflammatory cells into the lungs of OVA-sensitized mice.

We further analyzed whether AAV2/8-OVA can suppress cell infiltration and mucus secretion in the airways based on histological examination. Massive inflammatory cells infiltrated peribronchial and perivascular sites in the lungs of untreated and AAV2/8-EGFP control mice, whereas the number of inflammatory cells was much lower in AAV2/8-OVA-treated mice (Figure 3a). Quantitative analysis demonstrated a significant reduction in inflammatory cells in the lungs of AAV2/8-OVA-treated mice compared to the non-treatment and AAV2/8-EGFP control groups (Figure 3c). Similarly, decreased goblet cell hyperplasia and mucus production were observed in the airways of AAV2/8-OVA-treated mice compared to the two control groups (Figure 3b and d). To confirm this result, *gob5*, a molecule associated with mucin gene regulation and goblet cell hyperplasia, and *Muc5ac* expression were analyzed by qPCR (Figure 3e and f). Mice treated with AAV2/8-OVA expressed lower levels of *gob5* and *Muc5ac* than mice in the non-treatment and AAV2/8-EGFP control groups. Thus, AAV2/8-OVA administration was

able to attenuate the accumulation of inflammatory cells and decrease mucus production in the lungs of asthmatic mice.

Significant reduction of Th2 cytokine and chemokine gene expression in the lungs of AAV2/8-OVA-treated mice

Due to the substantial suppressive effect of AAV2/8-OVA on lung inflammation, we analyzed the expression levels of cytokine and chemokine genes in the lungs. OVA-sensitized mice treated with AAV2/8-OVA expressed significantly lower levels of Th2 cytokines compared to untreated and AAV2/8-EGFP control mice (Figure 4a). However, the RNA expression of Th1-related cytokines, such as IFN- γ , IL-12p35, and IL-12p40, was unchanged (Figure 4a). To determine whether the decreased number of lymphocytes in the lungs is attributed to the abolishment of Th2 cell recruitment, we measured CCL17 and CCL22, which selectively induce the chemotaxis of CD4⁺ Th2 cells into inflamed tissues in allergic diseases. The lungs of untreated and AAV2/8-EGFP control mice expressed large amounts of CCL17 and CCL22 compared to mice receiving AAV2/8-OVA (Figure 4b). The expression of the main chemokines for eosinophil recruitment (CCL11 and CCL24) was also reduced in AAV2/8-OVA-treated mice (Figure 4b). These results indicate that reduced chemokine production in response to AAV2/8-OVA treatment may prevent inflammatory cells from migrating into the lungs and alleviate the pathological features of OVA-sensitized mice.

AAV2/8-OVA repressed regional and systemic Th2-driven immune responses in the lungs of OVA-sensitized mice

To determine whether AAV2/8-OVA treatment suppressed local Th2 cell-derived

cytokines, culture supernatants of OVA-stimulated lymphocytes from mediastinal lymph nodes (mLNs) were examined. OVA-stimulated lymphocytes from mice receiving AAV2/8-OVA secreted less IL-4, IL-5, and IL-13 than lymphocytes from untreated and AAV2/8-EGFP control mice (Figure 5a). Similarly, OVA-stimulated splenocytes from AAV2/8-OVA-treated mice expressed significantly lower levels of Th2 cytokines compared to untreated and AAV2/8-EGFP control mice (Figure 5b). No significant differences were observed in IFN- γ secretion by OVA-stimulated lymphocytes from mLNs and spleens among the different groups (data not shown). Thus, AAV2/8-OVA inhibited Th2 cell-mediated immune responses and ameliorated the pathogenesis of allergic asthma.

We also examined whether AAV2/8-OVA administration inhibited antibody production against OVA in serum. Before encountering allergen, serum OVA-specific antibodies were hardly detected in all mice (data not shown), even in mice infected with AAV2/8-OVA. Significantly lower levels of serum OVA-specific IgG1 and IgG2a were detected in OVA-sensitized mice treated with AAV2/8-OVA than untreated or AAV2/8-EGFP control mice (Supplementary Figure S3a and S3b). The ratio of OVA-specific IgG1 to IgG2a was also significantly reduced (Supplementary Figure S3d). However, hepatic OVA expression did not seem to affect serum OVA-specific IgE in asthmatic mice (Supplementary Figure S3c). These data suggest that the administration of AAV2/8-OVA suppressed the production of OVA-specific antibodies in OVA-sensitized mice.

Higher Foxp3 expression was detected in liver and lung tissues from AAV2/8-OVA-treated mice

