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CARsomes inhibit airway allergic inflammation in mice by inducing antigen-specific Th2 cell apoptosis

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Abstract

Background: Skewed T helper (Th)2 response plays a crucial role in the pathogenesis of allergic diseases. The therapeutic efficacy for allergic diseases is unsatisfactory currently. This study aims to regulate the skewed Th2 response with CARsomes.

Methods: The CARsome consisted of an epitope of *Dermatophagoides farinae*-1 (Derf1), a segment of the anti-DEC205 antibody, the scFv, and an open reading frame of perforin. This fusion protein binds to DEC205 molecule on the surface of exosomes derived from dendritic cells (DC). The effects of CARsome on inducing antigen (Ag)-specific Th2 cell apoptosis were assessed both in vivo and in vitro.

Results: Exposure to CARsomes in the culture induced Ag-specific Th2 cell apoptosis. Injection of CARsomes through the vein puncture also induced Ag-specific Th2 cell apoptosis in the lungs of sensitized mice. CARsomes could induce Ag-specific regulatory T cells. Administration of CARsomes efficiently inhibited experimental allergic airway inflammation.

Conclusions: The CARsomes can inhibit allergic airway inflammation by inducing Ag-specific Th2 cell apoptosis and induce Ag-specific regulatory T cells. The data suggest that CARsomes have the translational potential to be used to treat allergic airway inflammation.

KEYWORDS

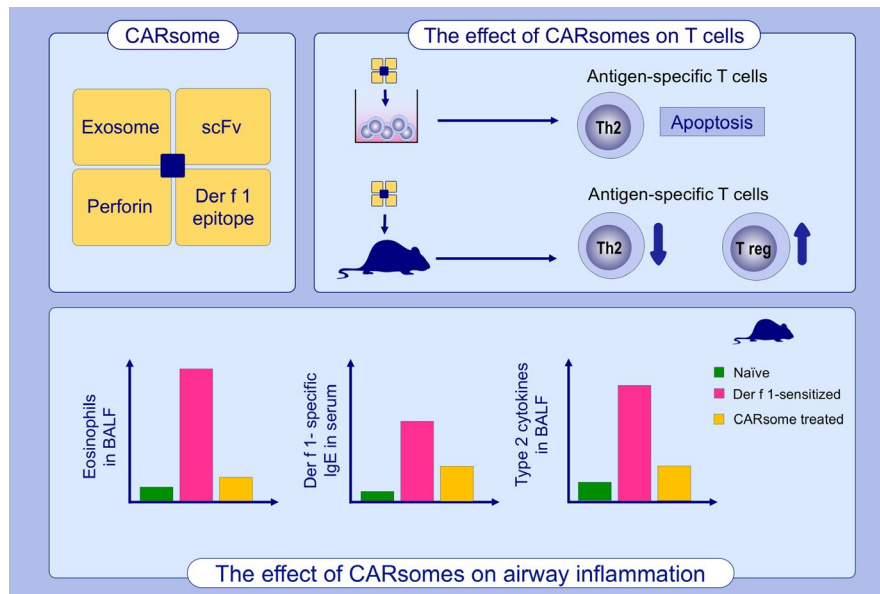
allergy, apoptosis, asthma, inflammation, T helper cells

1 | INTRODUCTION

Allergic asthma (asthma, in short) is an adverse response by the immune system in the airway tissues to airborne antigens, such as house dust mites (HDM). The prevalence of asthma is approaching to 10% worldwide¹; the underlying mechanism of asthma is to be further investigated. The clinical symptoms of asthma include spontaneous short of breathes, cough, and profound secretions in the airway tract.² If proper medical intervention is not applied for

asthma, complications may occur, patients can develop chronic airway obstruction, and pulmonary heart diseases or heart failure.³ To date, the therapeutic efficacy of asthma is not satisfactory currently.² Thus, it is urgent to invent novel and effective remedies for the treatment of asthma.

It is accepted that the skewed T helper (Th)2 polarization plays a key role in the pathogenesis of asthma.⁴ The Th2 polarization indicates a status that Th2 cells over populate in the local tissues. Thereby, the local tissues contain high levels of Th2 cytokines. The Th2 cytokines,



GRAPHICAL ABSTRACT

The regulatory function of CARsome, a fusion protein consisting of a dendritic cell-derived exosome, a segment of the anti-DEC205 antibody (scFV), an epitope of Der f 1 antigen, and an open reading frame of perforin, was assessed both in vitro and in vivo. In vitro CARsomes induce apoptosis of antigen-specific Th2 cells. In vivo they reduce the number of antigen-specific Th2 cells and increase Treg numbers. CARsomes efficiently inhibit allergic airway inflammation in mice.

especially the IL-4, induce plasma cells to produce a large amount of IgE. IgE binds to the high affinity receptors of IgE on the surface of mast cells to make mast cells sensitized. Re-exposure to specific antigens drives the sensitized mast cells to release chemical mediators to trigger an allergic response.⁵ Yet, we do not have satisfactory methods to regulate the skewed Th2 response in subjects with allergic diseases currently.⁶ Therefore, it is necessary to further investigate the pathogenesis of the skewed Th2 response and design proper methods to adjust it.

It is reported that CD4⁺ T cells of asthma patients have defects of apoptosis.⁷ Whether this phenomenon plays a role in the pathogenesis of asthma is to be further investigated. Apoptosis is a physiological process in the body to eliminate unnecessary cells, such as damaged, unrepairable cells or senescent cells via activating Fas/FasL, p53, or caspase pathways.⁸ The defects of apoptosis may extend the lifespan of the Ag-specific Th2 cells in asthma patients.⁷ The apoptotic process also can be regulated in the cell. Some exogenous factors, such as perforin, can induce target cell apoptosis.⁹ Therefore, we hypothesize that to induce Ag-specific Th2 cell apoptosis can correct the skewed Th2 polarization in asthma subjects and ameliorate asthma. In this study, prompted by the CAR-T (T cells carrying chimeric antigen receptor) system,¹⁰ we constructed the CARsome. It is a fusion protein consisting of a segment of the anti-DEC205 antibody, the scFv, an epitope of the specific antigen, *Dermatophagoides farinae*-1 (Derf1; a sub-antigen of HDM; one of the most common antigens of allergic asthma¹¹), and an open reading frame (ORF) of the perforin molecule (used to induce target cell apoptosis). The fusion protein binds to the DEC205 molecule on exosomes derived from dendritic cells (DCs). The effects of CARsome on inducing Ag-specific Th2 cell apoptosis and

suppressing allergic airway inflammation in a murine model were evaluated.

2 | MATERIALS AND METHODS

2.1 | CARsome construction

The CARsome consists of a DC-derived exosome, an MHC II molecule, an epitope of Derf1 (one of the allergens derived from house dust mite), an ORF of perforin molecule, and scFv (a segment of the anti-DEC205 antibody). The Derf1 gene was obtained at the website of <https://www.ncbi.nlm.nih.gov/nucleotide/AB034946>. Employing the Immune Epitope Database and Analysis Resource (http://www.iedb.org/home_v3.php), one MHC II binding dominant epitope (CATCAAAAATGAAAT) was designed from the Derf1 gene sequence; an epitope from ovalbumin, ATGTGTTTCCCTTA, was designed and used as a nonspecific epitope. The perforin gene was obtained from the NCBI website (Accession: NM_005041.5). A dominant open reading frame (ORF) of perforin was designed with the NCBI ORF Finder (<https://www.ncbi.nlm.nih.gov/orffinder/>) from 1010 to 1816 (including 807 nt, 268 aa). The gene of scFv, a segment of the anti-DEC205 antibody, was obtained from NCBI (ID: KC596074). The sequences were fused with a linker, Gly4Ser, and cloned into the vector PET32a by Sangon Biotech. Orientation and codon in-frame for all constructs were confirmed by restriction analysis and sequencing by Sangon Biotech. More information about the CARsome construction and the binding capacity between CARsomes and exosomes is presented in Figure S1.

2.2 | Assessment and isolation of Ag-specific Th2 cells

Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples of asthma patients by Percoll gradient density centrifugation and cultured in the presence of specific Ag (referred to the results of the Ag skin test and Ag-specific IgE assessment) overnight.¹² More than 95% of the CD4⁺ CD154⁺ T cells were isolated by flow cytometry cell sorting (FCCS). The isolated CD4⁺ CD154⁺ T cells were also IL-4⁺ and regarded as Ag-specific Th2 cells. Purity of isolated Ag-specific Th2 cells was greater than 95% as checked by flow cytometry.

2.3 | Isolation of Ag-specific iTregs

Sensitized mice were treated with CARsomes daily for 1 week. After sacrifice, the lungs were excised. Lung mononuclear cells (LMCs) were isolated from lung tissue and cultured in the presence of specific antigens, Derf1 (10 µg/mL), overnight. CD4⁺ CD25⁺ CD154⁺ CD127⁻ iTregs were sorted by flow cytometry cell sorting. Purity of isolated cells was greater than 95% as assessed by flow cytometry.

2.4 | Statistics

The difference between two groups was determined by Student's *t* test. ANOVA followed by Dunnett's *t* test or Bonferroni test was performed for multiple comparisons. *P* < .05 was considered statistical significance.

3 | RESULTS

3.1 | Characterization of CARsomes

A fusion protein molecule containing Derf1, scFv, and perforin was constructed (Figure 1A). Exosomes were generated from bone marrow-derived DCs (BMDC), purified and used to construct CARsomes with the fusion protein. The CARsomes contain DEC205, MHC II, and the fusion protein (Figure 1B-F). Exosomes from DEC205-deficient DCs (Figure 1H) were not bound by the fusion protein (Figure 1I), indicating the fusion protein binds the DEC205 in the exosomes. By immunoelectron microscopy, the exosomes are 60-100 nm in size and are bound by the fusion protein (Figure 1J).

3.2 | CARsomes induce Ag-specific Th2 cell apoptosis

This study aims to employ CARsomes to eliminate Ag-specific Th2 cells from subjects with allergic disorders by inducing the cell apoptosis. CD4⁺ T cells were prepared from asthma patients and healthy

control (HC) subjects. The cells were cultured in the presence of CARsomes for 24 hours with cisplatin to test their responsiveness to apoptosis inducer. We observed that exposure to cisplatin (a nonspecific apoptosis inducer¹³) markedly induced naive CD4⁺ T-cell apoptosis, while significantly less apoptotic cells were induced in CD4⁺ T cells collected from asthma patients (Figure S2), indicating that CD4⁺ T cells of asthma patients are resistant to apoptosis. Exposure to CARsomes in the culture markedly increased the number of apoptotic CD4⁺ T cells in samples collected from asthma patients (asthma CD4⁺ T cells, in short) sensitized to mites in a CARsome dose- and time-dependent manner (Figure S3A-D), but not in those collected from asthma patients sensitized to other antigens or from HC subjects (Figure 2A,B). CARsomes lacking of either scFv or perforin or Ag (Derf1) did not induce Ag-specific CD4⁺ T-cell apoptosis (Figure S3E,F). Over 90% of the apoptotic CD4⁺ T cells expressed CD154, while less than 6% annexin v⁻ CD4⁺ T cells showed CD154⁺ (Figure 2C-F). Exposure to CARsomes did not induce the CD154⁻ T-cell apoptosis (Figure 2G-J). The data indicate that the apoptotic asthma CD4⁺ T cells are Ag-specific as the increase in CD154 expression is a specific feature of Ag-specific T-cell activation.¹² The results demonstrate that CARsomes can specifically induce Ag-specific Th2 cell apoptosis.

