

ORIGINAL ARTICLE

Specific antigen-guiding exosomes inhibit food allergies by inducing regulatory T cells

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Abstract

The therapies for food allergy (FA) need to be improved. The generation of inducible regulatory T cells (Tregs) can support immune tolerance in the body. This study aims to suppress experimental FA by inducing Tregs through the employment of modified exosomes (mExosomes). In this study, mExosomes were prepared by incubating dendritic cells with interleukin (IL)-2 and ovalbumin (OVA, used as a specific antigen) in the culture. Exosomes were purified from culture supernatant and used as the mExosomes. A murine FA model was developed to test the effects of mExosomes on the generation of Tregs in the mouse intestinal tissues and inhibiting FA. The results showed that mExosomes, which carried IL-2 and a complex of OVA peptide–major histocompatibility complex class II on the surface of exosomes, bound to OVA-specific CD4⁺ T cells and induced CD4⁺ T cells to differentiate into Tregs. In the FA mouse intestinal tissues, we found low IL-2 levels that were positively correlated with the number of Tregs. Depletion of IL-2 in mice prevented the generation of Tregs. The levels of peroxisome proliferator-activated receptor- γ were increased in the FA intestinal tissues with inhibited IL-2 production. Administration of mExosomes induced Tregs in the intestinal tissues and efficiently suppressed FA in mice. We conclude that the mExosomes can suppress FA in mice through inducing Tregs. The data suggest that the mExosomes have translational potential in the treatment of FA and other allergic disorders.

INTRODUCTION

Food allergy (FA) is a common disease in which aberrant responses to food antigens (Ags) occur in the intestine.¹ The prevalence of FA is about 2–4% in adults and 4–6% in children worldwide,² and FA pathogenesis is not yet fully understood. The clinical symptoms of FA vary from mild abdominal discomfort to life-threatening anaphylactic shock.² Current therapies for FA are not satisfactory.^{2–4} Therefore, it is necessary to further understand FA pathogenesis and develop novel and effective remedies for FA.

Pathologically, FA is an aberrant T helper-2 (Th2) dominant immune response in the intestinal tissues.⁵ When

the epithelial barrier is dysfunctional, macromolecular protein Ags are absorbed into the deep regions of the intestinal tissues where the Ags contact immune cells and induce Ag-specific immunoglobulin E (IgE) production.⁶ IgE binds the high-affinity IgE receptors on the surface of mast cells to sensitize the mast cells. Re-exposure to specific Ags can trigger sensitized mast cells to release allergic mediators and induce FA attacks.^{5,7} Under physiological conditions, the immune response is tightly regulated by the immune regulatory system in the body.⁸ The Th2 polarization status of FA subjects mirrors a dysfunctional status of the immune regulatory system.⁹ The underlying mechanism remains to be further investigated.

The immune regulatory system in the body includes immune regulatory cells [e.g. regulatory T cells (Tregs), regulatory B cells] and immune regulatory mediators [e.g. transforming growth factor- β (TGF- β), interleukin (IL)-10].¹⁰ Cumulative reports indicate that the dysfunctional status of the immune regulatory system can be observed in various immune diseases, including FA.⁹ Because the major functions of the immune regulatory system are to regulate abnormal immune activities, the immune regulatory system dysfunction may result in abnormal immune responses, such as FA.⁹ The underlying mechanism from which the intestinal immune regulatory system becomes dysfunctional is not yet fully understood. IL-2 is required in Treg differentiation,¹¹ and inhibition of IL-2 facilitates FA development.¹² Thus, the production or administration of exogenous IL-2 has the potential to inhibit FA by upregulating Treg differentiation.

It has been reported that exosomes can be used as carriers for drug administration.¹³ Exosomes are membrane-bound extracellular vesicles that are produced by eukaryotic cells containing cellular components.¹⁴ In addition, exosomes can be engineered to carry special substances for therapeutic purposes.¹³ In this study, we modified exosomes (mExosomes) to carry IL-2 and to express a specific Ag on the surface. The effects of the mExosomes on Treg generation and FA inhibition were tested with a murine model.

