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Chimeric antigen-guiding extracellular vesicles eliminate antigen-specific Th2 cells in subjects with food allergy

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ABSTRACT

Background: Antigen (Ag)-specific T helper (Th)2 cells play a central role in food allergy (FA) pathogenesis. Methods can be used to eliminate Ag-specific Th2 cells that are currently lacking. This study aims to eliminate the Ag-specific Th2 cells with a novel nanoparticle, the mEV (modified extracellular vesicles, that carry a chimeric antigen peptide, MHC II and caspase 3) in a murine FA model.

Methods: mEVs were generated by exposing dendritic cells (DC) to ovalbumin (OVA, a specific Ag) and recombinant caspase 3 (Casp3) in the culture overnight. Exosomes were purified from culture supernatant by the magnetic antibody approach. A murine FA model was developed with OVA as the specific Ag.

Results: Purified mEVs had the molecular markers of extracellular vesicle, CD81, CD63, and CD9, cleaved Casp3 and MHC II/OVA complexes. mEVs specifically bound to the surface of Ag-specific $CD4^+$ T cells, induced Ag-specific $CD4^+$ T cell apoptosis both *in vitro* and *in vivo* as well as increased regulatory T cells in the intestinal tissues. Administration of mEV efficiently suppressed experimental FA.

Conclusions: mEVs carry Ag/MHC II complexes and Casp3, that can induce Ag-specific Th2 cell apoptosis. Administration of mEV can efficiently suppress experimental FA. The results suggest that the mEVs have the translational potential to be used in the treatment of FA and other allergic diseases.

Keywords: Nanoparticle, Extracellular vesicle, Allergy, Immunotherapy, Apoptosis

INTRODUCTION

Food allergy (FA) is one of the allergic diseases that is an aberrant response to the innocuous food antigens (Ag) by the immune system in the intestinal tissues.[1] It is known that Food Ags can pass through the impaired intestinal epithelial barrier to get into the deep regions of the intestinal

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tissues, where the Ags can be captured by dendritic cells (DC). After processing, DCs present the Ag information (usually are small peptides) to induce the Ag-specific T helper 2 (Th2) cells and further induce Ag-specific IgE production by plasma cells. IgE binds to the high affinity IgE receptors on the surface of mast cells that makes mast cell sensitized. Re-exposure to specific Ags triggers FA attacks by activating the sensitized mast cells.[2] Therefore, Ag-specific Th2 cells play a central role in the FA pathogenesis. However, methods that can be employed to eliminate the Ag-specific Th2 cells from subjects with FA or other allergic diseases are lacking currently.[3]

Although the therapeutics for FA have advanced greatly in recent years, the therapeutic effects on FA are not satisfactory yet. The specific allergen immunotherapy is the specific remedy for allergy treatment, but it takes a lone time – 3 years in general; the therapeutic effects are to be improved.[3] Nanoparticles are a new device employed for the treatment of allergic disorders. Recent studies show that R848/specific allergen-carrying nanoparticles can induce regulatory T cell development and inhibit experimental allergy.[4] Extracellular vesicles (EV) are another carrier employed in allergy treatment. Using specific antigen carrying EVs can inhibit experimental FA[5] and induces antigen-specific immune tolerance.[6,7] However, whether these therapeutic designs can eliminate Ag specific Th2 cells remains to be further investigated.

The chimeric Ag receptor (CAR) carrying T cell (CAR-T cell) therapy has been employed in the treatment of cancers for years.[8-10] The effects of CAR-T cell-therapy on cancers, such as the $CD19^+$ B cell leukemia, have been recognized.[11] The rationale of CAR-T cell therapy is that CAR-T cells can target specific molecules, such as CD19 in B cell leukemia, on cancer cells.[12] The interaction between target cells and the CAR in CAR-T cells can activate the CAR-bearing cells, eg, $CD8^+$ cytotoxic T cells. The $CD8^+$ T cells release cytotoxic mediators, eg, perforin and granzyme B, to kill the targeted cancer cells.[13] Following the concept of CAR-T cell therapy, we designed a modified EV (mEV), that carried an MHC II/Ag complex and casapas-3 (casp3). The mEV has similar function to CAR-T cells; CAR-T cells can directly kill targeted cells (e.g. tumor cells),[8-10] while mEV can activate the cell death pathways to indirectly induce target cell death.

MATERIALS AND METHODS

Reagents

TCRα and Fas shRNA reagent kits (sc-37273-SH), antibodies of CD3 (Alexa Fluor® 488; PC3/188A), CD4 (Alexa Fluor® 546; MT310), IL-4 (Alexa Fluor® 594; OX81), CD154 (Alexa Fluor® 647; F-1), CD9 (C-4), CD63 (MX-49.129.5), CD81 (B-11), MHC II (Y-Ae), ovalbumin (2D-11), Fas (G-9), and TCR (R73) were purchased from Santa Cruz Biotech (Santa Cruz, CA). Biotinylated anti-CD9 Ab and anti-biotin magnetic micro beads were purchased from Miltenyi Biotech (San Diego, CA). Casp3 protein, ELISA kits of Casp3, IgE, mMCP1, IL-4, IL-5 and IL-13 were purchased from R&D Systems (Minneapolis, MN). Ovalbumin, FITC-annexin V kit and FITC-dextran were purchased from Sigma Aldrich (St. Louis., MO). Immunoprecipitation kit and materials for Western blotting were purchased from Invitrogen (Carlsbad, CA). Magnetic beads coated with anti-CD9 antibody was purchased from AMS Biotechnology (Abingdon, UK).

Mice

BALB/c mice were purchased from Guangdong Experimental Animal Center (Guangzhou, China). DO11.10 mice were purchased from Jackson Laboratory (Bar Harbor, Maine). Mice were maintained in a specific pathogen-free facility at with accessing water and food freely. The animal experimental procedures were approved by the Animal Ethics Committee at Shenzhen University.

Protein extraction

Cells were collected from relevant experiments and incubated with a lysis buffer (10 mM HEPES; 1.5 mM $MgCl_2$; 10 mM KCl; 0.5 mM DTT; 1 mM EDTA; 0.05% NP40) for 30 min and centrifuged at 13,000 *g* for 10 min. The supernatant was collected and used as cytosolic protein extracts. The remained pellets were resuspended and incubated with a nuclear lysis buffer (5 mM HEPES; 1.5 mM $MgCl_2SO_4$; 4.6 M NaCl; 0.2 mM EDTA; 0.5 mM DTT; 26% glycerol) for 30 min and centrifuged at 13,000 *g* for 10 min. The supernatant was collected and used as nuclear protein extracts. All the procedures were carried out at 4 °C.