3.3 | CARsomes reduce the number of Ag-specific Th2 cells in sensitized mice

We then developed an asthma mouse model. The sensitized mice were treated with CARsomes through the tail vein injection daily for 6 days. After sacrifice, the lung was excised for isolation of lung mononuclear cells (LMC). LMCs were cultured in the presence of specific Ag for 24 hours. The LMCs were harvested and analyzed by flow cytometry. The results showed that the frequency of CD4⁺ CD25⁺ CD154⁺ IL-4⁺ Th2 cells was significantly higher in sensitized mice than that of control mice (treated with saline). Treatment with CARsomes markedly reduced the numbers of Ag-specific Th2 cells in the lung, but did not alter the number of non-Ag-specific Th2 cells (Figure 3A-D). CARsomes also induced apoptosis in Ag-specific Th2 cells, while apoptosis was scarcely detected in non-Ag-specific Th2 cells (Figure 3E-I). The results indicate that administration of CARsomes can reduce the numbers of Ag-specific Th2 cells in the lung of sensitized mice, in which induction of Ag-specific Th2 cell apoptosis plays an important role.

3.4 | CARsomes induce Ag-specific Tregs in sensitized mice

Published data indicate that phagocytosis of apoptotic cells by phagocytes promotes the induction of Tregs.^{14,15} We inferred that the apoptotic Th2 cells induced by CARsomes might induce Ag-specific Tregs in sensitized mice. To test this, sensitized mice were treated with CARsomes by vein injection daily for 6 days.

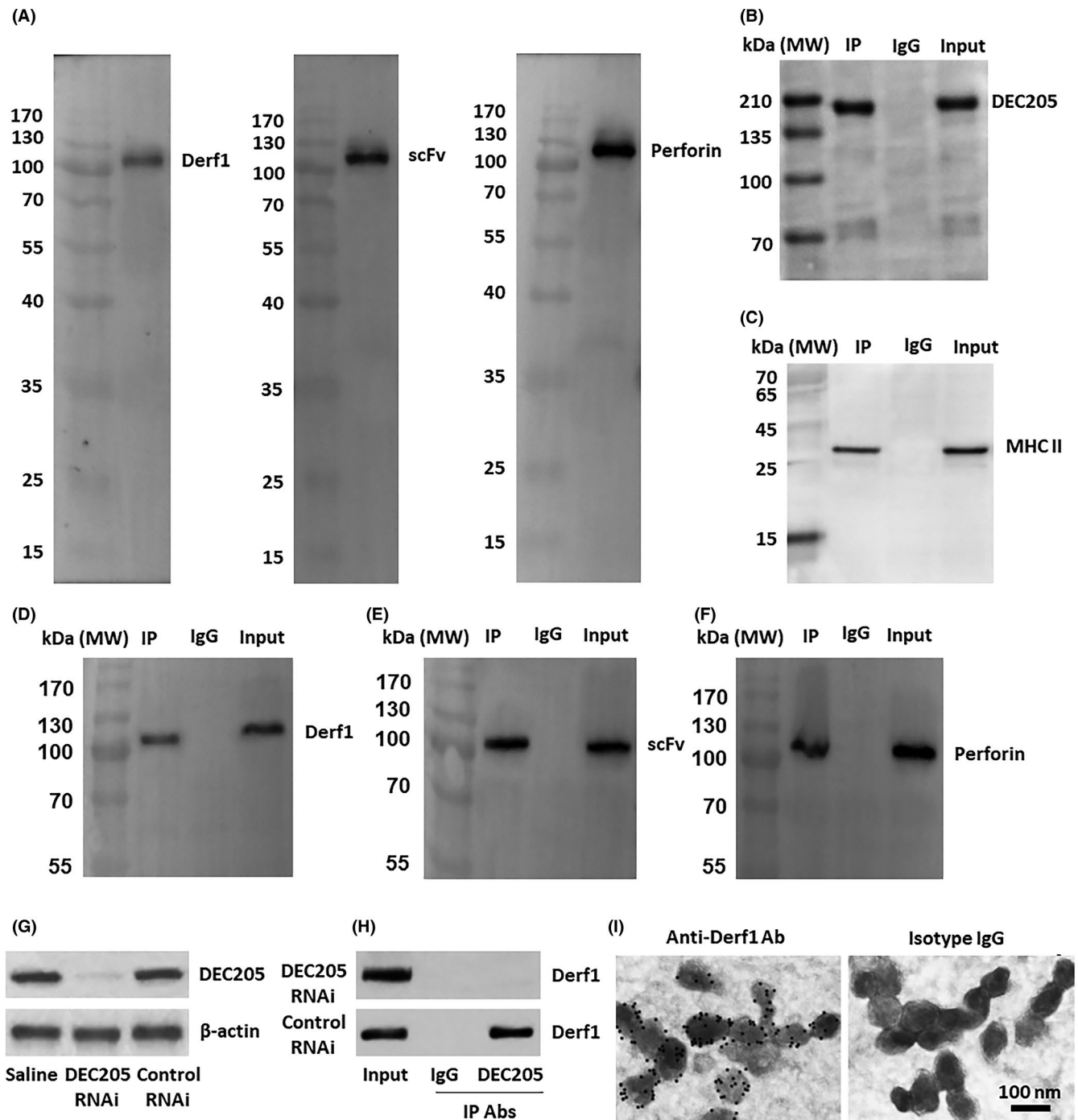


FIGURE 1 Characterization of CARsomes. A, immunoblots show that the fusion protein reacts to antibodies of Derf1, scFv and perforin, respectively. B-F, immunoprecipitation (IP) results show five components of a complex on DC-derived exosomes. G, results of DEC205 RNAi of DCs. H, the fusion protein cannot bind to DEC205-deficient exosomes. I, representative electron photomicrographs show DC-derived exosomes. The gold particles (6 nm) indicate anti-Derf1 antibody binding. Original magnification: $\times 40\,000$. IP: Samples were prepared by IP. Input: Samples collected in lysates before IP using as controls. IgG: Samples obtained by IP using isotype IgG as IP antibodies. The data represent six independent experiments

After sacrifice, the lungs were excised, LMCs were prepared and analyzed by flow cytometry. Indeed, the number of Ag-specific $CD4^+ CD25^+ Foxp3^+$ Tregs was induced in the lung by treating with CARsomes. About 85.6% of the inducible Tregs (iTreg) were also $TGF-\beta^+$, indicating that most iTregs express $TGF-\beta$. In addition, 87.9% of the iTregs were $CD154^+$, indicating that the iTregs

are Ag-specific¹² (Figure 4A-C). The Ag-specific Tregs had the immune suppressive functions in a specific Ag-dependent manner (Figure 4D,E). The results indicate that CARsomes have the capacity to induce Ag-specific iTregs in the lung of mice with airway allergic inflammation. The iTregs have immune suppressive functions.

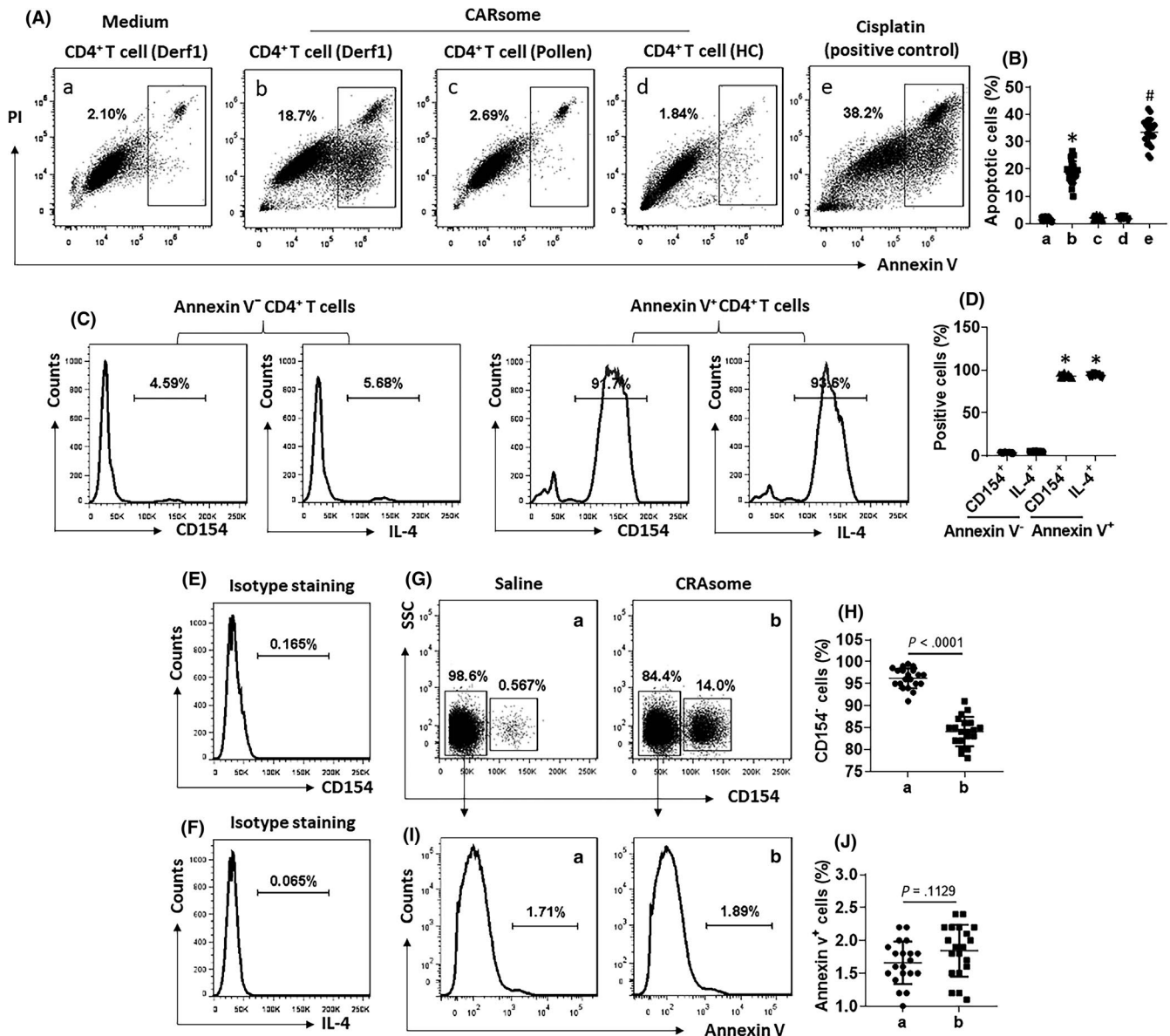


FIGURE 2 CARsomes induce Ag-specific Th2 cell apoptosis. CD4⁺ T cells were isolated from blood samples collected from asthma patients ($n = 20$; sensitized to Derf1) or patients sensitized to pollen ($n = 20$; collected in in-season) and healthy control (HC) subjects ($n = 20$). The cells were cultured in the presence of CARsomes (10 $\mu\text{g/mL}$) for 24 h. A, gated dot plots show apoptotic cells. B, scatter plots show summarized apoptotic cells. Each dot present data obtained from one subject. Data of bars are presented as mean $\pm$ SEM. * $P < .01$, compared with group a. C-D, gated histograms show frequency of CD154⁺ cells (C) and IL-4⁺ cells (D) in gated cells of panel A. E-F, isotype staining of CD154 and IL-4. G-J, asthma CD4⁺ T cells were treated with CARsomes or saline overnight. G, gated dot plots show CD154⁺ and CD154⁺ CD4⁺ T cells. H, summarized CD154⁺ and CD154⁺ CD4⁺ T cells. I, gated histograms show annexin v⁺ CD154⁻ CD4⁺ T cells. J, summarized annexin v⁺ CD154⁻ CD4⁺ T cells. Each sample was assessed in duplicate. Cisplatin (60 $\mu\text{mol/L}$) used as a positive control

3.5 | CARsomes increase amounts of Phosphatidylserine (PS) on the surface of Ag-specific Th2 cells to facilitate the induction of iTregs