RESULTS

IL-2 levels are negatively correlated with skewed Th2 polarization in the FA mouse intestinal tissues

An FA mouse model was developed (Supplementary figure 1). Jejunal segments were excised from FA and naïve control (NC) mice. Protein extracts were prepared with the intestinal tissues and analyzed by ELISA. The results showed that the IL-2 levels were lower in FA mice than those in NC mice (Figure 1a). The levels of Th2 cytokines, including IL-4, IL-5 and IL-13, were higher in FA mice than those in NC mice (Figure 1b–d). A negative correlation was detected in the data between IL-2 levels and Th2 cytokines of FA mice (Figure 1e–g). The results suggest that there may be an association between the insufficient production of IL-2 and the overproduction of Th2 cytokines in FA intestinal tissues.

IL-2 levels are associated with Treg frequency in the intestine of FA mice

To determine the association between IL-2 levels and Treg frequencies in the FA mouse intestine, lamina propria mononuclear cells were prepared from FA mice and NC

mice and analyzed by flow cytometry. We found that the CD4⁺CD25⁺Foxp3⁺ Treg frequency was significantly lower in the FA group than in the NC group (Figure 2a, b), and the TGF- β levels in the intestinal tissues followed this same pattern (Figure 2c). A positive correlation was detected in the data between IL-2 and Tregs or TGF- β (Figure 2d, e). In addition, we also performed immunohistochemistry to visualize Tregs in the intestinal tissues. The results showed significantly fewer Treg counts in FA mice than those in NC mice (Figure 2e, f). The results suggest that the low IL-2 levels are associated with the low Treg frequency and the low TGF- β levels in the FA mouse intestine.

Depletion of IL-2 retards Treg generation in the intestine

The data reported above suggest that IL-2 may be required in Treg generation in the intestine. To test this, naïve BALB/c mice received large doses of ovalbumin (OVA) gavage daily for 7 days. We found that the Treg frequency was markedly increased in wild-type mice, whereas the Treg frequency in IL-2-deficient mice was not altered by the OVA feeding (Figure 3). The results indicate that IL-2 deficiency prevents Treg generation in the intestine.

Peroxisome proliferator-activated receptor- γ suppresses IL-2 in CD4⁺ T cells in the FA intestinal tissues

As published data indicate that peroxisome proliferator-activated receptor- γ (PPAR γ) can inhibit IL-2 in T cells,¹⁵ we wondered whether the lower levels of IL-2 in FA mice were associated with the PPAR γ changes in the intestinal tissues. To test this, intestinal tissues were excised from FA and NC mice. Proteins were extracted from the tissues and analyzed by ELISA. Indeed, we found that the PPAR γ levels were markedly higher in FA intestinal tissues as compared with those in NC intestinal tissues (Figure 4a). A negative correlation was detected between the PPAR γ protein levels and IL-2 protein levels in the FA intestinal tissues (Figure 4b). Treating naïve mice with a PPAR γ agonist, rosiglitazone, every other day for 1 week significantly suppressed the IL-2 levels in the intestinal tissues (Figure 4c). Administration of GW9662, an antagonist of PPAR γ , during the sensitization course maintained the IL-2 levels (Figure 4d) as well as the Treg numbers in the FA mouse intestinal tissues (Figure 4e, f).

Characterization of IL-2-carrying mExosomes

Bone marrow-derived dendritic cells were generated and cultured in the presence of OVA and IL-2 overnight. Supernatant was collected, and exosomes were purified