To explore the possible mechanisms involved in modulating the hypo-immune responses to allergen, we examined the proportions and activation phenotypes of various lymphocyte subpopulations in the liver, spleen, liver-draining lymph nodes, and mLNs. The frequencies of NK, NKT, CD3⁺, CD4⁺, and CD8⁺ T cells were similar in all groups (Supplementary Figure S4a). The activation profiles of CD4⁺ and CD8⁺ T cells were comparable among the different groups (Supplementary Figure S4b). Hepatocyte-restricted transgene expression was previously reported to promote immune tolerance by inducing Foxp3 expression in CD4⁺CD25⁺ Treg cells.^{7,10} Thus, we investigated whether Foxp3 expression by CD4⁺ T cells from asthmatic mice was affected by AAV2/8-OVA treatment. The frequency of CD4⁺CD25⁺Foxp3⁺ T cells did not differ significantly in AAV2/8-OVA-treated mice and control mice (data not shown). The mean fluorescence intensity (MFI) of Foxp3 in liver-draining lymph node-isolated CD4⁺ T cells was elevated by AAV2/8-OVA treatment compared to the non-treatment group (Supplementary Figure S5). We further applied qPCR, a more sensitive method, to detect the Foxp3 RNA expression levels in CD4⁺ cells. The CD4⁺ cells from mice treated with AAV2/8-OVA expressed significantly increased levels of Foxp3 in the liver and lungs (Figure 6a and b). Higher TGF- β and IL-10 RNA levels were also observed in the livers of mice treated with AAV2/8-OVA compared to control groups (Figure 6c and e). In the lungs, IL-10 expression was greatly decreased in mice treated with AAV2/8-OVA compared to control groups (Figure 6f), but the level of TGF- β was not different between the groups (Figure 6d). These results imply that liver-specific allergen expression may create a tolerogenic microenvironment through enhanced amounts of TGF- β and IL-10 in the liver, promoting Foxp3 expression in CD4⁺ T cells.

Discussion

Allergic asthma has become a global public health issue that has been considerable burden on health services and incurs expensive social costs. Despite a growing number of pharmaceutical and immunotherapy strategies for asthma, the development of an active, effective, and long-term immunomodulatory approach has been difficult. Here, our results demonstrate for the first time that specific expression of allergen in hepatocytes by rAAV-mediated gene transfer induces tolerogenic immunity, which alleviates allergic airway inflammation and inhibits Th2 cell-driven immune responses. Pseudotyped AAV2/8 vector with hepatocyte-restricted promoter specifically transduced the allergen gene in the liver. OVA-specific Th2 immune responses and antibody production were significantly suppressed in asthmatic mice receiving AAV2/8-OVA. These results suggest that ectopic allergen expression in hepatocytes resulted in local and systemic hypo-immune responses to allergen re-challenge. Consequently, the application of the liver tolerance effect may be a novel strategy for therapeutic modulation and intervention in allergic asthma.

Antigen-specific tolerance has been demonstrated by transgene expression in hematopoietic stem cells, immature APCs, and tolerogenic tissues, such as the liver and thymus.⁵ Gene transfer-mediated immune tolerance has been applied in the treatment of hereditary protein deficiencies²⁵ and autoimmune diseases¹⁰ in animal models and clinical trials.²⁶ In a murine model of hemophilia B, hepatocyte-restricted factor IX expression sustained therapeutic protein levels and evaded unwanted immune responses.²⁷ In addition, hepatocyte-specific gene transfer of α -galactosidase and acid sphingomyelinase has been shown to trigger tolerogenic immunity and improve pathological features of Fabry and Nieman-Pick disease.^{18,28} In an animal model of human multiple sclerosis,

neural autoantigen-expressing hepatocytes prevented and repressed CD4⁺ T cell-driven neuroinflammation.¹⁰ Some potential mechanisms of immune tolerance by hepatic gene transfer have been proposed that involve the elimination and unresponsiveness of effector T cells or the induction of Tregs.^{7,8,10} The development of transgene-specific CD4⁺CD25⁺Foxp3⁺ Tregs has also been reported following hepatic gene transfer.^{7,10} For allergic disease, the current study demonstrates that allergen-expressing hepatocytes effectively protect against asthma development.

How lung inflammation was alleviated by allergen expression in the liver is currently not clear. The phenomenon of “liver tolerance” has been shown to be local and systemic tolerance of self and foreign antigens presented by non-parenchymal cells or expressed in hepatocytes. Several non-parenchymal cells are involved in the tolerogenic properties of the liver, including dendritic cells,²⁹ liver sinusoidal endothelial cells (LSECs),³⁰ Kupffer cells,³¹ and hepatic stellate cells.³² These unique cells regulate tolerant responses via the production of anti-inflammatory cytokines, such as IL-10, TGF-β, or programmed cell death ligand-1.³³ Furthermore, hepatocytes directly contact circulating T cells through the specialized fenestrations of LSECs. The immunological functions of hepatocytes are facilitated by the polarized expression of MHC and ICAM-1 molecules towards the sinusoidal lumen.³⁴ A previous study suggested that T cells activated by hepatocytes die prematurely, leading to immune tolerance.³⁵ Thus, it is likely that antigen presented by hepatocytes or other cell populations is able to modulate dysregulated immune responses.

The induction of CD4⁺CD25⁺ Tregs in tolerance to the transgene product or autoantigen has been reported following hepatic *in vivo* gene transfer.^{7,10} Based on the aberrant number of Tregs and Foxp3 mRNA levels in patients allergic to house dust mites

(HDMs),^{36,37} the increased Foxp3 gene expression in the liver and lungs of AAV2/8-OVA-treated mice suggested the possibility of the development of Tregs. Liver-generated CD4⁺Foxp3⁺ cells are recruited to or circulate in the inflamed lung tissue to suppress Th2 cell-driven inflammation. Thus, the increased Foxp3⁺ gene expression levels in the liver and lungs (Figures 6a and b) suggest that hepatic gene transfer-induced CD4⁺Foxp3⁺ Tregs partially contributed to the tolerogenic effect. Similar to oral tolerance, the reduction of AHR, lung inflammation, and Th2 cytokines is associated with an increased proportion of airway CD4⁺Foxp3⁺ Tregs in asthmatic mice.³⁸ IL-10 and TGF- β are well documented to be crucially involved in the maintenance of immune tolerance to transgene products and peripheral Treg conversion.^{10,39} In the allergen milieu of the liver, significantly increased expression of TGF- β and IL-10 was detected in OVA-sensitized mice treated with AAV2/8-OVA (Figure 6). Allergen stimulation combined with immunosuppressive cytokines in the liver probably facilitated Foxp3 acquisition of CD4⁺ cells with both cytokines. Nevertheless, IL-10 expression was significantly decreased following AAV2/8-OVA treatment in the lungs. Although the reduction in IL-10 may be attributed to reduced lymphocyte recruitment into the lungs, whether IL-10 directly suppresses lung inflammation or indirectly targets allergen-mediated responses through enhanced Foxp3 expression in the lung tissues is not clear. Nevertheless, we cannot formally prove which mechanism is really involved in the suppression of lung inflammation in asthmatic mice, though it is clear that the alleviated allergic responses occurred when the allergen gene was expressed in the liver.