We then investigated the underlying mechanism by which CARsomes induce Tregs. Published data indicate that phagocytes phagocytose apoptotic T cells to induce iTregs,¹⁴ in which PS or PD-L1 plays an important role in the process of phagocytosis.^{14,16} Data of Figures 2-4 suggest that CARsomes may increase the

levels of PS on the surface of Ag-specific Th2 cells to facilitate the induction of iTregs. To test this, we prepared LMCs from the lung tissue of sensitized mice. Pre-existing CD154⁺ cells in LMCs were selected out by FCCS. Remained LMCs were cultured in the presence of CARsomes or CARsomes without carrying perforin (CARsomes#) overnight. The cells were analyzed by flow cytometry. We found that, after exposure to CARsomes in the culture, a portion of CD4⁺ T cells show high levels of CD154 (Figure 5A); these CD4⁺ CD154⁺ T cells were also CD25⁺ IL-4⁺, indicating

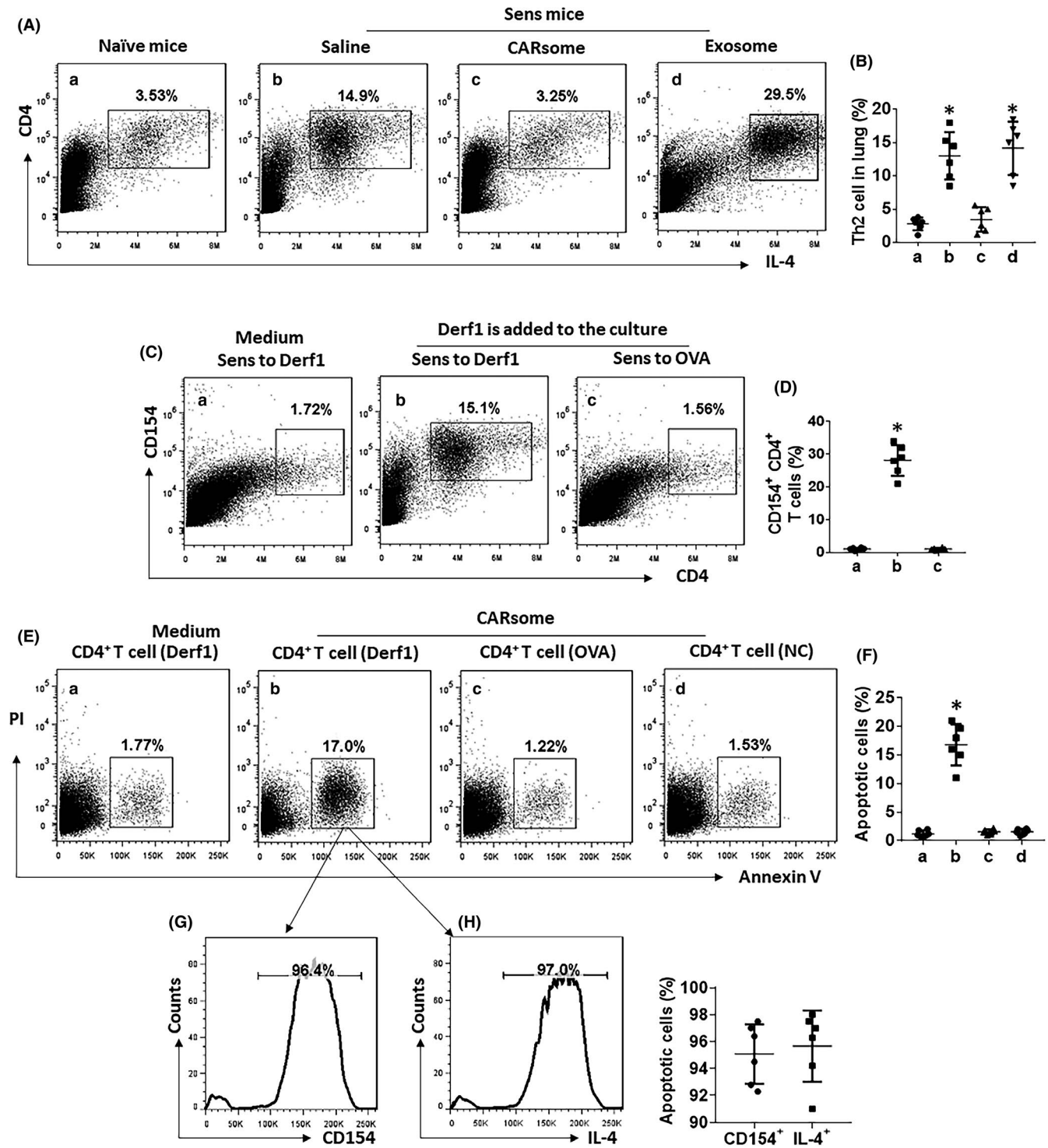


FIGURE 3 CARsomes reduce Ag-specific Th2 cells in the airway of sensitized mice. Mice were sensitized (sens) to Derf1 or OVA to develop an asthma animal model. A, the mice were treated as denoted above each subpanel. Upon sacrifice, the lungs were excised. Mononuclear cells were isolated from the lungs and analyzed by flow cytometry. Gated dot plots show frequency of Th2 cells. B, scatter plots show summarized Th2 cells in the lung. C-D, mononuclear cells were isolated from the lung and cultured in the presence Derf1 (10 µg/mL) or OVA (10 µg/mL; nonspecific antigen control) for 48 h. E-F, Gated dot plots show Ag-specific Th2 cells. Scatter dot plots show summarized Ag-specific Th2 cells. NC: Naïve control. G-I, gated histograms show the Ag-specific Th2 cells are also CD154⁺ and IL-4⁺. Scatter dot plots show summarized data of panels G and H. Each group consists of 6 mice. In panels B, D, and F, each dot present data obtained from one mouse (assessed in duplicate); data of bars are presented as mean ± SEM. *P < .01, compared with group a

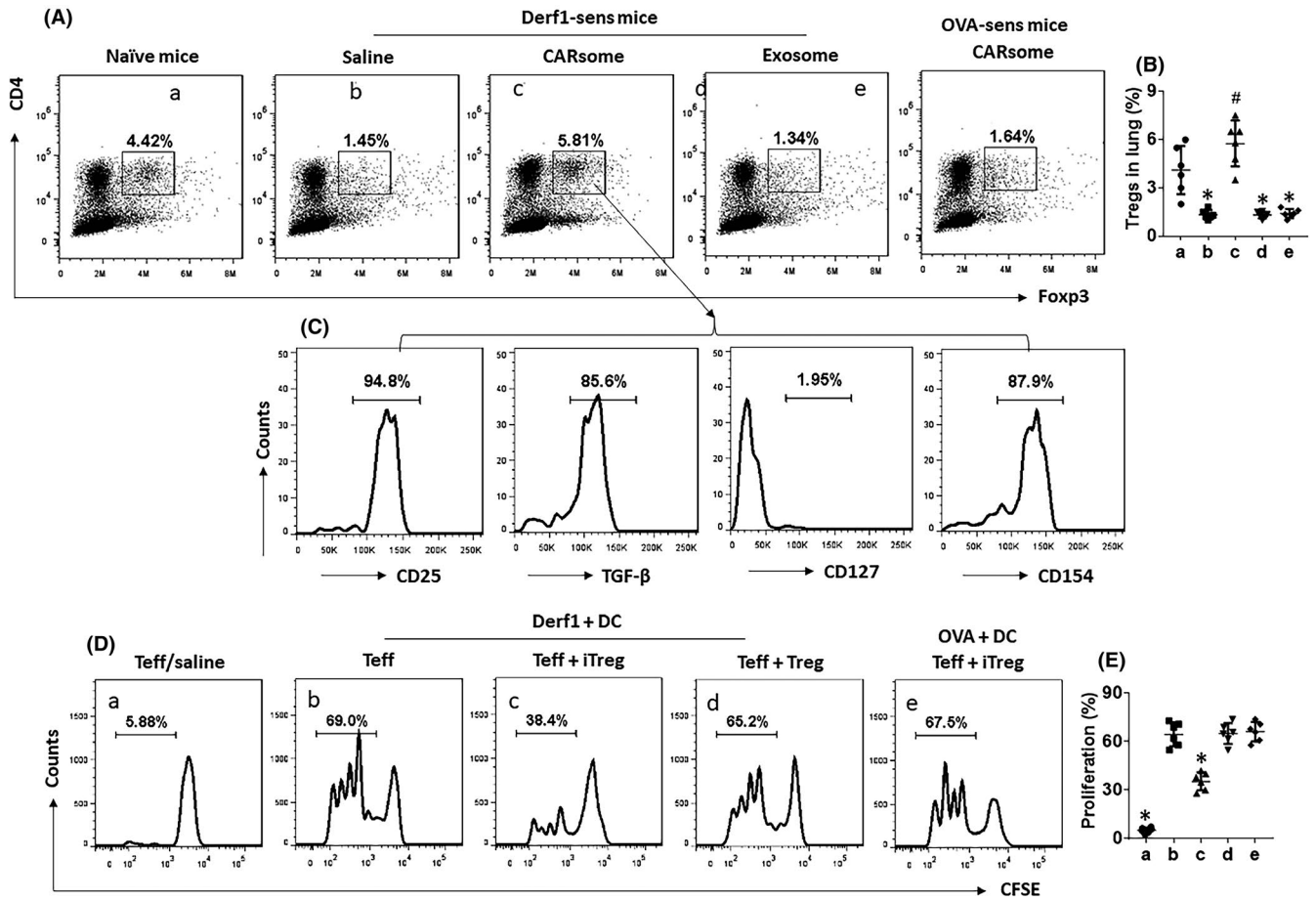


FIGURE 4 CARsomes induce iTregs in the lung of sensitized mice. Sensitized mice (6 mice per group) were injected with CARsomes (20 μ g/mouse in 0.1 mL saline) daily for 6 d. Lung mononuclear cells were isolated from each mouse and analyzed by flow cytometry. Teffs were isolated from the naïve mouse spleen. A–B, gated dot plots show the frequency of iTregs. Scatter plots show summarized iTregs. C, gated histograms show frequency of cells of CD25⁺, TGF- β ⁺, CD127⁺, and CD154⁺ in CARsome-induced lung iTregs (Ac) as denoted below each subpanel. D–E, gated histograms show proliferating Teffs after the treatment denoted above each subpanel. Scatter plots show summarized data of Teff proliferation. In B and E, each dot present data obtained from one mouse (assessed in duplicate). Data of bars are presented as mean $\pm$ SEM. * P < .01, compared with group a (B) or group b (E). # P < .01, compared with group b (B). iTreg: CARsome-induced Treg. Treg: Tregs isolated from the naïve mouse spleen. Derf1 (or OVA): Derf1 (or OVA; used as a control antigen) was presented in the culture at 10 μ g/mL. Ratio of Teff:Treg:DC = $10^5:2 \times 10^4:2 \times 10^4$

these cells are Ag-specific Th2 cells.¹² Less than 8% CD25⁺ IL-4⁺ T cells were detected in CD4⁺ CD154⁺ T cells after exposure to CARsomes (Figure 5B–E). Higher levels of PS were observed in these Ag-specific Th2 cells after treatment with CARsomes as compared with those treated with CARsome# (Figure 5F). The data demonstrate that CARsomes can activate and increase PS on the surface of Ag-specific Th2 cells.

On the other hand, the results of Figure 5 were reproduced by an *in vivo* experiment. After treatment with CARsome or CARsome# for 6 days, LMCs were isolated from the lung of mice and analyzed by flow cytometry. The results showed that the CARsome-primed Ag-specific Th2 cells had much higher levels of PS as compared with those treated with CARsome# (Figure S4). We also observed CARsome-increased PS facilitated DCs to phagocytose Ag-specific Th2 cells in an *in vivo* experiment (Figure S5), the DCs then gained the capacity to induce Ag-specific iTregs (Figures S6 and S7).