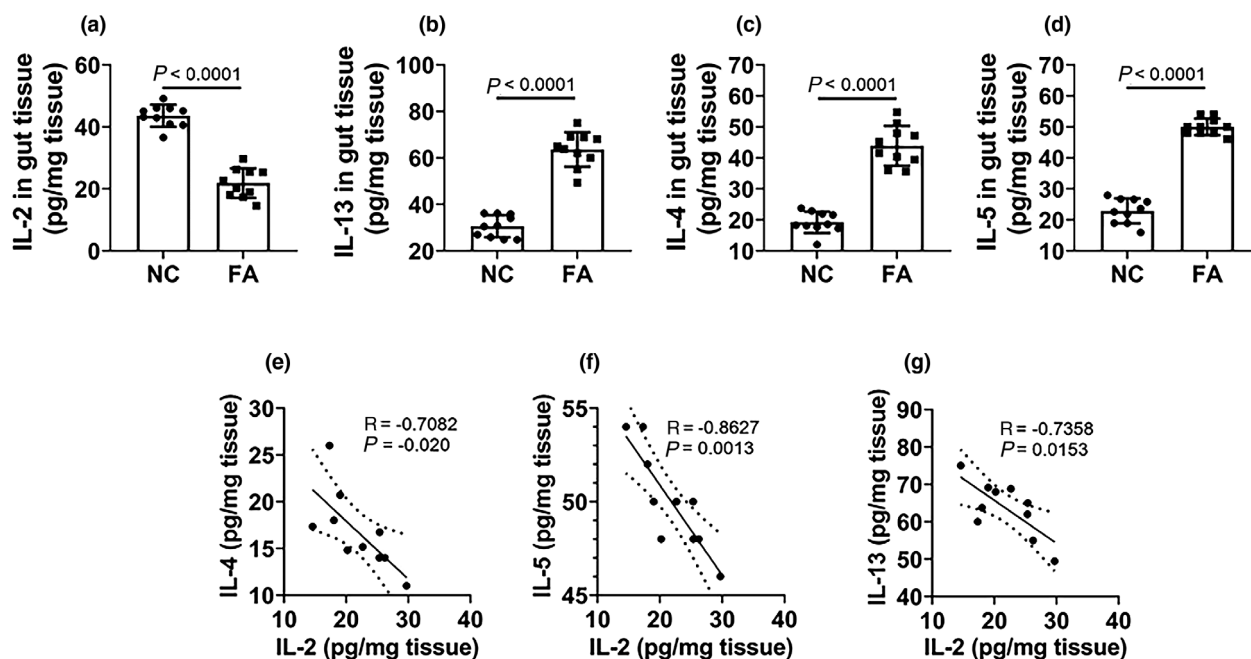


Figure 1. Interleukin (IL)-2 contents in food allergy (FA) intestinal tissues are negatively correlated with T helper-2 (Th2) cytokine contents. Proteins were extracted from intestinal tissues excised from FA mice and naïve control (NC) mice, and analyzed by ELISA. (a–d) Bars show levels of (a) IL-2, (b) IL-13, (c) IL-4 and (d) IL-5 in the intestinal tissue protein extracts. (e–g) Negative correlation between IL-2 and Th2 cytokines. The data of a–d are presented as mean ± s.e.m. Statistics used were as follows: (a–d) the Student's *t*-test and (e–g) Pearson's correlation assay. Each group consisted of 10 mice. Each sample was analyzed in triplicate. Each dot in bars represents data obtained from one sample.

from the supernatant with established procedures.¹⁶ As expected, OVA–major histocompatibility complex class II (MHC II) complexes (Figure 5a) as well as IL-2 were detected in protein extracts of the exosomes; the extracts also contained CD9, CD63 and CD81 (Figure 5b), indicating that the proteins were extracted from exosomes.¹⁷ These exosomes were designated mExosomes. The mExosomes could specifically bind to the surface of OVA-specific CD4⁺ T cells (Figure 5c, d). After exposing to mExosomes in the culture for 2 days, the expression of Bcl-6, TGF- β and Foxp3 was markedly increased in the OVA-specific CD4⁺ T cells (Figure 5e–g). Neuropilin, a specific marker of thymus-derived Tregs,¹⁸ was not detected (Figure 5h), whereas the expression of CTLA-4, an immune-suppressive molecule, was detectable in these OVA-specific CD4⁺ T cells, but not significantly upregulated by mExosome (Figure 5i). IL-10, another immune regulatory cytokine, was not detected in these OVA-specific CD4⁺ T cells (not shown). The data suggest that mExosomes can generate inducible Tregs.

Administration of mExosomes induces Ag-specific Tregs and suppresses experimental FA

We then treated FA mice with mExosomes by peritoneal injection daily for 7 days. We found that FA mice had

the expected FA response, including a drop in the core temperature, diarrhea, increase in serum-specific IgE and mouse mast cell protease-1, high Th2 cytokine levels in the intestinal tissues and damaged intestinal epithelial barrier dysfunction. Administration of mExosomes efficiently suppressed the FA response, and this suppression was abolished by concurrent treatment with anti-CD25 antibody or rosiglitazone (the PPAR γ agonist). Administration of control exosomes (i.e. exosomes without IL-2 or OVA) or bovine serum albumin-carrying exosomes did not show any suppressive effects on FA response (Figure 6a–h). Finally, Tregs were significantly induced in the intestinal tissues in FA mice that received the mExosome therapy (Figure 7 and Supplementary figure 2).