Various factors, including dose, nature, and expressed form of the antigen, exposure route, delivery system, and mouse strain determine the development of immune activation or tolerance. The location where antigen encounters antigen-presenting cells

(APCs) is also important.^{40,41} We tested whether different subcellular antigen localizations will influence immune tolerance by hepatic gene transfer. Although both membrane-anchored and secreted antigens induce antigen-specific antibodies,^{42,43} the activation of CD4⁺ T cells was greater in mice receiving membrane-anchored antigen than those receiving secreted antigen.⁴² Other studies have indicated that mice immunized with soluble antigens encoded by DNA-based vaccine provoke a strong IgG1 response and greater levels of IL-4, but this does not occur with the membrane-bound antigen.^{44,45} Our results indicate that mice receiving secreted OVA (treated with AAV2/8-sOVA) had higher eosinophilia and Th2 cytokine RNA expression in the lungs than mice treated with vector carrying membrane-anchored OVA (Supplementary Figure S6). Highly efficient immune tolerance was also induced by the transmembrane form of neural autoantigen in hepatocytes in autoimmune neuroinflammation.¹⁰ On the other hand, low doses of antigen have been demonstrated to efficiently induce and expand Tregs compared to high doses of antigen.^{46,47} Lower levels of OVA transgene expression were observed in the livers of mice receiving vector carrying membrane-anchored OVA compared to those receiving secreted OVA (data not shown). Thus, we speculate that membrane-bound OVA provides an environment for less effective activation of Th2 cells and less antigen exposure promotes Treg function compared to secreted OVA.

AAV is a favorable vector for gene transfer due to its non-pathogenic nature, widespread infectivity, and stable transgene expression.¹² Over the past few decades, pseudotyped AAV vectors have been developed extensively and exerted therapeutic efficacy in disease models. A growing number of successful gene therapies have also been reported in clinical trials of AAV for inherited disorders and acquired diseases.²⁶ Our previous studies successfully applied an AAV2/9 vector carrying CC10 to efficiently

target lung epithelial cells and restore CC10 levels, alleviating lung inflammation in OVA-sensitized mice.¹⁵ In addition, AHR, eosinophilia, and airway remodeling were significantly mitigated in HDM allergen-sensitized mice with AAV2/9-delivered CCL11 small hairpin RNA.¹⁷ Compared to overall immune suppressive chemokine-modulatory gene sequences, antigen-specific immune tolerance may have fewer side effects. In addition, the liver is a more tolerogenic organ and evidence has demonstrated that hepatic gene transfer using AAV vector may promote transgene-specific systemic immune tolerance.^{7,48,49} The AAV8 serotype preferentially targets the liver to support sustained transgene products and induce active tolerogenic responses.^{50,51} AAV2/8 vector carrying therapeutic transgenes predominantly assists tolerant responses to transgenes in mice with lysosomal storage disorders.^{18,28} The livers of patients with hemophilia B have been transduced successfully by AAV2/8-Factor IX vectors with no lasting toxicity and transiently achieving therapeutic levels.⁵² We established liver-restricted allergen expression with AAV2/8 vectors, which resulted in immune hypo-responsiveness to OVA-induced antibody production and Th2 cytokine secretion in OVA-sensitized mice. Thus, AAV2/8 vector combined with a hepatocyte-specific promoter was a beneficial vehicle for hepatic gene transfer to suppress hypersensitive lung responses to allergen. Similar strategies may be applied for other allergic diseases that do not have a localized target, such as atopic dermatitis. Furthermore, liver-mediated immune tolerance may be beneficial for systemic or organ-specific autoimmune diseases.

In conclusion, ectopic expression of antigen in the liver by AAV-directed gene transfer has been applied gradually to induce tolerant responses against transgene products. For the first time, our study indicates that hepatocyte-restricted allergen expression by pseudotyped AAV2/8 vector effectively suppressed asthmatic pathogenesis

in allergen-sensitized mice. AAV2/8 vector carrying the OVA transgene up-regulated the expression of immunosuppressive cytokines, which may facilitate Foxp3 acquisition of CD4⁺ cells in the liver. Therefore, targeting allergen expression to the liver serves as a novel approach to induce active tolerance for the treatment of allergic disease.

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Author Disclosure

None of the authors has any financial conflict of interest related to this manuscript.