3.6 | Administration of CARsomes inhibits allergic airway inflammation in mice

Finally, we tested the effects of CARsomes on inhibition of experimental allergic airway inflammation. A mouse model of airway allergic inflammation was developed with Derf1 as the specific antigen. Sensitized mice showed asthma-like signs. Profound infiltration of mononuclear cells in the lung tissues we observed, which mainly surrounded the bronchioles (Figure 6A). Hypersensitivity was detected in sensitized mice (Figure 6B). Bronchial lavage fluids (BALF) were collected. Cellular components were isolated from BALF and analyzed by flow cytometry. The results showed that eosinophils and neutrophils were the major cell type in BALF, which were significantly more in the asthma group than that of the saline group (Figure 6C,D). Frequency of IFN- γ ⁺ and IL-17⁺ cells was slightly lower in LMC of sensitized mice (Figure S8). Serum levels of OVA-specific

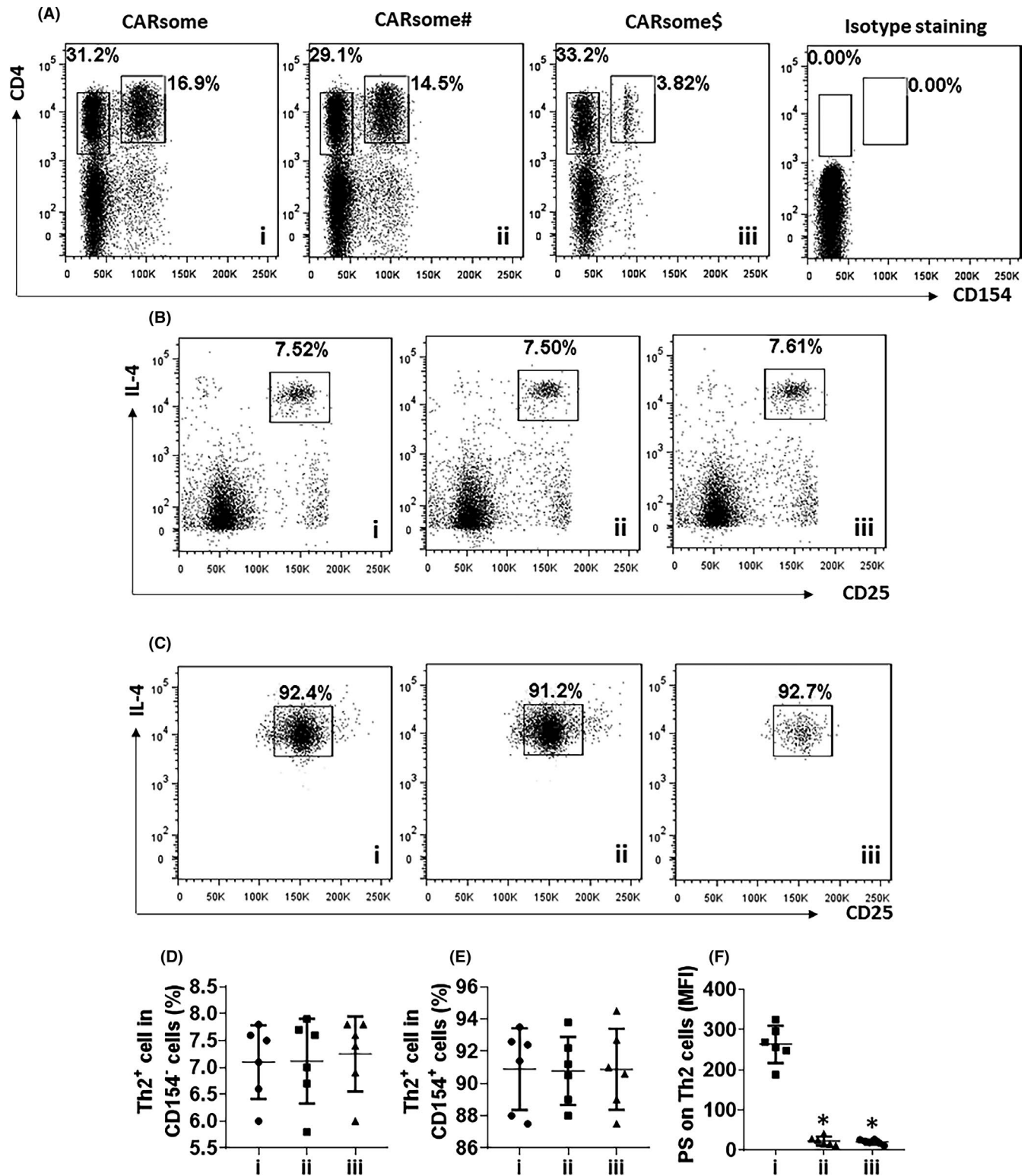


FIGURE 5 CARsomes activate Ag-specific Th2 cells. LMCs were isolated from sensitized mice; the pre-existing CD154⁺ cells were selected out; the remained cells were cultured in the presence of CARsomes or CARsomes without perforin (#), or CARsomes containing scFv, perforin, and OVA epitope (CARsome\$), for 48 h. A, gated flow cytometry dot plots show CARsome-induced CD4⁺ CD154⁺ cells (left gate) and CD4⁺ CD154⁺ T cells (right gate). B, gated dot plots show CD4⁺ CD154⁺ T cells in the right gated of panel A are also CD25⁺ IL-4⁺. C, gated dot plots show CD25⁺ IL-4⁺ T cells in the CD4⁺ CD154⁺ cells in the left gate of panel A. D-E, summarized CD25⁺ IL-4⁺ T cells in panels A and B. F, scatter dot plots show median fluorescence intensity (MFI) of PS on the surface of Ag-specific Th2 cells. Data of bars are presented as mean $\pm$ SEM. * $P < .01$, compared with the group i. Each dot present data obtained from one sample (assessed in duplicate)

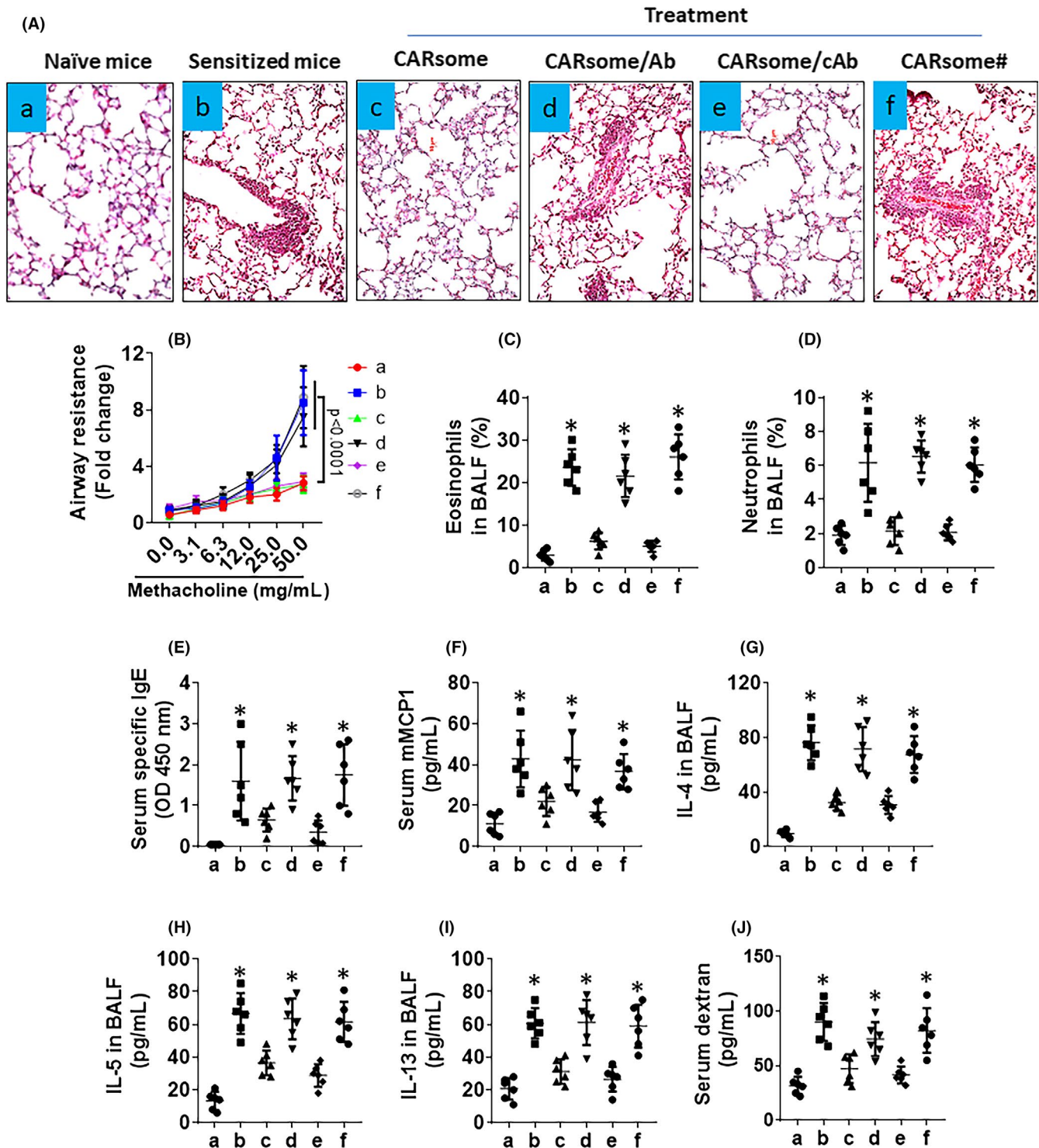


FIGURE 6 CARsomes suppress experimental allergic asthma. Sensitized mice were prepared with Derf1 as the specific antigen. Mice were treated with the procedures denoted above each image of panel A. Ab (cAb): Mice were treated with anti-CD25 antibody (or a control antibody, an isotype IgG) at 0.25 mg/mouse 1 d prior to the beginning using CARsomes. CARsome (or saline, or CARsome#): Sensitized mice were treated with CARsomes (or saline, or nonperforin CARsome) at 20 μ g/mouse through tail vein injection daily for 6 d (started 1 d after the last challenge with specific antigen). A, representative images of lung histology (original magnification: $\times 200$). B, airway resistance of mice challenged with methacholine. C-D, summarized frequency of eosinophils (C) and neutrophils (D) in BALF. E-F, serum levels of Derf1-specific IgE (E) and mouse mast cell protease-1 (mMCP1; F). G-I, levels of IL-4 (G), IL-5 (H), and IL-13 (I) in BALF. J, serum levels of TRITC-dextran used as an indicator of airway epithelial barrier functional status. Data of bars are presented as mean $\pm$ SEM. Each dot present data obtained from one mouse (assessed in duplicate). * $P < .01$, compared with group a

IgE (Figure 6E) and mouse mast cell protease-1 (mMCP1) (Figure 6F), Th2 cytokines (IL4, IL-5 and IL-13) (Figure 6G-I) in BALF were higher in sensitized mice than that in control mice. The airway epithelial barrier was dysfunctional in sensitized mice (Figure 6J). Treatment with CARsomes efficiently suppressed the asthma-like signs in the asthma group, such effects were abolished by injection of anti-CD25 Ab or when the Derf1 epitope in CARsome was replaced by an OVA epitope (CARsome#). Depletion of iTregs abolished the CARsome-induced suppressive effects (Figure S9 and Figure 6A-J). The results demonstrate that CARsomes can inhibit experimental asthma.

4 | DISCUSSION

In this study, we constructed the CARsomes, which consist of a fusion protein with the components of an epitope of Derf1, an ORF of perforin and a segment of the anti-DEC205 antibody, the scFv. The fusion protein binds to the DEC205 on DC-derived exosomes to be the CARsomes. The results showed that administration of CARsomes efficiently inhibited experimental airway allergic inflammation by reducing the number of Ag-specific Th2 cells and increasing the number of Ag-specific Tregs. The data suggest that the CARsomes have translational potential to be used in the treatment of allergic asthma.

We used DC-derived exosomes as a carrier to construct the CARsomes. Cumulative studies showed that exosomes have immune regulatory capacity.¹⁷ Our early work showed that intestinal epithelial cell-derived exosomes played an important role in the establishment of Ag-specific immune tolerance in the intestine.¹⁸ Immune active substances carried by exosomes are the basis by which exosomes fulfill the immune regulatory functions.^{17,18} In the present study, we employed a novel approach to construct CARsomes following the concept of exosomes.¹⁷ The chimeric fusion protein is the backbone of the CARsome. Such a design satisfies the purpose of inhibiting allergic asthma by the well-organized components of CARsome. The peptide of Derf1 specifies the targets that are Derf1-specific T cells. The ORF of perforin aims to initiate the apoptotic process in target cells. The results demonstrate that CARsomes reach the goal of the original design.