Western blotting

Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 50 μg/well and transferred onto a PVDF membrane. After blocking by incubating with 10% skim milk for 30 min, the membrane was incubated with the primary antibodies (diluted in 1:500) of interest overnight, washed with TBST (Tris-buffered saline containing 0.05% Tween-20) 3 times, incubating with the secondary antibodies (labeled with peroxidase; diluted to 1:5000) for 2 h at room temperature, washed with TBST 3 times. Immunoblots on the membrane were developed with the enhanced chemiluminescence and photographed with an imaging device (iBright 1500; Invitrogen).

Immunoprecipitation

Proteins were prepared as described above. Pre-existing immune complexes in the samples were precleared by incubating with protein G sepharose beads for 2 h. The beads were eliminated from samples by centrifugation at 5000 *g* for 10 min. Supernatant was collected and incubated with antibodies of interest (diluted in 1:500) or isotype IgG overnight to form immune complexes. Immune complexes in samples were precipitated by incubating with protein G sepharose beads for 2 h. The beads were collected by centrifugation for 10 min at 5000 *g*. Proteins on beads were eluted using an eluting buffer (10 mM Tris-Cl, pH 8.5) and analyzed by Western blotting.

Immune cell isolation

Immune cells were isolated from the mouse spleen or the intestinal tissues to be used in relevant experiments. Intestinal segments were excised from mice upon the sacrifice. The tissues were cut into small pieces and incubated with collagenase IV (1 mg/ml) for 30 min at 37 °C. Single cells were filtered through a cell strainer (100 μm first, then 70 μm). Lamina propria mononuclear cells (LPMCs) were further isolated from single cells by Percoll gradient density centrifugation. To purify certain immune cells, the cells were labeled with fluorescence conjugated antibodies of interest (diluted in 1:100) and isolated by flow cytometry cell sorting (FCCS) with an Aria flow cytometer (V.6·1; Becton Dickinson, San José, CA).

Cell culture

Cells were cultured with RPMI1640 medium. The medium was supplemented with 10% fetal bovine serum (FBS. To deplete exosomes from FBS, FBS was treated with the centrifugation procedures of mEV preparation before using for BMDC culture), 100 U/ml penicillin, 0.1 mg/ml streptomycin and 2 mM glutamine. Cell viability was greater than 99% as assessed by Trypan blue exclusion assay.

Flow cytometry

Single cells were collected from relevant experiments. On the surface staining, cells (10^6 cells/sample) were stained with fluorescence-labeled antibodies (diluted in 1:100) of interest (detailed in figure legends) or isotype IgG for 30 min at 4 °C. After washing with phosphate buffered saline (PBS) 3 times, the cells were analyzed with a flow cytometer (FACSCanto II, BD Bioscience). In the intracellular staining, cells were fixed with 1% paraformaldehyde (containing 0.05% Triton X-100 to increase the cell membrane permeability) for 1 h. After washing with PBS, the cells were processed with the procedures of surface staining. The data were analyzed with a software package (FCS Express; De Novo Software, Pasadena, CA) with the data of isotype IgG staining as gating references. Gating strategy: $CD3^+$ $CD4^+$ T cells were gated first; from the gating cell population, IL-4^+ T cells (Th2) cells, or $Foxp3^+$ TGF-β^+ T cells (Treg) were determined.

Bone marrow-derived dendritic cell (BMDCs) preparation

The femur bones were excised from mice. The bone marrows were flushed out with saline. Red blood cells in the bone marrow were lysed with a red blood cell lysis buffer (Sigma Aldrich) following the manufacturer's instruction. The remained bone marrow cells were cultured in complete RPMI1640 medium in the presence of IL-4 (20 ng/ml) and GM-CSF (25 ng/ml). On day 9, cells were harvested, from which $CD11c^+$ DCs were purified by FCCS.

Preparation of mEV

BMDCs were cultured in the presence of OVA (50 μg/ml) and caspase 3 (casp3) (50 μg/ml)

overnight. Supernatant was collected and subjected to exosome purification following established procedures.[14] Briefly, the supernatants were collected and centrifuged at 300 *g* (10 min), 1200 *g* (20 min), and 10,000 *g* (30 min), respectively, to remove cell debris. mEVs were pelleted from the supernatant at 100,000 *g* for 1 h and resuspended in PBS, and were purified by magnetic isolation. Biotinylated anti-CD9 Ab was added to the samples at 100 ng/ml, incubated at room temperature overnight; followed by adding magnetic anti-biotin magnetic beads for 2 h at room temperature. mEVs on the beads were eluted with an eluting buffer (10 mM Tris-Cl) and resuspended in PBS for further experiments (pH was adjusted to 7 by acetic acid). The protein in mEVs was quantified using a Bradford assay. The content of casp3 in exosomes was determined by ELISA. The OVA/MHC II complexes were assessed by immunoprecipitation. In addition, about 30% BMDCs were apoptosis after exposure to Casp3 as assessing by flow cytometry, that did not affect the mEV preparation.

Electron microscopy

mEVs were prepared as described above and fixed with a fixative (containing 0.75% glutaraldehyde and 2% paraformaldehyde) for 2 h, and followed by washing with PBS 3 times. mEV suspension was placed on a Formvar/carbon-coated grid and dried at room temperature overnight. The grids were counterstained with 2% aqueous uranyl acetate, followed by 0.2% lead citrate. mEVs were examined using a JEOL JEM-1200 EX transmission electron microscope.

Assessment of mEV binding Ag-specific CD4$^+$ T cells

Before the last centrifugation of exosome purification, a portion of mEVs was stained with Carboxyflourescein diacetatesuccinimidyl ester (CFSE; 1 μg/ml) for 10 min. Unbound CFSE was removed by centrifugation with the Corning® Costar® Spin-X® centrifuge tube filters (0.45 μm) at 13,000×*g* for 5 min. DO11.10 CD4$^+$ T cells and BALB/c CD4$^+$ T cells were prepared and cultured. mEVs were added to the culture at 20 μg protein/ml. The cells were harvested 30 min later and analyzed by flow cytometry.