DISCUSSION

In this study, we prepared mExosomes that carried IL-2 and a complex of OVA (the specific Ag in this study) and MHC II. This complex of OVA–MHC II is designed to target on the T-cell receptor on OVA-specific CD4⁺ T cells, and the IL-2 to facilitate Treg differentiation. Indeed, the data show that mExosomes specifically bound to OVA-specific CD4⁺ T cells and induced Treg differentiation. The data also show that the insufficient

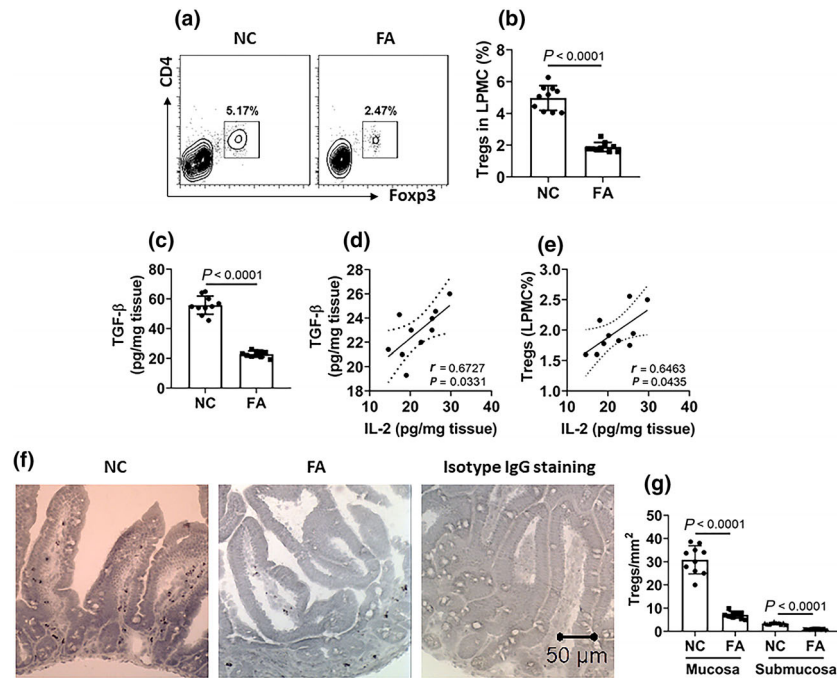


Figure 2. Food allergy (FA) intestinal regulatory T cells (Tregs) are positively correlated with the interleukin (IL-2) levels in the intestinal tissues. **(a)** Jejunal segments were excised from naïve control (NC) mice and FA mice; lamina propria mononuclear cells (LPMCs) were prepared from the segments and analyzed by flow cytometry. Gated dot plots show CD4⁺ Foxp3⁺ Tregs. **(b)** Bars show summarized Treg frequency in the intestine. **(c)** Proteins were extracted from each intestinal tissue sample and analyzed by ELISA. Bars show transforming growth factor- β (TGF- β) levels in intestinal tissue protein extracts. **(d, e)** Positive correlation between TGF- β protein or Treg frequency and IL-2 protein levels in the FA mouse intestinal tissues (IL-2 data are presented in Figure 1). **(f, g)** Each intestinal tissue sample was processed by immunohistochemistry examination. **(f)** Representative images show Foxp3⁺ Tregs in the intestine. **(g)** Bars show summarized data of Treg counts in the intestinal mucosa and submucosa. The data of bars are presented as mean $\pm$ s.e.m. Each dot in bars represents data obtained from one sample. Statistics used were as follows: **(b, c)** the Student's *t*-test, **(d)** Pearson's correlation assay and **(g)** ANOVA + the Bonferroni test. Each group consisted of 10 mice. Each sample was analyzed in triplicate. The gated Tregs in **a** are also CD25⁺ (not shown). Ig, immunoglobulin.