The data show that administration of CARsome regulates Th2 polarization in the airway of sensitized mice as well as induces apoptosis of Ag-specific Th2 cells collected from asthma patients. It is the consensus that skewed Th2 response plays a central role in the pathogenesis of allergic diseases.¹⁹ Various approaches have been tried to reconcile the skewed Th2 response. Such as allergen-specific immunotherapy,²⁰ mast cell stabilizers,²¹ anti-histamines²² and corticosteroids.²³ The therapeutic efficacy of these approaches is still to be improved. The present study provides a novel remedy for suppressing the skewed Th2 response, based on which the experimental airway allergic inflammation was efficiently suppressed. Although IL-4 plays the most important role in the initiation and maintenance of skewed Th2 response,²⁴ simply inhibit IL-4 expression may not be appropriate because IL-4 has multiple functions in immunity.²⁵ The

CARsomes specifically target to reduce Ag-specific Th2 cells that do not have the possibility to affect the immune function of the pan Th2 cell compartment. This feature is dependent on that a Derf1 peptide is carried by CARsomes. The Derf1 forms a complex with MHC II (provided by the DC-derived exosomes), by which CARsomes specifically target to Derf1-specific Th2 cells.

The CARsomes contain an ORF of perforin. The original purpose of this is to induce target cell apoptosis. Indeed, exposure to CARsomes induced Ag-specific Th2 cell apoptosis. The basis of Th2 polarization or the dominant Th2 response is that the local tissue is over populated by Th2 cells.²⁴ The pathogenesis of such a phenomenon has not been fully understood yet.²⁴ On the one hand, excessive development of Th2 cells in the local tissue can be one of the major reasons²⁶; on the other hand, defects of apoptosis in Th2 cells can be another. Jiang et al found that Th2 cells of asthma patients showed the defects of apoptosis.⁷ Our recent findings show that CD4⁺ T cells from patients or mice with allergic disorders are resistant to apoptosis inducers due to over expression of Bcl2L12.^{27,28} Therefore, to recover the apoptosis machinery in Ag-specific Th2 cells or specifically induce Ag-specific Th2 cell apoptosis can be a remedy to reduce the number of Th2 cells to alleviate the Th2 polarization in allergic asthma. The present data demonstrate that CARsomes are an effective remedy to reconcile the skewed Th2 polarization by inducing Ag-specific Th2 cell apoptosis.

The data show that administration of CARsomes can induce Tregs. A variety of approaches have been described in the generation of Tregs such as exposure to TGF- β 1,²⁹ metabolites derived from commensal bacteria,³⁰ allergen-specific immunotherapy,³¹ and through phagocytosing apoptotic T cells to generate Tregs is one of them.¹⁴ The present data demonstrate that CARsomes also induce generation of Tregs through this mechanism. CARsomes induce Ag-specific Th2 cell apoptosis, which are phagocytosed by DCs; the DCs then gain the capacity to induce Ag-specific Tregs. Comparing inducing pan Tregs, to induce the Ag-specific Tregs has a valuable merit; these Tregs only suppress the immune activities of other Ag-specific T cells as shown by the present data. The Derf1-specific Tregs only suppress Derf1-specific Th2 cells, but not suppress OVA-specific Th2 cells. The data show that administration of CARsomes reduces the number of Th2 cells, but does not reduce the cells below the baseline, indicating that CARsomes do not interfere with Th2 lineage differentiation. This feature is attributed to that CARsomes carry a peptide of Ag that enables CARsomes specifically to target Ag-specific Th2 cells and do not affect other Th2 cells. The significance of this feature is to ensure that CARsomes fulfill the immune regulatory functions without affecting the normal immune components. It is noted that the Treg generation is relatively low in the present study, which may be because we used male mice in the experiments. Taken together, employing CARsomes can precisely regulate the immune response by inducing Ag-specific Tregs targeting Ag-specific Th2 cells.

The data show that administration of CARsomes efficiently suppressed experimental airway allergic inflammation in a murine model. Asthma significantly affects human health and social

economy.² Current therapies for asthma are not satisfactory.² Although the allergen-specific immunotherapy is a specific remedy for allergic asthma, its therapeutic efficacy needs to be improved.² Employing anti-histamine or corticosteroids can get good inhibitory effects on asthma symptoms, but the effects only last for a short time. The present data show that employing CARsomes target to suppress Ag-specific Th2 cells in subjects with asthma. Since Ag-specific Th2 cells are the foundational pathogenic factor in allergic diseases,³² it is expected that CARsomes can suppress the symptoms of asthma. Indeed, the data show that administration of CARsome gain satisfactory therapeutic efficacy in experimental asthma.

In summary, the major product of the present study is that we constructed the CARsome; it can regulate skewed immune activities of allergic asthma by reducing Ag-specific Th2 cells and increasing Ag-specific Tregs in the airway. The CARsomes have the translational potential in the treatment of allergic diseases in the airway.

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CONFLICT OF INTEREST

None to declare.

AUTHOR CONTRIBUTIONS

HPZ, YXS, GY, JQL, LHM, XRG, YNS, ZL, and HTZ performed experiments, analyzed data, and reviewed the manuscript. PCY, ZGL, and DGW organized the study and supervised experiments. PCY designed the project and wrote the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Supplemental materials

Reagents

RNAi kit of DEC205, antibodies of DEC205, MHC II, perforin were purchased from Santa Cruz Biotech (Santa Cruz, CA). Derf1 protein was prepared in our laboratory (Am J Transl Res. 2017; 9 (5): 2143–2152). ELISA kits of IgE, mMCP1, IL-4, IL-5 and IL-13 were purchased from Dakewe Biomart (Beijing, China). Fluorescence-labeled antibodies for flow cytometry, including CD4 (PerCP), IL-4 (FITC), CD154 (Cy7), Foxp3 (PE), CD25 (Cy3), TGF- β (APC), CD127 (APC-Cy5.5) were purchased from BD Biosciences (Franklin Lakes, NJ). Annexin V kit, FITC-dextran (40 kDa), FITC-labeling kit, lipopolysaccharide (LPS), collagenase IV and protein G were purchased from Sigma Aldrich (St. Louis., MO). Reagent and materials for RT-qPCR, Western blotting and immunoprecipitation (IP) were purchased from Invitrogen (Carlsbad, CA). pHrodo was purchased from Thermo Fisher Scientific (Waltham, MA).

Animals

Male BALB/c mice (6-8-week-old) and male New Zealand rabbit (6-8-month-old) were purchased from the Beijing Experimental Animal Center (Beijing, China). The mice and rabbits were maintained in a specific pathogen free facility with accessing food and water freely. The experimental procedures were approved by the Animal Ethical Committee at Shanxi Medical University (SXMUAE20160010). Not using female animals in the study was to avoid a possible effect of menstruation on experiments.

Human subjects and collection of blood samples

Two groups of patients with asthma were recruited into this study at the Department of Respiriology, First Hospital of Shanxi Medical University. One group of patients sensitized to mites (at remission period; serum specific IgE, sIgE, ≥ 0.35 kU/L) but not to pollen (sIgE < 0.35 kU/L); another group of patients sensitized to pollen (out-season; sIgE ≥ 0.35 kU/L) but not to mites (sIgE < 0.35 kU/L). The diagnosis and management of asthma were carried out by our physicians following our routine procedures. The demographic data of human subjects are presented in Table S1 in supplemental materials. Patients had any of the following conditions were excluded from the study: Cancers, allergic diseases other than allergic asthma, autoimmune diseases, or under treatment with corticosteroids/immune suppressive agents for any reasons. None of the patients were undergoing and had never undergone specific immunotherapy. Age- and gender-matched healthy control (HC) subjects were also recruited into this study at our Hospital. The experimental procedures were approved by the Human Ethical Committee at Shanxi Medical University (#SXMUHE2016008) and is in line with the principles of the Declaration of Helsinki. A written informed consent was obtained from each human subject. Blood samples were collected from each subject through the ulnar vein puncture.

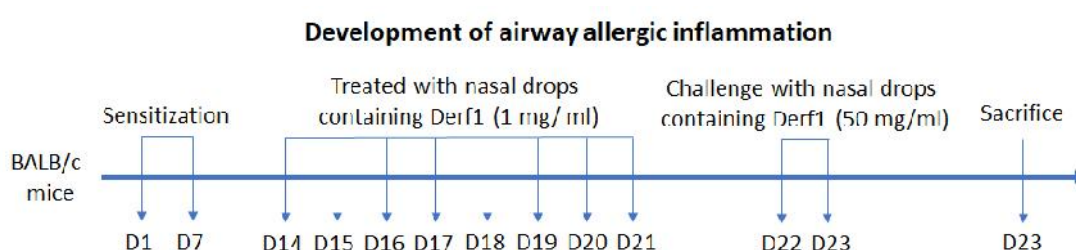
Assessment of human serum IgE

The sera were isolated from blood samples obtained from human subjects. Serum total- and specific IgE levels (mites and pollens) were determined by the ImmunoCAP technology with the reagent kits purchased from ThermoScientific (Phadia AB, Uppsala Sweden) using an immunoassay analyzer (ImmunoCAP1000) to perform the

immunoassay following the manufacturer's instruction and referred to published procedures [Luo W, Pan G, Huang H, Zheng P, Wei N, Zhang Y, Zeng G, Sun B. A Component-resolved Diagnostic Approach for a Study on Grass Pollen Allergens in Chinese Southerners with Allergic Rhinitis and/or Asthma. J Vis Exp. 2017 Jun 4; (124)].

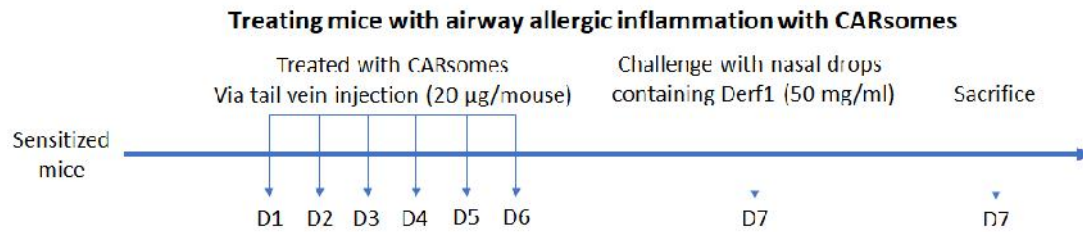
Development of airway allergic inflammation in mice

Male BALB/c mice (6-8-week-old) were immunized by cutaneous injection with Derf1 (100 µg/mouse in 0.1 ml alum) on day 0 and day 7, respectively. The mice were treated with nasal drops (50 µl per side) containing Derf1 (1 mg/ml) daily from day 14 to day 21. Mice were challenged with Derf1 (50 mg/ml) at 50 µl/mouse on day 22 and sacrificed on day 23. Airway resistance, serum specific IgE, mMCP1, lung histology, a collection of bronchoalveolar lavage fluid (BALF) of the mice were assessed following our established procedures (Sci Rep. 2017 Aug 17;7(1):8519). The procedures are illustrated below:



Treatment of sensitized mice with CARsomes

Sensitized mice were treated with the procedures denoted in the scheme below. The mice were treated with CARsomes (or saline, or non-perforin CARsome) at 20 µg/mouse through tail vein injection daily for 6 days. On day 7, mice were challenged with nasal drops containing Derf1 (50 mg/ml) and sacrificed 3 h later.