Assessment of apoptotic cells induced by mEVs

CD4$^+$ T cells were exposed to mEV (20 μg/ml) in the culture for 24 h and then stained with propidium iodide (PI) and annexin v-FITC reagent kit following manufacturer's instructions. The cells were analyzed by flow cytometry (FACSCanto II, BD Bioscience). In the *in vivo* study, mice were intraperitoneally injected with mEVs (0.1 mg/mouse) every other day for 1–3 times. Mice were sacrificed 1 day after the last injection. LPMCs were prepared, stained with PI/annexin v reagents and analyzed by flow cytometry. CD4$^+$ T cells were gated first, in which apoptotic cells were then counted.

RNA sequencing

CD4$^+$ T cells were harvested from the experiments. Total RNA was extracted by the TRIzol reagents. The RNA sequencing with the RNA samples was carried out by the EGene Biotech.

Food allergy mouse model development and treatment with mEVs

Following our established procedures,[4] BALB/mice were immunized by subcutaneously injected with OVA (0.1 mg in 0.1 ml alum) at the back skin on day 0 and day 3, respectively. Mice were challenged by gavage-feeding with OVA (1 mg/mouse in 0.3 ml saline) on day 7, 9, 11, 13, respectively. From day 20 (one week after the last Ag challenge), FA mice were intraperitoneally injected with mEVs or mEV lysates at 0.1 mg/mouse every other day for 3 times. A group of FA mice was treated with saline used as controls. Two days after the last injection, mice were challenged by gavage-feeding with a large dose of OVA (0.3 ml per mouse; stock: 50 mg/ml). The FA response parameters were assessed following established procedures.[4]

Assessment of Ag-specific Th2 cells in the mouse intestine

FA mice (had been sensitized to OVA) were challenged with a large dose of OVA (the specific Ag; 0.3 ml per mouse; stock: 50 mg/ml). LPMCs were prepared and analyzed by flow cytometry. CD3$^+$ CD4$^+$ T cells were gated first and CD154$^+$ (activating marker[15]) IL-4$^+$ T cells were counted in

the gated CD4$^+$ T cells and regarded as Ag (OVA)-specific Th2 cells.

RNA interference (RNAi) of TCR

CD4$^+$ T cells were isolated from the spleen of DO11.10 mice and BALB/c mice by FCCS. The cells were treated with TCR or Fas RNAi reagents or control RNAi reagents following the manufacturer's instruction to deplete the TCR or Fas. Cells were harvested 48 h later and analyzed by Western blotting and flow cytometry to assess the RNAi effects.

Statistics

Each experiment was repeated at least 3 times. Each sample was analyzed in triplicate. The data are presented as mean ± SEM. SPSS was used in statistical analysis. The difference between two groups was determined by the Student *t*-test. ANOVA followed by the Bonferroni post hoc test or the Dunnett's post hoc test was performed for multiple comparisons. $P < 0.05$ was set as a significant criterion.

RESULTS

Characteristics of mEV

To construct the mEVs, bone marrow-derived dendritic cells (BMDCs) were prepared and exposed to ovalbumin (OVA, used as a specific Ag in the development of an FA mouse model) and casp3 in the culture overnight. Supernatant was collected and processed for mEV purification by centrifugation and magnetic isolation following published procedures.[16] The purified mEVs were verified by Western blotting as CD9, CD63 and CD81, the mEV markers, were detected (Fig. 1A). Cleaved Casp3 was also detected in the protein extracts (Fig. 1A), indicating that caspase 3 was cleaved in BMDCs. Co-immunoprecipitation (co-IP) assay showed that MHC II/OVA complexes were detected in mEV protein extracts (Fig. 1B and C). Casp3 was also detected in the protein extracts with ELISA (Fig. 1D). Electron photomicrographs show the isolated mEVs about 100 nm in size surrounding by a double layer membrane (Fig. 1E).

mEVs specifically bind to Ag-specific CD4$^+$ T cells

The structure of MHC II/OVA in mEVs is expected to bind to the OVA-specific T cell receptors (TCR). To verify this, mEVs were labeled with carboxyfluorescein succinimidyl ester (CFSE) and added to naive CD4$^+$ T cell or OVA-specific (DO11.10) CD4$^+$ T cell culture for 30 min. We found that mEVs specifically bound to the surface of OVA-specific CD4$^+$ T cells, but not naive CD4$^+$ T cells. The binding was abolished in DO11.10 CD4$^+$ T cells after depletion of the TCR by RNAi (Fig. 2). The results demonstrate that mEVs can specifically bind to Ag-specific TCR on the surface of CD4$^+$ T cells that makes the T cells as specific targets.

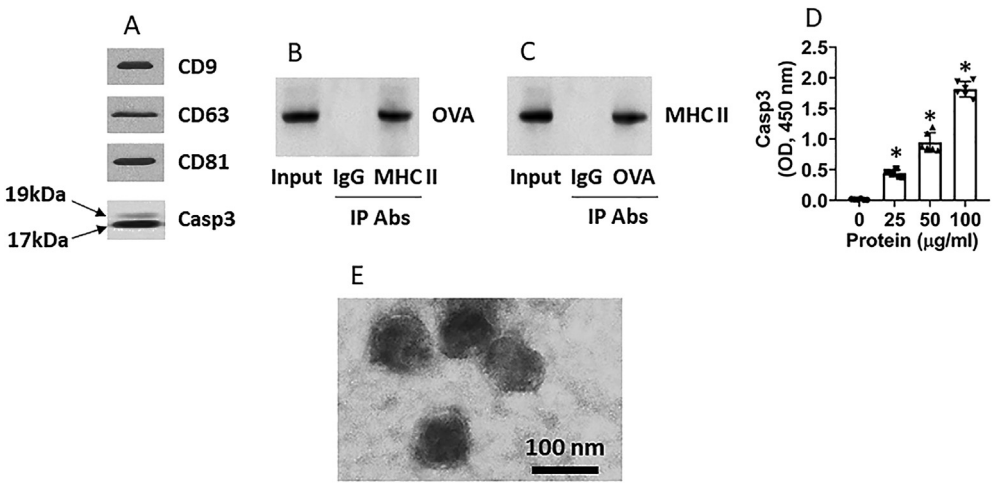

Fig. 1 Characterization of mEV. The mEVs were constructed as described in the text. A, immunoblots show exosome markers, CD9, CD63, CD81 and caspase 3 (Casp3) in mEV protein extracts. B–C, co-IP results show the OVA/MHC II complexes in mEV protein extracts. D, by ELISA, casp3 was detected in mEV protein extracts. E, a representative electron photomicrograph shows isolated exosomes. The data represent 3 independent experiments. Statistics of D: ANOVA followed by the Dunnett's test. *$p < 0.01$, compared to dose "0" group.