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Figure legends

Figure 1. AAV2/8-OVA ameliorated airway hyper-responsiveness (AHR) and inhibited OVA-specific antibody secretion in OVA-sensitized mice. **(a)** Schematic diagrams of plasmids encoding OVA and EGFP transgenes, which are composed of the hepatocyte-specific alpha 1 antitrypsin (hAAT) promoter, a 9-amino-acid influenza virus HA tag, and a polyadenylation sequence. The membrane-bound OVA expression cassette was constructed by fusing the murine B7-1 transmembrane domain (B7TM) with a cytosolic tail. ITR, inverted terminal repeat of AAV. **(b)** Experimental procedure for rAAV2/8 vector treatment and OVA sensitization. Mice were treated with AAV2/8 vector and sensitized with OVA. IV, intravenous; IP, intraperitoneal; IH, inhalation. **(c)** Methacholine-induced AHR. OVA-sensitized mice were analyzed in response to increasing concentrations of inhaled methacholine on day 85, after the last OVA challenge. Data were represented as enhanced pause (Penh) values (mean $\pm$ SEM; n=10-12 per group). Bronchoalveolar lavage fluid (BALF) samples were collected at the end of the experiment. **(d)** The levels of OVA-specific IgE (1:2 dilution) were determined by ELISA. Data are presented as mean $\pm$ SEM (n=10-12 per group). S, sensitized control; VC, AAV2/8-EGFP vector control; O, AAV2/8-OVA vector. ** $P < 0.01$ vs. S in (a) or vs. VC in (b), *** $P < 0.001$.

Figure. 2. AAV2/8-OVA reduced the infiltration of eosinophils and lymphocytes in the BALF from OVA-sensitized mice. Lung tissues were lavaged with PBS and **(a)** the percentages of different cells and absolute numbers of **(b)** total leukocytes, **(c)** eosinophils, and **(d)** lymphocytes were assessed by light microscopy using Liu staining. Data are presented as mean $\pm$ SEM (n=10-12 per group). Eo, eosinophils; Ne, neutrophils;

Ly, lymphocytes; Mo, monocytes. S, sensitized control; VC, AAV2/8-EGFP vector control; O, AAV2/8-OVA vector. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure. 3. Lung histopathology of OVA-sensitized mice treated with AAV2/8-OVA. Lung tissues were harvested and fixed in formaldehyde at the end of the sensitization protocol. Paraffin-embedded lung tissues were sliced and subjected to **(a)** hematoxylin and eosin (H&E) and **(b)** periodic acid-Schiff (PAS) staining. Representative sections are shown for each group. Scale bar = 200 μm . **(c)** Inflammatory cell infiltration and **(d)** goblet cell hyperplasia were quantified by MetaMorph and presented as percentages (mean $\pm$ SEM). Quantitative results in untreated mice are defined as 100%. The RNA expression levels of **(e)** gob5 and **(f)** Muc5ac were analyzed by quantitative PCR (n=9 per group) and normalized to β -actin. The fold change was contrasted to untreated mice and presented as a percentage (mean $\pm$ SEM). S, sensitized control; VC, AAV2/8-EGFP vector control; O, AAV2/8-OVA vector. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure. 4. Expression of Th2-related cytokines and eotaxin chemokines was suppressed in the lungs of OVA-sensitized mice treated with AAV2/8-OVA. Total RNA was extracted from homogenized lung tissues and the RNA expression levels of **(a)** Th2 or Th1 cytokines and **(b)** chemokines detected by quantitative PCR (n=9 per group). The relative quantity of each RNA was normalized to β -actin and the fold change determined relative to the untreated group. Data are presented as mean $\pm$ SEM. S, sensitized control; VC, AAV2/8-EGFP vector control; O, AAV2/8-OVA vector. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure. 5. Inhibited Th2 cytokine secretion by OVA-stimulated lymphocytes from the

mediastinal lymph nodes (mLNs) and spleens of OVA-sensitized mice treated with AAV2/8-OVA. Lymphocytes from **(a)** mLNs (n=4-6 samples in each group, each sample was pooled cells from 2-3 mice) and **(b)** spleens (n=10-12 per group) were collected and re-stimulated with 100 µg/ml OVA for 6 days. Culture supernatants were measured for IL-4, IL-5, and IL-13 using ELISA. Data are presented as mean ± SEM. S, sensitized control; VC, AAV2/8-EGFP vector control; O, AAV2/8-OVA vector. * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

Figure. 6. AAV2/8-OVA significantly enhanced Foxp3 expression in CD4⁺ cells from the livers and lungs of OVA-sensitized mice. The livers (n=6-8 per group) and lungs (n=10 per group) were homogenized for RNA extraction at the end of the sensitization protocol. Expression levels of **(a, b)** Foxp3, **(c, d)** TGF-β, and **(e, f)** IL-10 mRNA were determined by quantitative PCR. The relative quantity of each mRNA was normalized to β-actin. Foxp3 expression levels in CD4⁺ cells were calculated as the normalized levels of Foxp3 expression divided by the normalized levels of CD4 expression. The fold change was relative to the untreated group. Data are presented as mean ± SEM. S, sensitized control; VC, AAV2/8-EGFP vector control; O, AAV2/8-OVA vector. * $P<0.05$; ** $P<0.01$.