Airway epithelial barrier permeability assay

Mouse airway epithelial barrier permeability was assessed by treating with nasal drops (containing FITC-dextran at 10 mg/ml) at 50 µl per side. One day after the last challenge with antigen (Derf1), mice were treated FITC-dextran 2 h before sacrifice. The blood was collected at the time of sacrifice. The serum was isolated. Levels of FITC-dextran in the serum were determined in triplicate on a fluorescent plate reader, with serial dilutions of FITC-dextran used as a standard curve.

Cell culture

Cells were cultured in RPMI1640 medium supplemented with 5% fetal calf serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 2 mM glutamine. The medium was changed in 2-3 days. Cell viability was greater than 99% as assessed by Trypan blue exclusion assay.

Generation of bone marrow-derived dendritic cells (BMDC)

The bone marrows (BM) were isolated from naive BALB/c mice. BMDCs were generated from the bone marrows following our established procedures (Immunol Invest. 2008;37(8):782-97). Briefly, bone marrow cells were cultured in RPMI1640 medium supplemented with heat-inactivated 10% fetal bovine serum, 100 U/ml of penicillin, 0.1 mg/ml of streptomycin, granulocyte-macrophage colony stimulating

factor (40 ng/ml) and IL-4 (20 ng/ml). The medium with supplements were changed in every 3 days. Cells were harvested on day 9. The CD11c⁺ DCs were purified by flow cytometry cell sorting. The purity of BMDC was greater than 98% as assessed by flow cytometry.

Isolation of lung mononuclear cells (LMCs)

Lungs were excised from mice upon the sacrifice and cut into small pieces (2 × 2 × 2 mm). The tissues were incubated with collagenase IV (1 mg/ml) at 37 °C for 2 h with mild agitation. Single cells were collected by passing the lysates through a cell strainer (70 µm first, then 40 µm) and centrifuged at 2,000 g for 5 min. The cell pellets were resuspended in fresh medium. LMCs were isolated by Percoll gradient density centrifugation. The viability of isolated cells was greater than 98% as assessed by Trypan blue exclusion assay.

Generation and isolation of exosomes

BMDCs were pulsed with LPS at 10 µg/ml overnight (exposure to LPS is to activate DCs to produce more exosomes). Exosomes were isolated from the supernatants by differential centrifugations, 300 g for 10 min, 3000 g at 4 °C for 30 min, and 10,000 g for 40 min to remove cell debris and micro vesicles. The supernatant was collected and centrifuged for 90 min at 100,000 g to precipitate the exosomes. The pellets were washed in PBS, resuspended in PBS and stored at –80 °C until use

Preparation of recombinant fusion protein of Derf1, perforin and scFv, and the individual peptides

Plasmids of the fusion protein of Derf1, perforin and scFv, or individual peptides of Derf1, perforin and scFv, were constructed by Sangon Biotech (Shanghai, China). The plasmids were transformed into *E. coli* BL21 and selected by the presence of kanamycin. The expression of the fusion protein or individual peptide was induced by the addition of isopropyl- β -D-thiogalactopyranoside (IPTG, 1 μ M). The proteins were purified by Ni-NTA-agarose column. The total protein was determined by the Bradford assay.

Generation of polyclonal antibodies of Derf1 and scFv

Rabbits were subcutaneously injected with Derf1 or scFv at 1 mg/rabbit in 1 ml PBS together with the same amounts of complete Freund adjuvants on day 0 and day 7, respectively. The injection was rebooted on day 43, 85, 127, 169, 211, respectively. The specific antibodies were purified from the serum by affinity chromatography.

Bioactivity assessment of the fusion protein

The fusion protein (consisting of Derf1, perforin and scFv) bioactivities were assessed by Western blotting. The membranes were stained with antibodies of Derf1, or perforin, or scFv. On the other hand, microplates were coated with the fusion protein, followed by incubation with antibodies of Derf1, or perforin, or scFv. After incubating with peroxidase-labeled secondary antibodies, the plates were developed with Tetramethylbenzidine (TMB) and read with a microplate reader.

Electron microscopy

Exosomes were fixed with 0.75% glutaraldehyde and 2% paraformaldehyde for 2 h and incubated with the first antibody for 30 min at 4 °C and washed with PBS for 3 times. One drop of the exosome suspension was placed on a Formvar/carbon-coated grid and incubated with a 6 nm gold particle-conjugated anti-IgG (1/30 dilution; Jackson ImmunoResearch Laboratories) for 1 h. The grids were washed 6 times and counterstained with 2% aqueous uranyl acetate, followed by 0.2% lead citrate. Exosomes were observed using a JEOL JEM-1200 EX transmission electron microscope.

Flow cytometry

On the surface staining, cells were stained with antibodies of interest (labeled with fluorochromes) for 30 min at 4 °C. In the intracellular staining, cells were treated with fixative/permeating solution (BD Bioscience) first, then stained with fluorochrome-labeled antibodies of interest for 30 min at 4 °C. Isotype IgG was used as negative control staining. After washing with PBS, the cells were analyzed with a flow cytometer (FACSCanto II; BD Bioscience). Dead cells were excluded by gating before acquiring data. The data were analyzed by a software package FlowJo (Tree Star, Inc. Ashland, OR). Data obtained from isotype IgG staining were used as gating references.

Assessment of T cell proliferation

DCs, Tregs and CD4⁺ CD25⁻ T cells were isolated from lung mononuclear cells or spleen cells by flow cytometry cell sorting. Cells were labeled with fluorescence-labeled antibodies and sorted by flow cytometry. Purity of isolated cells was greater

than 95% as assessed by flow cytometry. CD4⁺ CD25⁻ T cells were labeled with carboxy fluorescein succinimidyl ester (CFSE) and cultured in CD3 antibody-coated 96-well plates in the presence of CD28 antibody (5 µg/ml) for 3 days. The cells were analyzed with a flow cytometer.

Protein extracts

Cells were collected from relevant experiments and incubated with a lysis buffer for 30 min. The lysates were centrifuged at 10,000 g for 10 min. The supernatant was collected and used as cytosolic extracts. The pellets were resuspended in a nuclear lysis buffer; 30 min later, the lysates were centrifuged at 10,000 g for 10 min. The supernatant was collected and used as nuclear extracts. All the procedures were performed at 4 °C.

Western blotting

Proteins were fractioned by SDS-PAGE and transferred onto a PVDF membrane. The membrane was blocked with 5% skim milk for 30 min, incubated with the primary antibodies (diluted at 1:300-500) of interest overnight at 4 °C, washed with TBST (Tris-buffered saline containing 0.1% Tween 20) for 3 times, followed by incubating with the second antibodies (labeled with peroxidase) for 2 h at room temperature and washed with TBST for 3 times. Immunoblots on the membrane were developed by the enhanced chemiluminescence and photographed with an imaging device. Thermo 26616 marker was used as the molecular marker.

Depletion of DEC205 in BMDCs

BMDCs were treated with RNAi reagent kit of DEC205 following the manufacturer's instructions. The effects of RNAi were assessed by Western blotting 48 h later.

Immunoprecipitation (IP)

Proteins were extracted from CARsomes and precleared by incubating with protein G agarose beads for 2 h. The beads were removed by centrifugation. Supernatant was incubated with antibodies of interest or isotype IgG overnight to form immune complexes, which were precipitated by incubating with protein G agarose beads for 2 h. The beads were collected by centrifugation. Proteins on the beads were eluted using an eluting buffer and analyzed by Western blotting. All the procedures were performed at 4 °C.

Assessment of cell apoptosis

Cells were collected from relevant experiments and stained with propidium iodide (PI) and annexin v. The cells were analyzed with a flow cytometer. Cells stained annexin v⁺ or PI⁺ annexin v⁺ were regarded as apoptotic.

Immune cell isolation

Besides Ag-specific Th2 cells and Ag-specific Tregs, other immune cells were isolated from LMCs by flow cytometry cell sorting (FCCS). CD4⁺ T cells were labeled with antibodies of CD3 and CD4, and then sorted by FCCS. Purity of isolated cells was greater than 95% as assessed by flow cytometry.

Enzyme-linked immunosorbent assay (ELISA)

Levels of cytokines in the serum and BALF were determined by ELISA with commercial reagent kits following the manufacturer's instructions.

Assessment of apoptotic Ag-specific Th2 cells phagocytosed in the lung

Ag-specific Th2 cells were isolated from LMCs of Derf1-sensitized mice by flow cytometry cell sorting (purity was greater than 95%) and exposed to CARsomes (10 µg/ml) in the culture for 48 h. The cells were collected, washed with fresh medium and labeled with pHrodo following the manufacturer's instructions. Asthma mice were injected with pHrodo-labeled Ag-specific Th2 cells (10^6 cells/mouse) through the tail vein. Mice were sacrificed 24 h later. LMCs were prepared and analyzed by flow cytometry. The frequency of pHrodo-containing phagocytes (including DCs and macrophages) was calculated.

Induction of T cell apoptosis

CD4⁺ T cells were isolated from blood samples collected from human subjects. The cells were incubated with cisplatin (60 µM) in the culture for 24 h, and then analyzed by flow cytometry with an Annexin V reagent kit following manufacturer's instruction. Cisplatin is one of the anti-cancer agents; it is also used as a tool agent to induce apoptosis in experimental research (Jiao X, Cai J, Yu X, Ding X. Paracrine Activation of the Wnt/ β -Catenin Pathway by Bone Marrow Stem Cell Attenuates Cisplatin-Induced Kidney Injury. *Cell Physiol Biochem*. 2017;44(5):1980-1994).

Assessment of binding capacity of CARsomes to exosomes

CARsomes (100 µg/ml; labeled with FITC with a FITC-labeling kit following the manufacturer's instructions) were mixed with exosomes at gradient concentrations (0, 10, 20, 40, 60 and 80 µg/ml) for 30 min at room temperature. The samples were passed through a filter (0.8 µm; ThermoFisher); the fluorescence intensity of the remnant CARsomes was measured with a fluorescence spectrometer (BioSpectrometer fluorescence).