mEVs induce Ag-specific CD4$^+$ T cell apoptosis

The mEVs carry both MHC II/Ag complexes and casp3. Casp3 is an apoptosis inducer.[17] Exposure to mEV is expected to induce Ag-specific Th2 cell apoptosis. To this end, CD4$^+$ T cells were isolated from the spleen of DO11.10 mice and BALB/c mice and cultured in the presence of mEVs at gradient concentrations for 48 h. The cells were analyzed by flow cytometry. We found that exposure to mEVs in the culture resulted in CD4$^+$ T cell apoptosis that was in a mEV concentration-dependent manner. Exposure to empty mEVs (EmEVs; without OVA and casp3) or adding mEV lysates (the lysates contained the same components of mEVs) did not induce Ag-specific CD4$^+$ T cell apoptosis. mEVs were not able to induce BALB/c CD4$^+$ T cell apoptosis, indicating that the mEVs only recognize Ag-specific T cells and induce the cell apoptotic. In addition, depletion of TCR in Ag-specific CD4$^+$ T cells abolished the apoptosis induction (Fig. 3A and B). mEVs not carrying Casp3 did not induce Ag-specific CD4$^+$ T cell apoptotic (Fig. 3C). The CD4$^+$ T cells were also analyzed by the whole genome transcriptome RNA sequencing assay. The results showed that the Fas and FasL gene activities were up regulated by mEVs in a dose-dependent manner. Empty mEVs (EmEVs) or mEV lysates did not alter the Fas or FasL levels in CD4$^+$ T cells (Fig. 3D), suggesting that casp3 plays a crucial role in the Fas and FasL elevation. The Fas expression in CD4$^+$ T cells was verified by Western blotting (Fig. 3E). To test the role of the Fas/FasL system in the mEV-induced Ag-specific CD4$^+$ T cell apoptosis, in separate experiments, the Fas expression was depleted by RNAi (Fig. 3F). Indeed, mEVs did not induce Fas-deficient CD4$^+$ T cell apoptotic (Fig. 3A and B). The results indicate that mEVs induce Ag-specific Th2 cell apoptotic through increasing the Fas/FasL expression.

We then tested the effects of mEVs on inducing Ag-specific CD4$^+$ T cell apoptotic *in vivo*. DO11.10 mice and BALB/c mice were peritoneally injected with mEVs every other day for 0–3 times. The mice were sacrificed one day after the last injection. Lamina propria mononuclear cells (LPMCs) were isolated and analyzed by flow cytometry. We found

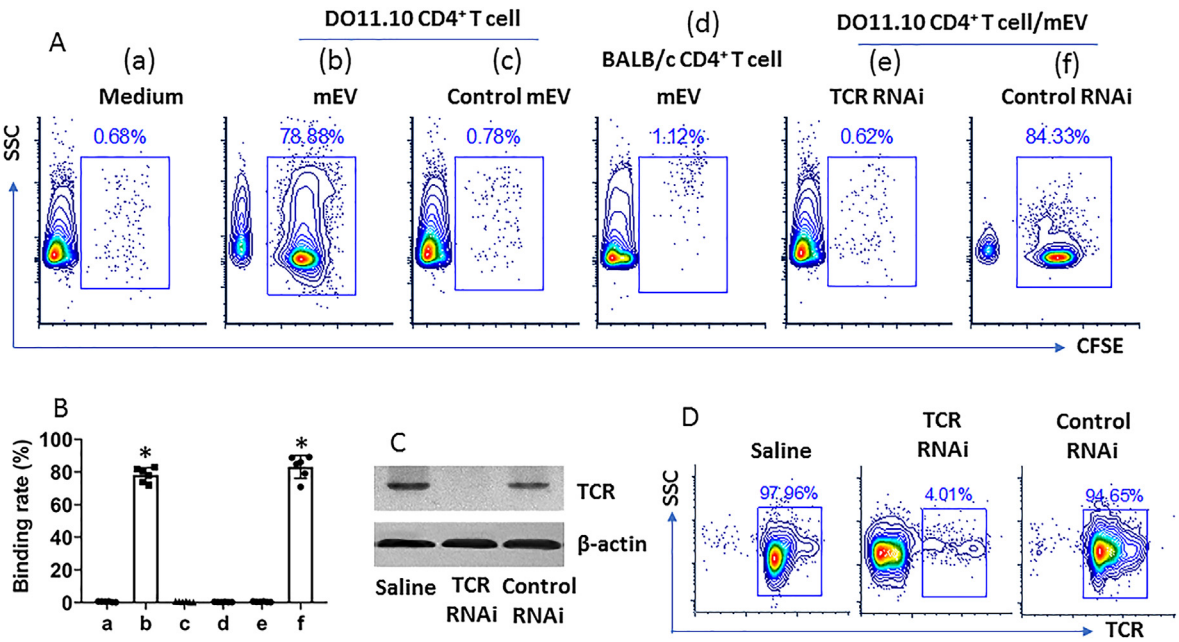

Fig. 2 MEVs specifically bind Ag-specific CD4$^+$ T cells. mEVs were labeled with carboxyfluorescein succinimidyl ester (CFSE). Ag-specific (DO11.10) CD4$^+$ T cells or naive (BALB/c) CD4$^+$ T cells were isolated from the mouse spleen and cultured in the presence of mEV or control mEV (containing BSA instead of OVA) for 30 min. A, gated flow cytometry plots show the frequency of CD4$^+$ T cells with mEV-binding. B, summarized mEV-bound CD4$^+$ T cells. C, immunoblots show TCR protein in DO11.10 CD4$^+$ T cells after the TCR RNAi. D, gated flow cytometry plots show TCR staining in DO11.10 CD4$^+$ T cells after the TCR RNAi. Statistics of B: ANOVA followed by the Dunnett's test. *$p < 0.01$, compared to group a.