Supplementary Figures

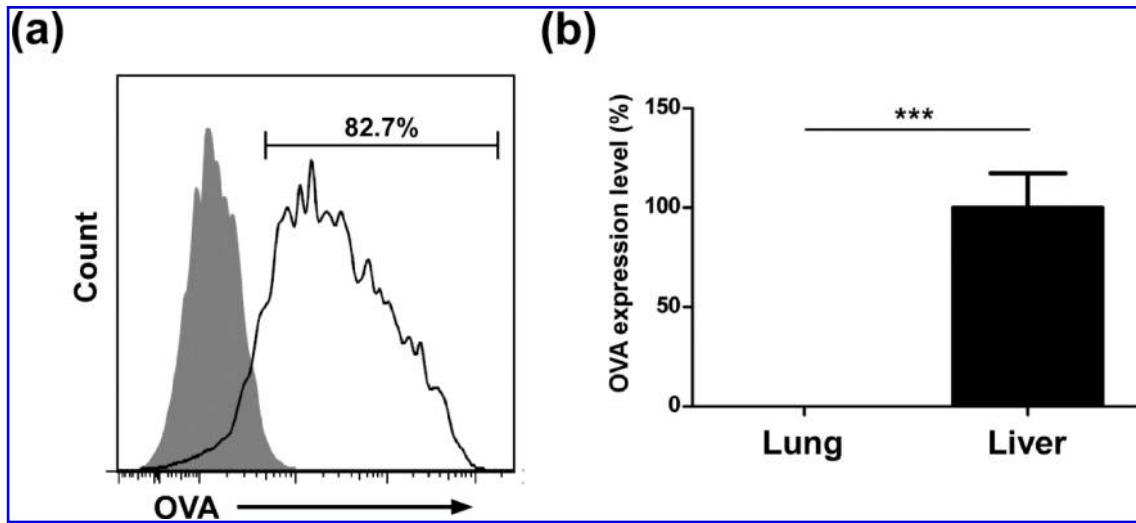


Figure S1. OVA transgene expression *in vitro* and *in vivo*. **(a)** Huh7 cells were transfected with pAAV/hAAT-EGFP (gray area) or pAAV/hAAT-OVA (black line) vectors. The expression of membrane-bound OVA was analyzed by flow cytometry using mouse anti-OVA mAb. **(b)** Total RNA was extracted from the livers or lungs of mice receiving recombinant AAV2/8-OVA and OVA transgene expression detected by quantitative PCR. The fold changes in the lungs were contrasted to those in the liver. Data are presented as mean $\pm$ SEM (n=10 per each tissue). *** P <0.001.

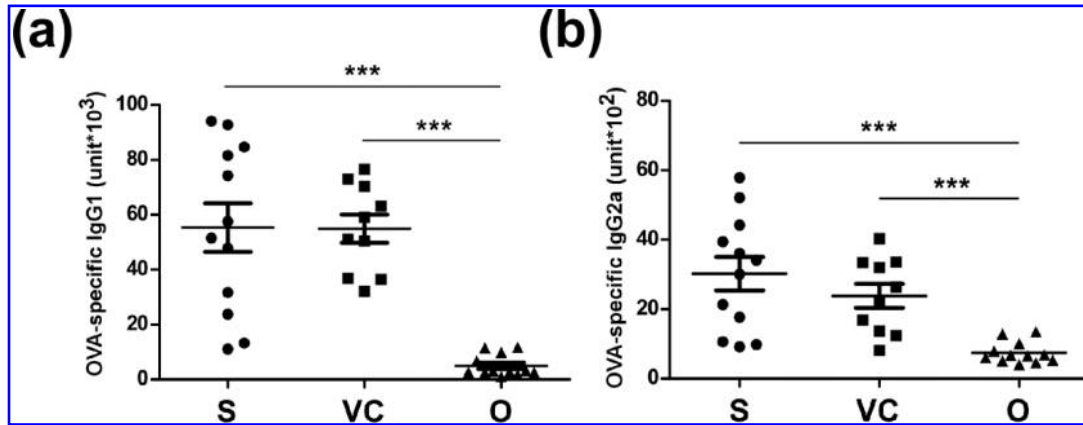


Figure. S2. The secretion of OVA-specific IgG1 and IgG2a was inhibited in the BALF of OVA-sensitized mice treated with AAV2/8-OVA. At the end of the sensitization protocol, the BALF supernatants were harvested and diluted (IgG1: 1:10,000; IgG2a: 1:500) to determine the levels of OVA-specific **(a)** IgG1 and **(b)** IgG2a by ELISA. Data are presented as mean $\pm$ SEM (n=10-12 per group). S, sensitized control; VC, AAV2/8-EGFP vector control; O, AAV2/8-OVA vector. *** $P<0.001$.