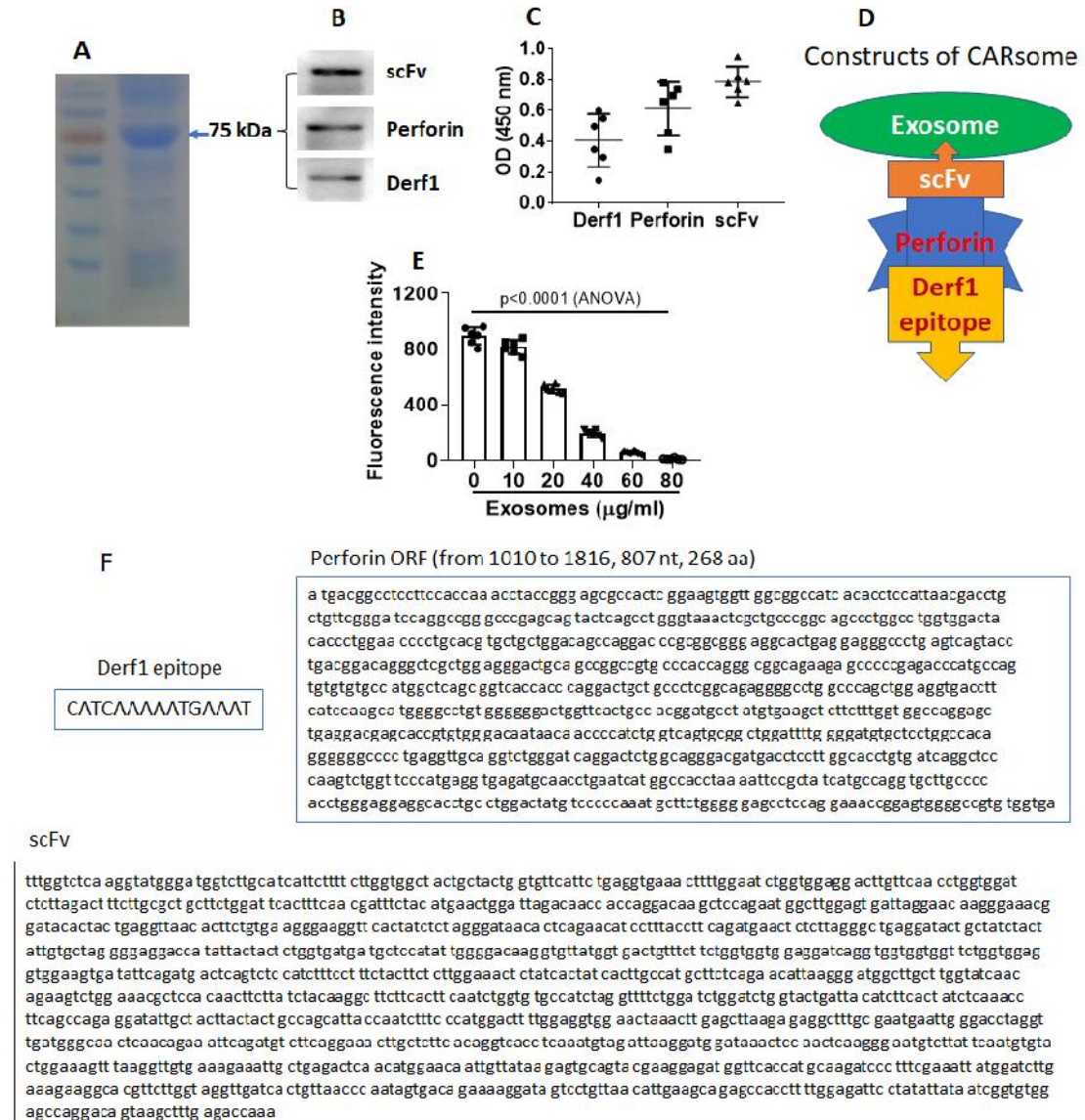


Figure S1. Construction of CARsomes. A fusion protein of scFv, perforin and Derf1 was constructed. A, isolation of the fusion protein. B, immunoblots show the fusion protein can be recognized by antibodies of Derf1, perforin and scFv, respectively, indicating that the fusion protein consists of scFv, perforin and Derf1. C, ELISA results show immune activities of scFv, perforin and Derf1 in the fusion protein. D, a schematic of CARsome structure. E, bars show the fluorescence intensity [CARsomes (100 μg/ml) were labeled with FITC] in remnant CARsomes after exposed to

exosomes at indicated amounts on the X axis. F, gene sequences of Derf1 epitope, perforin ORF and scFv.

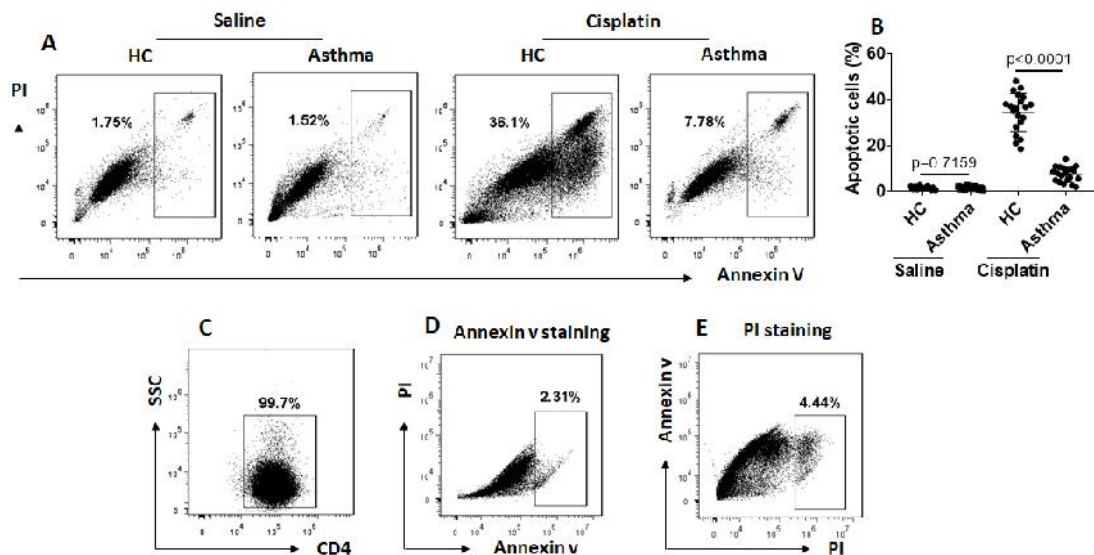


Figure S2. CD4⁺ T cells from asthma patients show defects of apoptosis. Blood samples were collected from 20 asthma patients and 20 healthy control (HC) subjects. CD4⁺ T cells were isolated from the blood samples and incubated with cisplatin (60 μ M) in the culture for 24 h. The cells were analyzed by flow cytometry. A, gated dot plots indicate apoptotic CD4⁺ T cells. B, scatter dot plots show summarized data of apoptotic cells. Each dot presents data obtained from one sample. Data of bars are presented as mean $\pm$ SEM. Statistics: ANOVA + Bonferroni test. C, dot plots show purity of isolated CD4⁺ T cells. D, single staining of Annexin V. E, single staining of PI.

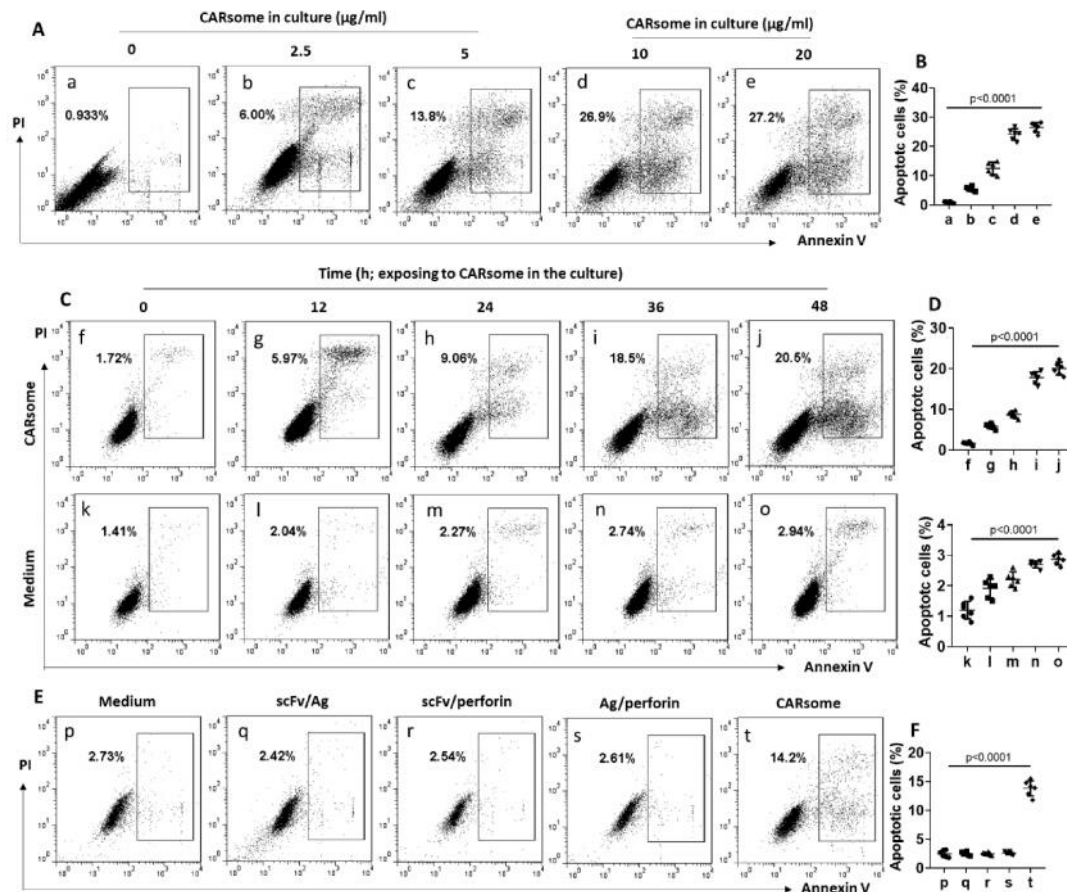


Figure S3. Assessment of the effects of CARsomes on inducing Th2 cell apoptosis.

Antigen-specific Th2 cells were isolated from blood samples obtained from asthma patients sensitized to mites. A-B, the cells were exposed to CARsome at indicated concentrations in the culture for 48 h and analyzed by flow cytometry. Gated dot plots indicate apoptotic cells. Scatter dot plots show summarized data of 6 independent experiments. C-D, CARsomes with incomplete components as denoted above each subpanel of panel C were used in the experiments. C, gated dot plots show apoptotic cells. D, scatter plots show summarized apoptotic cells. Each dot present data obtained from one subject. Data of bars are presented as mean $\pm$ SEM. Statistics: ANOVA.

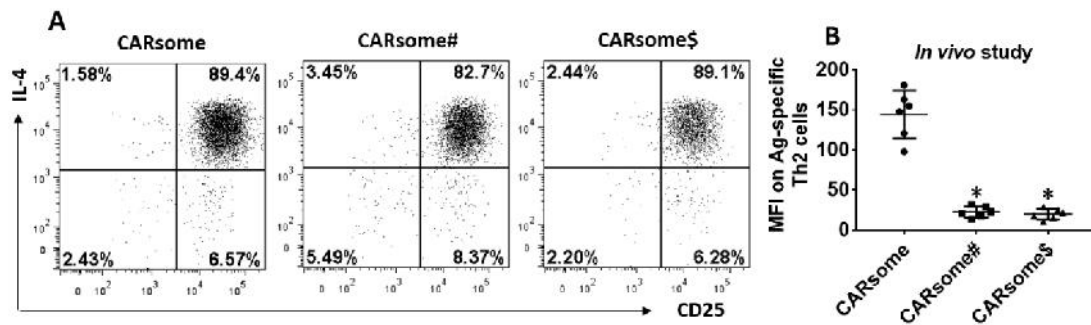


Figure S4. CARsomes increase amounts of PS on the surface of Ag-specific Th2 cells in the lung. Asthma mice (6 per group) were treated with CARsomes or CARsome# (without perforin) or CARsome\$ (consists of scFv, perforin and OVA epitope) at 10 μ g/mouse in 0.1 ml saline through tail vein injection daily for 6 days. After sacrifice, lungs were excised. LMCs were prepared with lung tissues, stained with annexin v (to show PS) and analyzed by flow cytometry. MFI of PS in Th2 cells was calculated by FlowJo. A, dot plots in right upper quadrants show Th2 cells. B, scatter plots show MFI of PS on the surface of Th2 cells. Data of bars are presented as mean $\pm$ SEM. Each dot present data obtained from one mouse (assessed by duplicate). * $p < 0.01$, compared with the CARsome group (ANOVA + Dunnett's test).

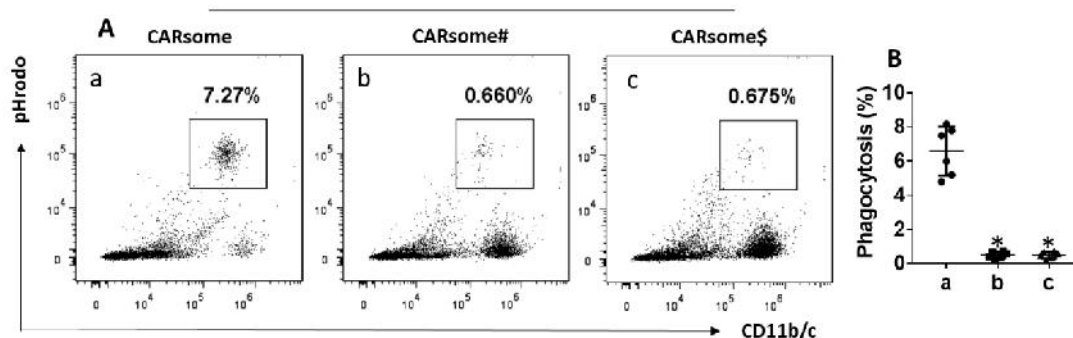


Figure S5. CARsomes facilitate to phagocytosis of Ag-specific Th2 cells in the lung. Ag-specific Th2 cells were treated with CARsomes (10 μ g/ml) in the culture for 48 h and stained with pHrodo, and then transplanted into naïve mice at 10^6 cells/mouse.