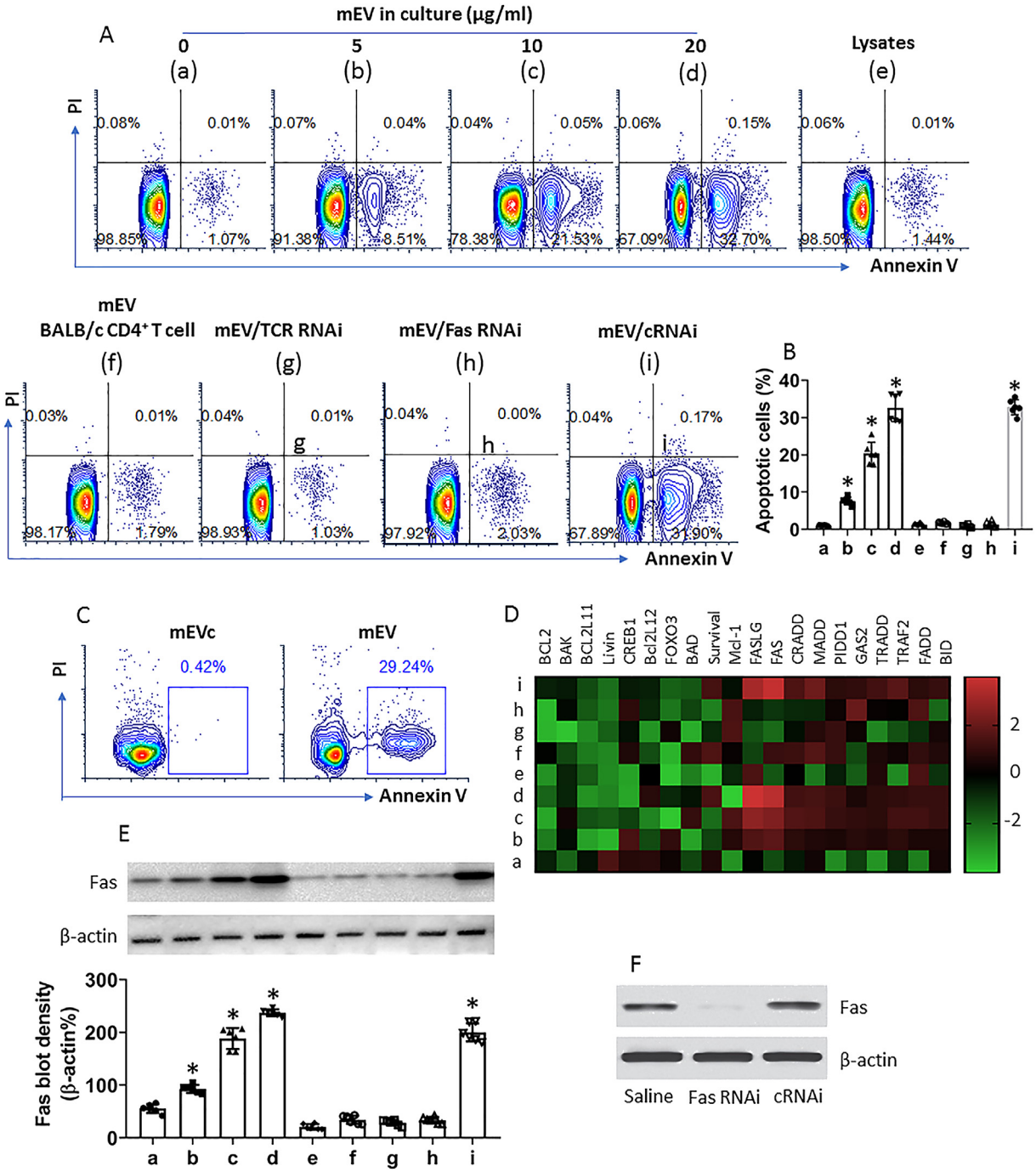

Fig. 3 MEVs specifically induce Ag-specific T cell apoptosis *in vitro*. CD4$^+$ T cells were isolated from the spleen of DO11.10 mice (a-e, g-i) or BALB/c mice (f). The cells were treated with the reagents denoted above each subpanel. The mEV dosage indicates the total proteins in subpanels a-d (other panels are 20 μg/ml). Lysates: Lysed mEV at 20 μg/ml. The Annexin V$^+$ or PI$^+$ Annexin V$^+$ cells are regarded as apoptotic cells (A). B, bars show summarized apoptotic cell counts from 6 independent experiments. C, gated plots show apoptotic CD4$^+$ T cells. mEVc: Control DAPC (not containing Casp3). D, the heatmap shows RNA sequencing results, the apoptosis-related gene levels in CD4$^+$ T cells. E, immunoblots show Fas protein levels in CD4$^+$ T cells. Bars below blots show the integrated density of Fas protein blots. F, immunoblots show the results of Fas RNAi. cRNAi: Cells were treated with control RNAi reagents. The data of bars are presented as mean ± SEM. Each dot presents data obtained from one sample. Statistics: ANOVA followed by the Dunnett's test. *$p < 0.0001$, compared with group a. The group labels of B, C and D are the same as those of flow cytometry plots.

that mEV administration could induce intestinal Ag-specific CD4$^+$ T cell apoptosis that was in a mEV administration time-dependent manner (Fig. 4A–C). The mEV-induced Ag-specific CD4$^+$ T cell apoptosis was also found in spleen cells and mesentery lymph node cells (Fig. 4D–I). The results demonstrate that mEVs can specifically induce Ag-specific CD4$^+$ T cell apoptotic *in vivo*.

Administration of mEV inhibits experimental FA through eliminating Ag-specific CD4$^+$ T cells

Finally, we developed an FA mouse model with OVA as the specific Ag. FA mice were treated with mEVs every other day for 3 times. We found that, upon challenging with specific Ag, OVA, FA mice showed FA-like responses, including diarrhea, drop of the core temperature, increase in serum specific IgE and mouse mast cell protease-1 (mMCP1), increase in Th2 cytokines in the intestinal tissues and caused the intestinal epithelial barrier dysfunctional. Administration of mEVs efficiently inhibited FA response. Empty mEVs or mEV lysates did not show any suppressive effects on FA response, mEVs containing BSA without OVA did not inhibit the OVA-specific FA (not shown), indicating that mEVs specifically suppress OVA-specific FA response (Fig. 5A–H). The data further showed that mEVs reduced OVA-specific Th2 cells in the intestine (Fig. 5I-L). In addition, since apoptotic Ag-specific CD4$^+$ T cells can be captured by phagocytes to promote regulatory T cell (Treg) development[18]; we assessed Tregs in the intestine. The results showed that the mEV