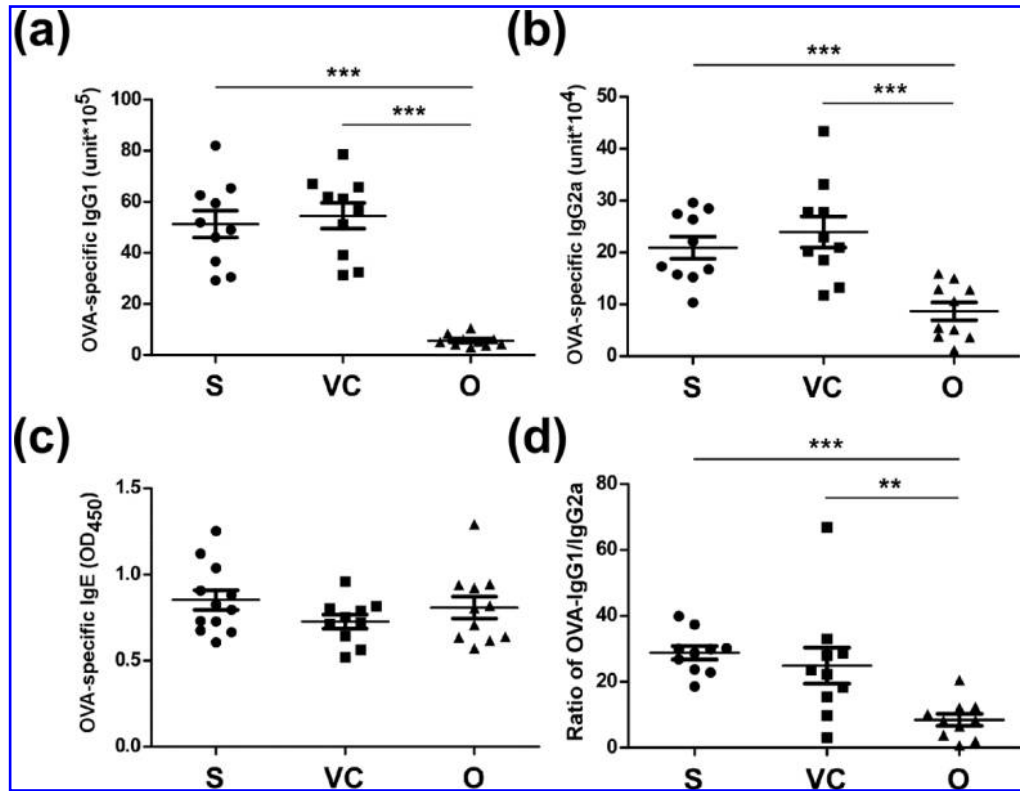


Figure. S3. Serum OVA-specific IgG1 and IgG2a levels were suppressed by AAV2/8-OVA treatment. Serum samples were collected and diluted (IgG1: 1:500,000; IgG2a: 1:10,000; IgE: 1:20). Levels of OVA-specific (a) IgG1, (b) IgG2a, and (c) IgE were determined by ELISA, and (d) the ratio of OVA-specific IgG1 to IgG2a was also determined. Data are presented as mean $\pm$ SEM (n=10 per group). S, sensitized control; VC, AAV2/8-EGFP vector control; O, AAV2/8-OVA vector. **P<0.01; ***P<0.001.