The mice were sacrificed next day. LMCs were prepared and analyzed by flow cytometry. A, gated dot plots show the frequency of phagocytes containing apoptotic Ag-specific Th2 cells (labeled with pHrodo). B, scatter dot plots show summarized data of gated phagocytes in panel A. Data of bars are presented as mean $\pm$ SEM. * $p < 0.01$, compared with group a (ANOVA + Dunnett's test). Each dot present data obtained from one experiment (assessed in duplicate). CARsome#: The CARsomes do not have perforin. CARsome\$: The CARsomes contain scFv, perforin and an OVA epitope (do not have a Derf1 epitope).

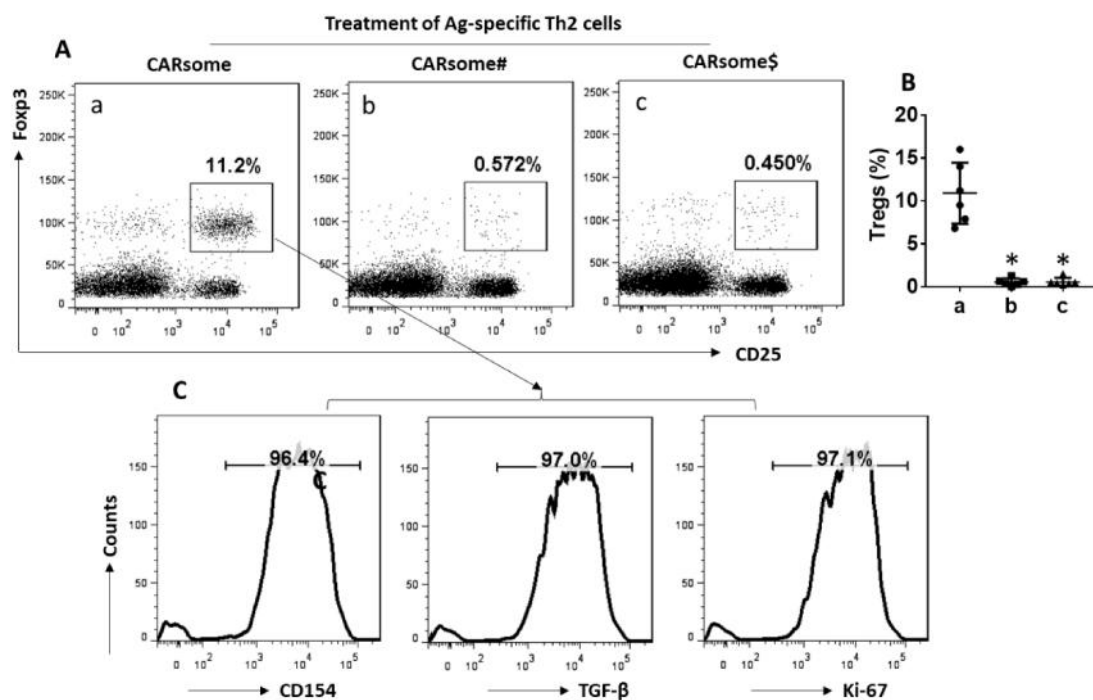


Figure S6. CARsomes facilitates generation of iTregs. LMCs were isolated from sensitized mice (treated with CARsomes daily for 6 days) and cultured in the presence of the specific antigen Derf1 (10 $\mu\text{g/ml}$) overnight. The cells were analyzed by flow cytometry. A, gated dot plots show the frequency of iTregs. B, scatter dot plots show summarized data of the iTregs in panel A. C, histograms show the iTregs

also express CD154, TGF- β and Ki-67, respectively. Each group consists of 6 mice. Each dot in panel B present data obtained from one sample (assessed in duplicate). Data of bars are presented as mean $\pm$ SEM. * $p < 0.01$, compared with group a (ANOVA + Dunnett's test). LMC: Lung mononuclear cells.

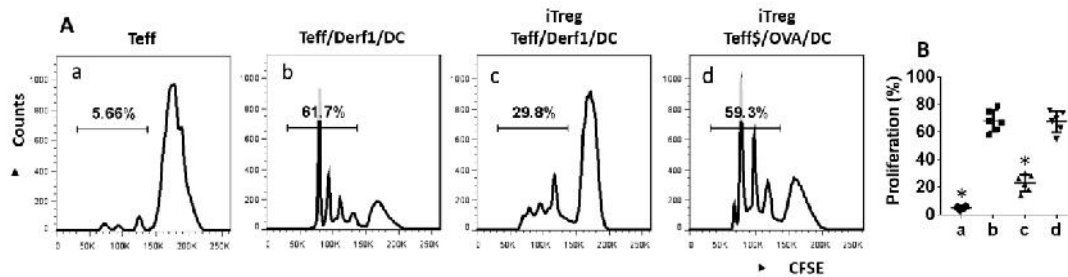


Figure S7. CARsome-induced iTregs show Ag-specific immune suppressive functions.

LMCs were isolated from the lungs of sensitized mice treated with or without CARsomes and cultured in the presence of specific antigen (Derf1, 10 μ g/ml) overnight to activate Ag-specific iTregs and Teffs. CD4⁺ CD25⁺ CD154⁺ CD127⁻ iTregs were sorted from LMCs isolated from CARsome-treated sensitized mice. CD4⁺ CD25⁻ CD127⁺ Teffs were sorted from LMCs isolated from sensitized mice without treating with CARsomes. DCs were sorted out from the spleen of naïve mice. iTregs, Teffs (labeled with CFSE) and DCs were cultured at a ratio of 10⁵:5 $\times$ 10⁵:10⁵ cells/ml as denoted above each subpanel of panel A. A, gated histograms show frequency of proliferating Teffs. B, scatter dot plots show summarized proliferating Teffs. Teff\$: Teffs were isolated from LMCs of sensitized mice sensitized to OVA (used as controls). Data of bars of panel B are presented as mean $\pm$ SEM. Each dot present data obtained from one experiment. * $p < 0.01$, compared with group b (ANOVA + Dunnett's test).

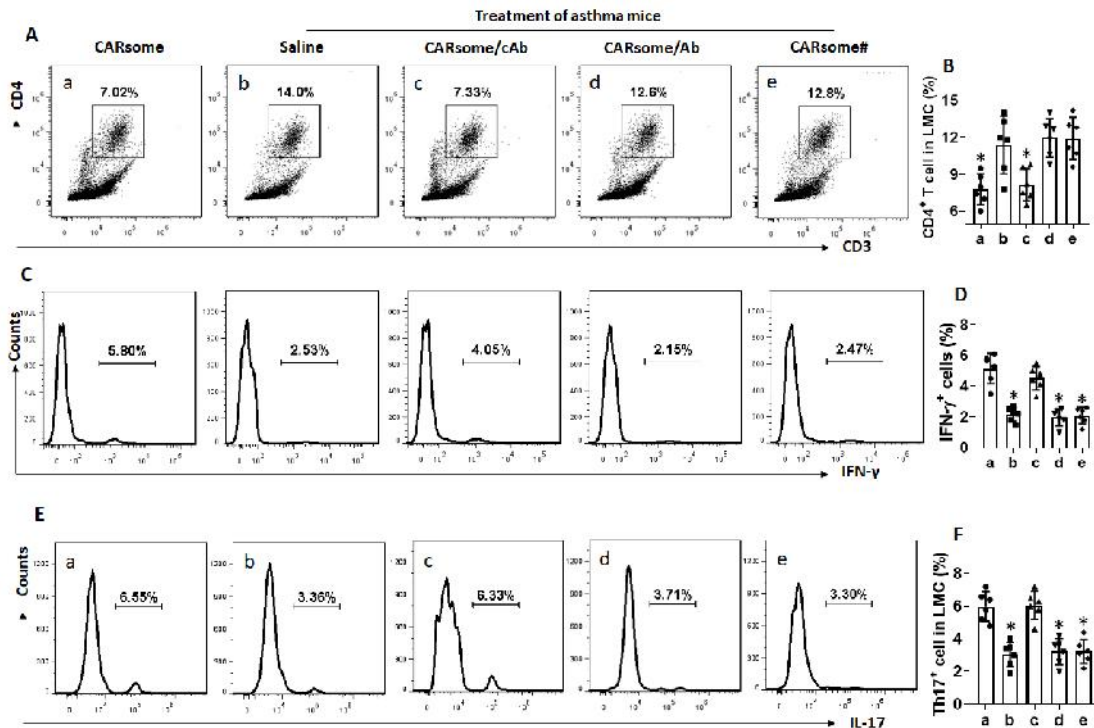


Figure S8. Assessment of CD4⁺ T cells, IFN-γ⁺ T cells and Th17 cells in the lung of mice with airway allergic inflammation. Mice with airway allergic inflammation were prepared. Lung mononuclear cells (LMCs) were prepared and analyzed by flow cytometry. A, gated dot plots show frequency of CD4⁺ T cells. B, summarized data of panel A. C, gated dot plots show frequency of IFN-γ⁺ T cells. D, summarized data of panel C. E, gated dot plots show frequency of Th17 cells. F, summarized data of panel E. Each group consists of 6 mice. *p<0.01, compared to group a (ANOVA + Dunnett's test).

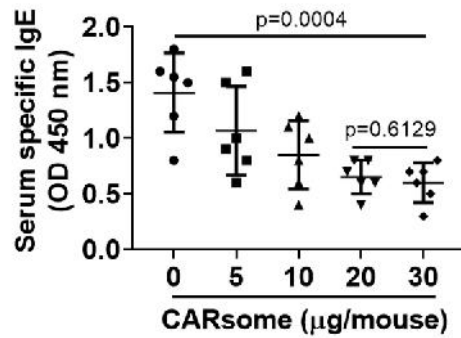


Figure S9. Role of CARsome in suppressing serum specific IgE in mice with airway allergic inflammation. A murine model of airway allergic inflammation was developed with Derf1 as the specific antigen. One day after the last challenge with Derf1, the mice were treated with CARsomes at indicated dosage daily for 6 days through the tail vein injection. Mice were challenged with Derf1 nasal drops one day after the last injection. The blood was collected from each mouse. The serum was prepared and analyzed by ELISA. The dot plots show serum specific IgE levels. Each group consists of 6 mice. Data of bars are presented as mean $\pm$ SEM. Each dot presents data obtained from one mouse. Statistics: ANOVA and Bonferroni test.

Table S1. Demographic data of human subjects

	Asthma (mite)	Asthma (pollen)	Healthy control
Total number	20	20	20
Gender (M/F)	10/10	10/10	10/10
Age (years)	32.5 ± 6.8	33.1 ± 5.6	31.8 ± 4.3
BMI (kg/m²)	23.3 (21.5-24.9)	23.7 (22.2-24.7)	
Total IgE (kU/L)	355 (213-505)	266 (186-443)	0.25 (0-0.31)
Specific IgE (kU/L)	25.2 (16.3-35.6)	18.6 (8.8-28.5)	0.21 (0-0.29)
FEV1 (% predicted)	102.5 (98.1–122.5)	103.5 (101.1–111.6)	
Eosinophils (cells/μL)	326 (228-451)	351 (212-465)	
Neutrophils (cells/μL)	3,166 (2,381–3,814)	3,144 (2,266–3,820)	
Monocytes (cells/μL)	363 (331–439)	359 (317–441)	
Lymphocytes (cells/μL)	1,904 (1,707–2,341)	1,921 (1,713–2,276)	
Mite skin test (+)	20	0	0
Pollen skin test (+)	0	20	0
Under medication	0	0	0

Data are presented as means with 95% CI. Geometric means are presented for total IgE and specific IgE; BMI, body mass index; IgE, immunoglobulin E; Mite, *Dermatophagoides farina*; FEV1, forced expiratory volume in 1 second.