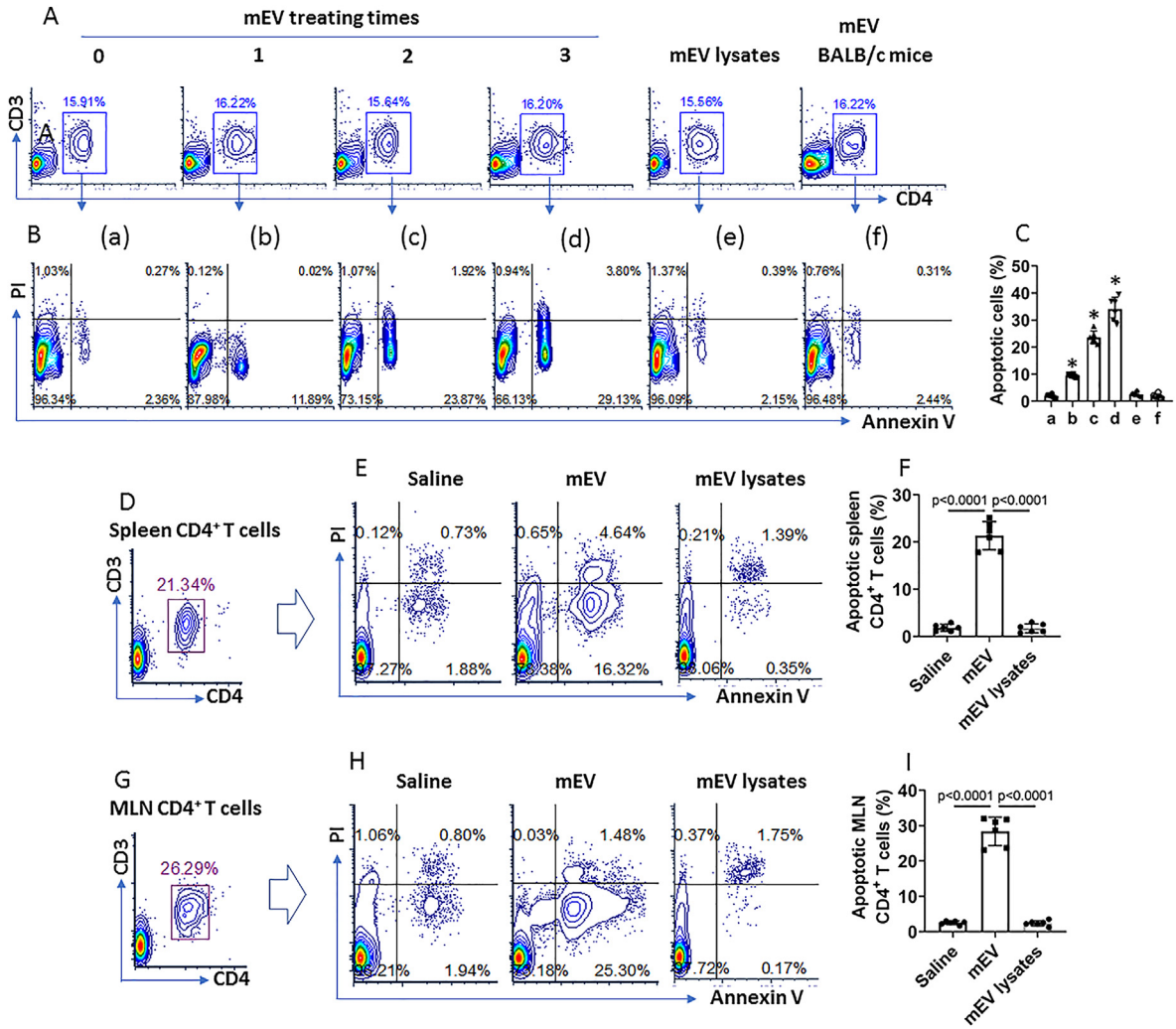

Fig. 4 MEVs induce Ag-specific CD4$^+$ T cell apoptosis *in vivo*. DO11.10 mice or BALB/c mice were peritoneally injected with mEV (0.1 mg protein/mouse) or mEV lysates every other day for 1–3 times. Mice were sacrificed one day after the last injection. LPMCs were isolated and analyzed by flow cytometry. A, CD3$^+$ CD4$^+$ T cells were gated from LPMCs. B, gated plots show apoptotic CD4$^+$ T cells. C, summarized apoptotic cell frequency in LPMCs. D-I, spleen cells and mesentery lymph node (MLN) cells were isolated from mice and analyzed by flow cytometry. D, CD3$^+$ CD4$^+$ T cells in spleen cells were gated first. E, apoptotic cells in spleen CD4$^+$ T cells were determined. F, summarized apoptotic CD4$^+$ T cells. G, CD3$^+$ CD4$^+$ T cells in MLN cells were gated first. H, apoptotic cells in MLN CD4$^+$ T cells were determined. I, summarized apoptotic CD4$^+$ T cells. The data of bars are presented as mean ± SEM. Each dot presents data obtained from one sample. Statistics: ANOVA followed by the Dunnett's test (C) or the Student *t*-test (F, I). *$p < 0.0001$, compared with group b (C) or the saline group (F, I). Each group consists of 6 mice.

administration increased Treg frequency in the intestinal tissues of FA mice (Fig. 6).

DISCUSSION

In this study, we constructed mEVs, that were DC-derived EVs carrying OVA (specific Ag)/MHC II complexes and casp3 molecules. Structurally, it likes the "CAR" in CAR-T cells.[8-10] The data show that mEVs could specifically bind to Ag-specific $CD4^+$ T cells and induced the Ag-specific $CD4^+$ T cell apoptotic and suppressed experimental FA.