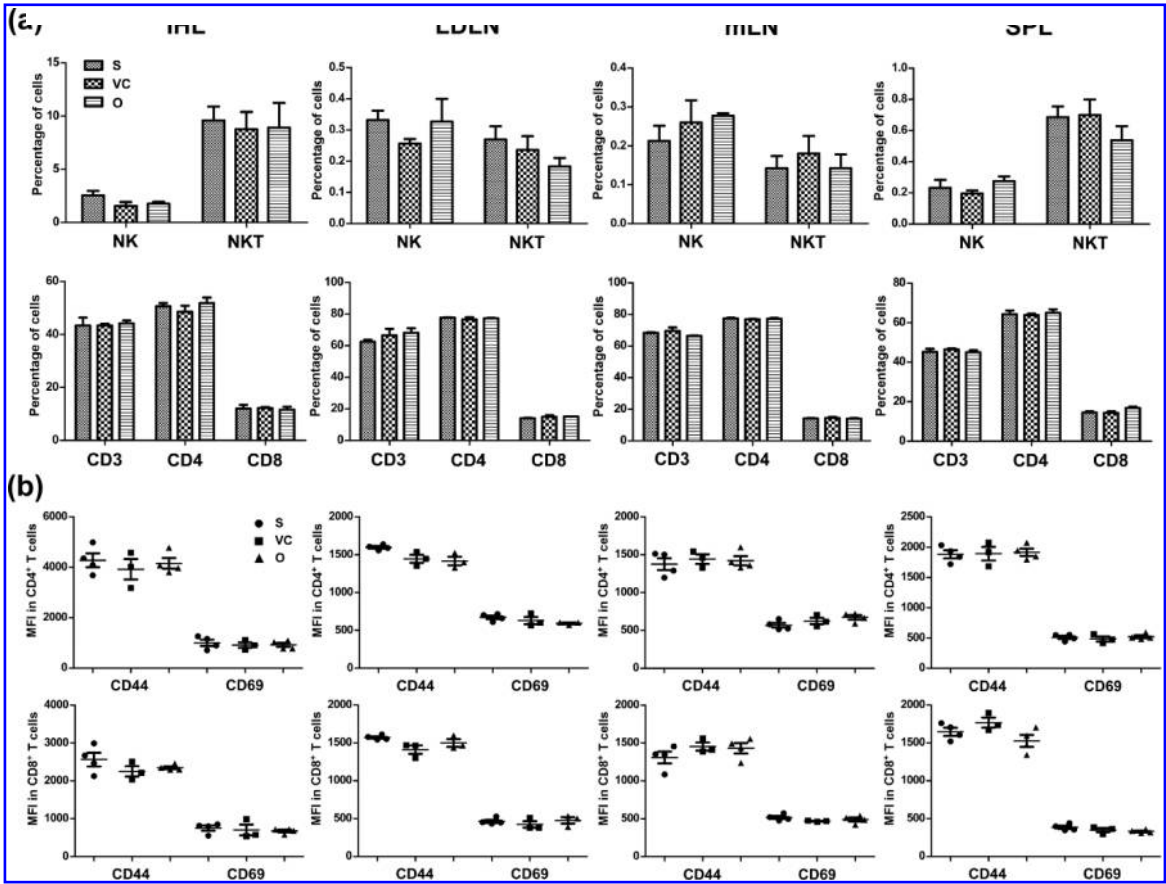


Figure. S4. AAV2/8-OVA did not influence the frequencies and activation profiles of various lymphocyte subpopulations in the livers, spleens, and draining lymph nodes of OVA-sensitized mice. At the end of the sensitization protocol, single cell suspensions from the liver, spleen, and liver-draining and mediastinal lymph nodes (LNs) were prepared as described in *Supplementary Materials and Methods*. **(a)** The frequency of different lymphocyte populations in the innate (NK and NKT cells) or adaptive (CD3, CD4, and CD8) immune system were evaluated by flow cytometry (n=4 per group). **(b)** For the activation profiles of CD4⁺ and CD8⁺ T cells, CD44 and CD69 expression was examined (n=4 per group). Data are presented as mean $\pm$ SEM. IHL, intrahepatic lymphocyte; LDLN, liver-draining LN; mLN, mediastinal LN; SPL, spleen lymphocyte; S, sensitized control; VC, AAV2/8-EGFP vector control; O, AAV2/8-OVA vector.

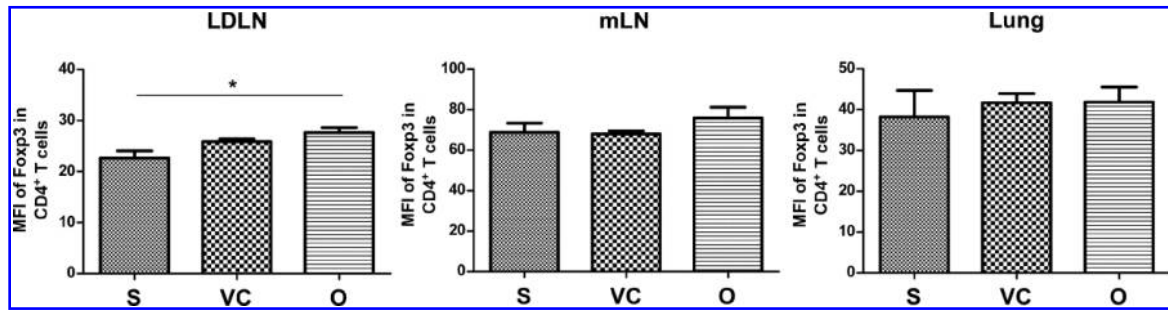


Figure. S5. Foxp3 intensity in CD4⁺ T cells from the lungs and draining lymph nodes of OVA-sensitized mice treated with AAV2/8-OVA. Foxp3 expression by CD4⁺ T cells from liver-draining lymph nodes (LDLNs), mediastinal lymph nodes (mLNs), and lungs was determined by flow cytometry for the CD4⁺ gated cell population (n=4 per group). Data are presented as mean $\pm$ SEM. MFI, mean fluorescent intensity; S, sensitized control; VC, AAV2/8-EGFP vector control; O, AAV2/8-OVA vector.

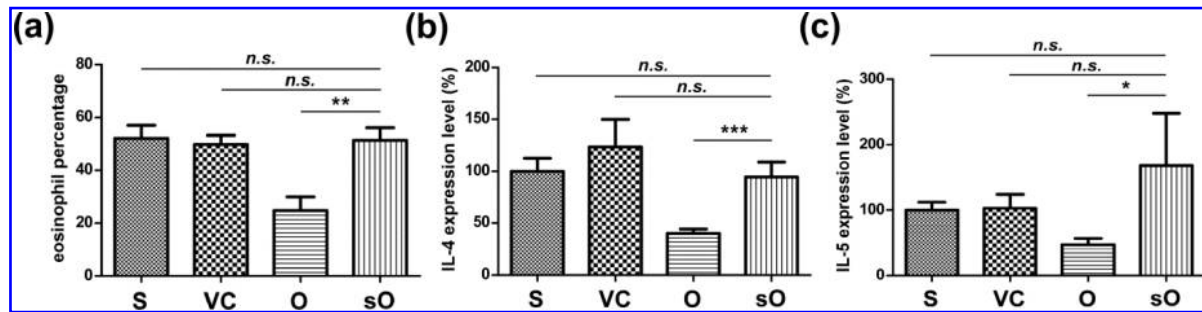


Figure. S6. Recombinant AAV vector carrying the secreted form of OVA did not reduce eosinophilic infiltration and Th2 cytokine expression in the lungs of OVA-sensitized mice.

(a) The percentage of eosinophils (n=10-12 in sensitized control (S), AAV2/8-EGFP vector control (VC), and AAV2/8-OVA vector with membrane OVA (O) groups; n=9 in AAV2/8-sOVA vector carrying secreted form of OVA (sO) group) and RNA expression levels of **(b)** IL-4 and **(c)** IL-5 (n=9 in S, VC, and O groups; n=6 in sO group) were examined as described in the *Materials and Methods*. The relative quantity of each RNA was normalized to β -actin, and the fold change was relative to the non-treatment group. Data are presented as mean $\pm$ SEM. n.s., no significance; * P <0.05; ** P <0.01; *** P <0.001.

Supplementary Table S1. Primer sequences for quantitative PCR

Gene		Sequence
CD4	forward	5'-AGGAAGTGAACCTGGTGGTG-3'
	reverse	5'-CTCCTGCTTCAGGGTCAGTC-3'
CCL22	forward	5'-AGGTCCCTATGGTGCCAATGT-3'
	reverse	5'-CGGCAGGATTTTGAGGTCCA-3'
OVA	forward	5'-CACAAGCAATGCCTTTCAGA-3'
	reverse	5'-GACTTCATCAGGCAACAGCA-3'

Supplementary Materials and Methods

RNA isolation and quantitative PCR

The extraction and reverse transcription of total RNA from the liver and lung was performed as described in the *Materials and Methods*. The complementary DNA were analyzed by quantitative PCR using specific primers (Supplementary Table S1) to detect OVA transgene expression.