Since Ag-specific Th2 cells play a central role in the FA pathogenesis,[19] to eliminate Ag-specific Th2 cells is expected to alleviate or stop FA response.[20] Ag-specific Th2 cells do not have specific molecular markers on the cell surface; this makes it difficult to employ specific antibodies to eliminate this cell fraction. On the other hand, we should not remove the pan $CD4^+$ T cells because Ag-specific Th2 cells are only a small fraction of $CD4^+$ T cells. Fortunately, the TCR on the Ag-specific Th2 cell surface is Ag-specific, it can only be recognized by specific Ag/MHC II complexes.[21]

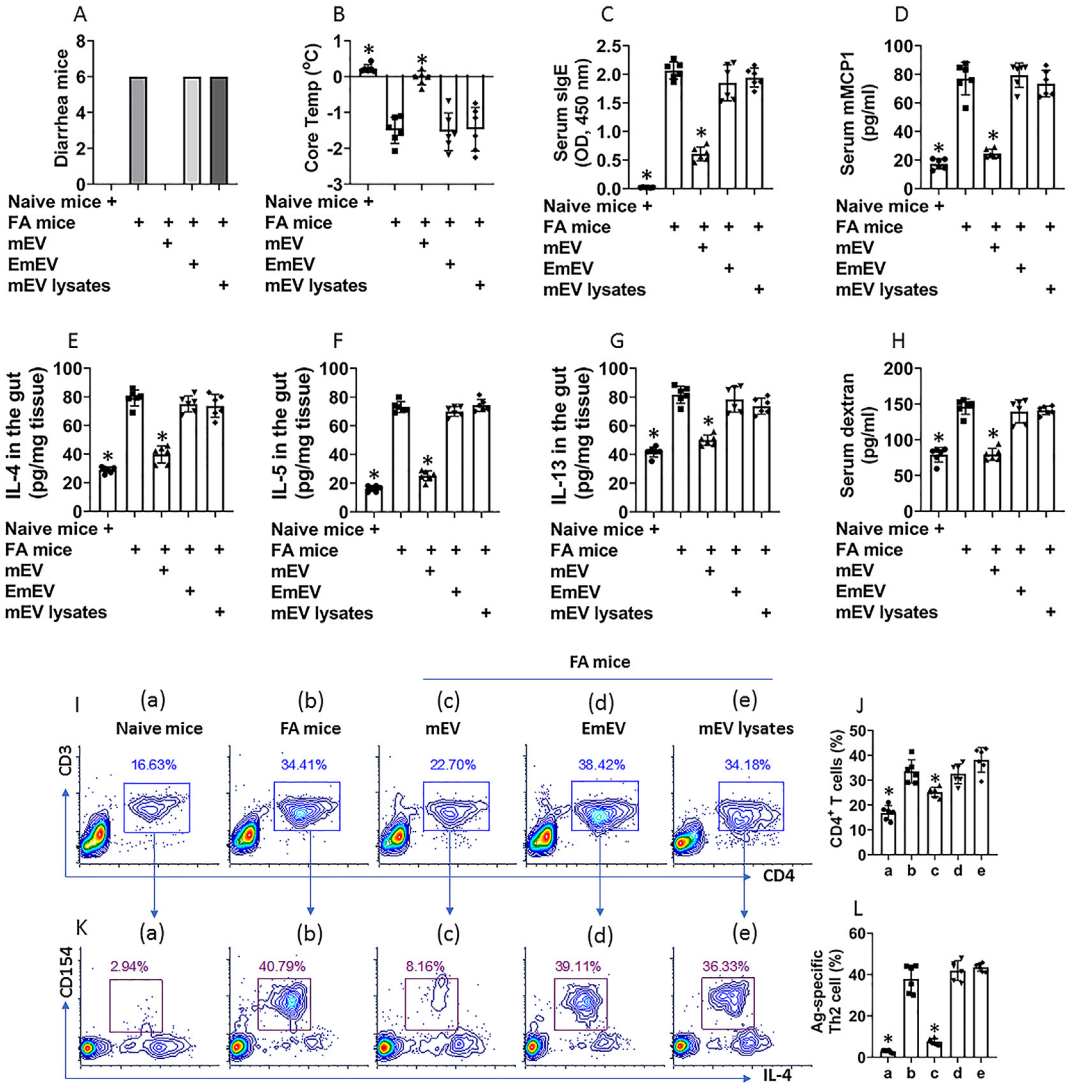

Fig. 5 Administration of mEV suppresses Ag-specific Th2 cells in the intestine and inhibit experimental FA response. From day 20 (one week after the last Ag challenge), FA mice were intraperitoneally injected with mEVs, or empty mEVs (EmEV), or mEV lysates, at 0.1 mg/mouse every other day for 3 times. A-B, number of diarrhea mice (A) and core temperature (Temp) changes (B). C-D, serum specific IgE (sIgE) and mouse mast cell protease-1 (mMCP1). E-G, Th2 cytokine levels in intestinal protein extracts. H, serum dextran levels. I, gated plots show $CD4^+$ T cell frequency in LPMCs. J, bars show summarized $CD4^+$ T cell frequency in LPMCs. K, gated plots show activated Ag-specific Th2 cells in LPMCs. L, bars show summarized Ag-specific Th2 cell frequency in LPMCs. The data of bars are presented as mean ± SEM. Each dot presents data obtained from one sample. Statistics: ANOVA followed by the Dunnett's test. *$p < 0.0001$, compared with the "FA mice" group (B–H) or group b (J, L). Each group consists of 6 mice.

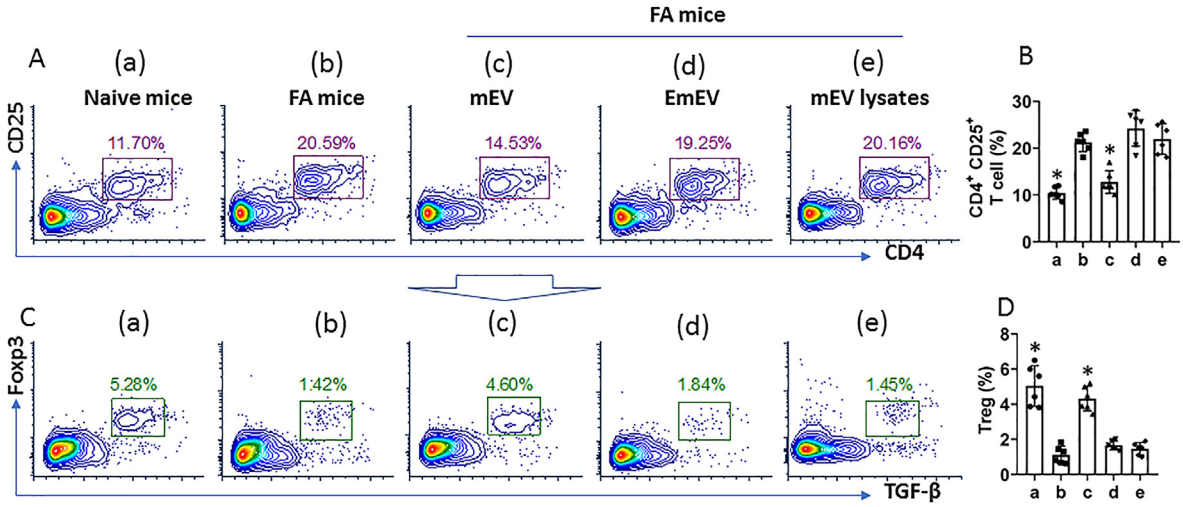

Fig. 6 MEV generates Tregs in FA mouse intestine. FA mice were treated with mEVs or empty exosomes (EmEV) or mEV Lysates as denoted above each subpanel. A, gated flow cytometry plots show $CD4^+$ $CD25^+$ T cell frequency in LPMCs. B, summarized $CD4^+$ $CD25^+$ T cell frequency in LPMCs. C, gated flow cytometry plots show $Foxp3^+$ TGF-β^+ Treg frequency in gated $CD4^+$ $CD25^+$ T cells of panel A. D, summarized $Foxp3^+$ TGF-β^+ Treg frequency in $CD4^+$ $CD25^+$ T cells. The data of bars of panels B and D are presented as mean ± SEM. Statistics: ANOVA followed by the Dunnett's test. *$p < 0.0001$, compared with group b. Each group consists of 6 mice.

This feature makes it possible to design specific ligands to bind the Ag-specific TCR. Published data indicate that the CAR-T cell technique is not only used to eliminate $CD19^+$ B cell leukemia, but it also can be used to counteract infectious disease, such as HIV infection, autoimmune diseases, and the rejection in organ transplantation.[22] Our previous work showed that by the molecular cloning approach, a specific fusion protein consisting of an MHC II molecule, an epitope of the specific Ag and a perforin peptide was constructed. This fusion protein could suppress experimental airway allergic response.[7] The present study expands our previous experimental strategy by finding the MHC II/Ag complexes can be carried by DC-derived exosomes, in which other substances can be on purposely loaded. In this case, casp3 was carried by the EVs that was designated mEV.

By the RNA sequencing assay, we found that mEVs significantly increased the Fas/FasL expression in $CD4^+$ T cells. The Fas/FasL system is the canonical apoptosis-inducer. This feature is exploited by many therapeutic designs; such as anti-cancer drug design. An example is cisplatin. Cisplatin is one of the first line anti-cancer drugs; it induces cancer cell apoptosis by up regulating the Fas expression in targeted cancer cells.[23] Our data are in line with the previous studies by showing that mEVs can induce Ag-specific Th2 cell apoptosis by up regulating the Fas expression in the cells. It is noteworthy that the empty mEV did not show any effects on Fas expression in Ag-specific $CD4^+$ T cells, nor induce target cell apoptotic. The results emphasize that OVA and casp3 carried by mEVs plays a critical role in the mEV-induced Ag-specific $CD4^+$ T cell apoptosis. Although the mEV lysates contain all the components of mEVs, the lysates did not induce Ag-specific Th2 cell apoptotic, nor suppressed FA response in mice, indicating the conformation of mEV is necessary for mEVs to fulfill the immune regulatory role.

The results showed that the Treg frequency in the FA intestinal tissues was lower than that of naïve control mice. Tregs are the canonical cell fraction in the immune regulatory activities in the body.[24] The Th2 polarization status in the intestinal tissue of FA as shown by the present data indicate that the immune regulatory system is dysfunctional. This is supported by the data, the Treg counts in the FA intestinal tissue are markedly fewer than that in the control group. Previous reports also showed similar phenomenon.[25] The present data show that mEVs can induce antigen-specific Th2 cell apoptotic. The apoptotic cells may be phagocytosed by macrophages or dendritic cells that promotes the Treg development as reported previously.[18] The present data are in line with this

by showing that the administration of mEVs significantly increases the Treg counts in the intestinal tissues. On the other hand, whether administration of mEVs generates a microenvironment like the physiological condition that is suitable for Treg development, this is worth to be investigated in the future.

The data show that administration of mEVs, that carry specific Ags, MHC II and Casp3, can efficiently alleviate experimental FA. Therefore, the data suggest that mEVs have the translational potential to be employed in the treatment of FA or other allergic diseases after completing the relevant pre-clinical assessment. On the other hand, whether mEVs or analogue EVs can induce other Ag-specific T cell, such as Ag-specific Th1 cell, apoptosis? This is an interesting topic and worth to be investigated in those Th1 dominant inflammatory disorders such as inflammatory bowel disease. In addition, Ag presenting cells, such as dendritic cells or macrophages, may phagocytose mEVs and become apoptotic that may also contribute to the suppressive effects on FA response.

Previous work showed that, by molecular cloning approach, a fusion protein containing a specific antigen epitope, a scFv segment to connect DEC205 molecule on the surface of DC and perforin.[7] On the other hand, another EV was constructed that carried IL-2 and could suppress experimental food allergy by inducing Tregs in the intestine.[5] The present study focused on another aspect of the pathogenesis of allergic disorders by targeting on eliminating the Ag-specific Th2 cells as Th2 polarization plays a critical role in the pathogenesis of airway allergy.[3] The results verified our study hypothesis by showing that administration of mEVs efficiently alleviated experimental food allergy. Therefore, it would be then considered that packaging combined cargos (IL-2 + IL-10 + caspase-3) into chimeric EVs may greatly increase their therapeutic activity by inducing the complex effects complementary to one another.

In summary, the present paper reports a novel nanoparticle, the mEV. By generating with DCs, mEVs carry MHC II/specific Ag complexes and casp3. Administration of mEVs induces Ag-specific Th2 cell apoptosis, generates Ag-specific Tregs and suppresses experimental FA.

Abbreviations
Antigen (Ag); food allergy (FA); mEV (an extracellular vesicle carrying chimeric antigen peptide, MHC II and caspase 3); dendritic cells (DC); ovalbumin (OVA); caspase 3 (Casp3).

Availability of data and materials
All data and materials are included in this paper.

Ethics approval
The experimental procedures were approved by the Animal Ethics Committee at Shenzhen University (#SZUAE2018036).

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Author contributions
YYZ, LHM, GY, JQL, ZQL and LTY performed experiments, analyzed data and reviewed the manuscript. PCY, ZGL and PXR organized the study and supervised experiments. PCY designed the project and prepared manuscript.

Authors' consent for publication
All authors have agreed with the publication of this work in World Allergy Organization Journal.

Declaration of competing interest
None to declare.

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