Measurement of serum and BALF OVA-specific IgG1 and IgG2a antibodies

The levels of OVA-specific IgG1 and IgG2a antibodies in the BALF or serum were analyzed by ELISA. Briefly, the plates (Costar, Corning, NY, USA) were coated with 10 µg/ml OVA at 37°C for 1 hour and then incubated with diluted samples at 37°C for another hour. Next, the biotinylated rat anti-mouse IgG1 and IgG2a monoclonal antibodies (BD PharMingen, San Diego, CA, USA) and streptavidin-conjugated horseradish peroxidase (HRP) (BD PharMingen) were sequentially added to the plates. Finally, the plates were incubated with substrate solution (TMB, BD Biosciences) for 20 minutes and the reaction stopped with 2N H₂SO₄. Absorbance was measured using an ELISA reader at 450 nm (SpectraMax M2, Molecular Devices, CA, USA). The concentration of either OVA-specific IgG1 or IgG2a was defined in arbitrary units according to each of the standard curves based on the pooled serum obtained from multiple OVA-sensitized mice.

Cell isolation

Intrahepatic lymphocytes (IHLs) were prepared as described previously.¹ Briefly, the

liver was perfused immediately with digestion buffer (HBSS containing 0.01% collagenase IV, Sigma) through the hepatic portal vein after sacrifice. Following perfusion, the liver was minced into small pieces and incubated in digestion buffer at 37°C for 40 minutes. Cell suspensions were obtained from homogenized liver gently passed through a cell strainer (100 μ m, BD Bioscience). Parenchymal cells were separated from the IHLs by centrifugation at 50 x g for 5 minutes. The remaining cells were suspended in a 33% Percoll gradient solution (GE Healthcare, Uppsala, Sweden) to purify the hepatic lymphocytes by centrifugation at 754 x g for 18 minutes.

Similar procedures were used to isolate pulmonary lymphocytes. The lung tissue was perfused with PBS via the right ventricle, excised from the thoracic cavity, and minced into small pieces in digestion solution (RPMI 1640 with 1% penicillin/streptomycin, 20 mM HEPES, 200 U/ml collagenase, and 0.05% DNase I) prior to incubation at 37°C for 30 minutes on a rotary agitator (190 rpm). After incubation, the digested lung tissue was gently grinded using a cell strainer (40 μ m) to generate cell suspensions. Lung lymphocytes were prepared in a 47% Percoll gradient solution by centrifugation at 1500 rpm for 10 minutes at 4°C. Cells were isolated from the spleens and draining lymph nodes as described in the *Materials and Methods*.

Flow cytometry

To detect the expression of the OVA transgene, human hepatoma Huh7 cells were transiently transfected with pAAV/hAAT-OVA vectors using Lipofectamine 2000 (Invitrogen). After 24 hours, cells were harvested and then incubated with mouse anti-OVA monoclonal antibody (mAb) (OVA-14, Sigma) and Alex Fluor 488-conjugated goat anti-mouse IgG (Invitrogen). For cell population and activation analysis, isolated

single cells from various tissues were suspended in staining buffer (1% BSA in PBS) and pre-incubated with anti-mouse CD16/32 mAb (clone 93) for 20 minutes on ice to block non-specific binding of Abs to Fc receptors. Surface marker expression was assessed by flow cytometry using phycoerythrin (PE)-conjugated anti-CD3 ϵ (145-2C11), allophycocyanin (APC)-conjugated or fluorescein isothiocyanate (FITC)-conjugated anti-CD4 (GK1.5), PE-conjugated or APC-eFluor 780-conjugated anti-CD8 (53-6.7), PE-Cy7-conjugated anti-CD44 (IM7), FITC-conjugated anti-CD69 (H1.2F3), and brilliant violet 421-conjugated anti-NK1.1 (PK136). Staining reactions were performed at 4°C for 30 minutes in the dark. For intracellular Foxp3 staining, cells were pre-incubated with relevant mAbs against cell surface markers and washed. Next, the cell pellets were re-suspended in fixation/permeabilization buffer (Foxp3 Staining Buffer Set, eBioscience, San Diego, CA, USA) for 16 hours at 4°C in the dark. Finally, the cells were stained with APC-conjugated anti-Foxp3 or isotype control Ab. All mAbs were obtained from BioLegend or eBioscience. Stained cells were analyzed by FACSCalibur or CantoII flow cytometry and the data processed using CellQuest or DIVA software (BD Bioscience).

Construction and preparation of AAV2/8 vector carrying the secreted OVA gene fragment

The recombinant AAV (rAAV) construct with the coding sequence for OVA (designated as AAV2/8-sOVA) was similar to membrane-bound OVA as described in the *Materials and Methods*, but this vector did not contain the 178 COOH terminal amino acids of the B7-1 domain including the transmembrane domain. The procedure to generate the AAV2/8-sOVA vector and determine copy number was performed similar to other vectors as described in the *Materials and Methods*.

Supplementary references

1. Chang CM, Lo CH, Shih YM et al. Treatment of hepatocellular carcinoma with adeno-associated virus encoding interleukin-15 superagonist. Hum Gene Ther 2010;21:611-621.