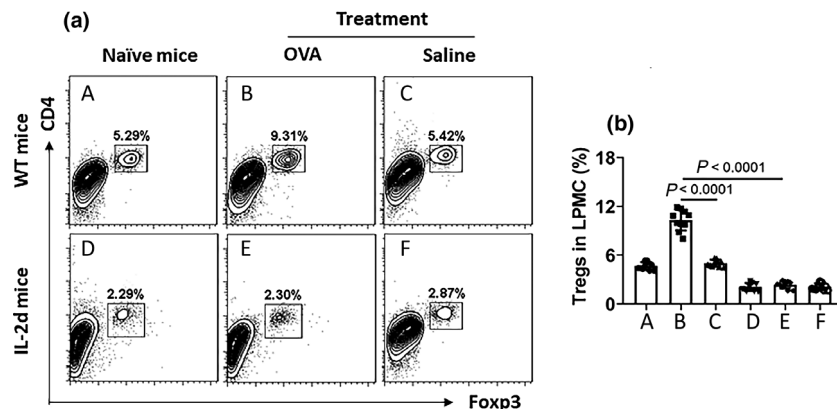


Figure 3. Interleukin (IL-2) deficiency retards generation of regulatory T cells (Tregs) in the intestine. IL-2-deficient (IL-2d) mice and wild-type (WT) mice were gavage fed with ovalbumin (OVA; 5 mg per mouse in 0.1 mL saline) or saline daily for 7 days. Lamina propria mononuclear cells (LPMCs) were prepared and analyzed by flow cytometry. **(a)** Gated dot plots show Treg frequency in LPMCs. **(b)** Bars show summarized Treg frequency from 10 mice per group. Data of bars are presented as mean $\pm$ s.e.m. Each dot in bars represents data obtained from one sample. Statistics used were ANOVA + the Bonferroni test. The gated Tregs in **a** are also CD25⁺ (not shown). Each group consists of 10 mice. Each sample was analyzed in triplicate.

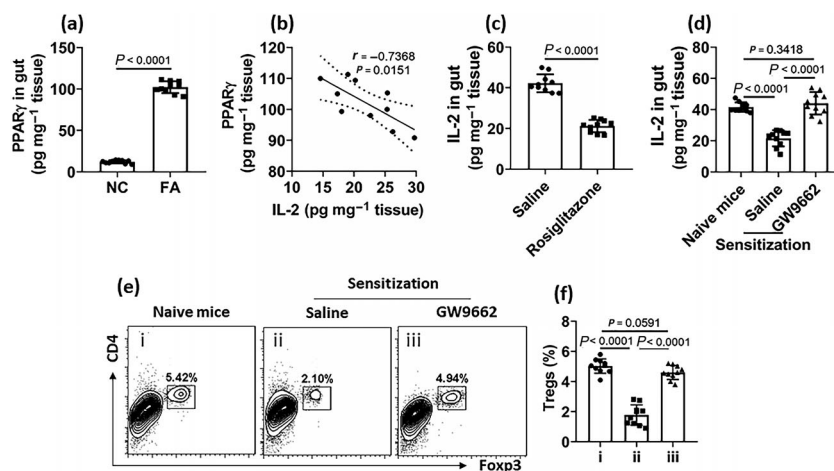


Figure 4. Peroxisome proliferator-activated receptor- γ (PPAR γ) suppresses interleukin (IL)-2 in food allergy (FA) intestinal tissues. Jejunal segments were excised from naïve control (NC) mice and FA mice. Proteins were extracted from the tissues and analyzed by ELISA. **(a)** Bars show PPAR γ protein levels in mouse gut tissue protein extracts. **(b)** Negative correlation between gut PPAR γ and gut IL-2 (original IL-2 data are presented in Figure 1) of the FA group. **(c)** Bars show IL-2 levels in mouse intestinal tissue protein extracts (by ELISA) after treating mice with rosiglitazone (a PPAR γ agonist; 10 mg kg⁻¹ in 0.1 mL saline, gavage) every other day for three times. **(d-f)** Mice were treated with GW9662 (0.1 mg in 0.1 mL saline, intraperitoneally; a PPAR γ antagonist) or saline each time upon exposing them to specific antigen in the course of sensitization. Proteins were extracted from the gut tissues and analyzed by ELISA. **(d)** Bars show IL-2 levels in mouse intestinal tissue extracts. Lamina propria mononuclear cells (LPMCs) were isolated from the gut tissues and analyzed by flow cytometry. **(e)** Gated plots show regulatory T cell (Treg) frequency in the intestine. **(f)** Bars show summarized Treg data from 10 mice per group. Data of bars are presented as mean $\pm$ s.e.m. Statistics used were as follows: **(a, c)** the Student's *t*-test, **(b)** Pearson's correlation assay and ANOVA + the Bonferroni test **(d, f)**. Each group consists of 10 mice. Each sample was analyzed in triplicate. Each dot in bars represents data obtained from one sample. Group labels in panel f are the same as those in panel e.

IL-2 expression was found in the FA mouse intestinal tissues, which was negatively correlated with low Treg frequency in the intestine. The IL-2 depletion prevented naïve CD4⁺ T cells from differentiating into Tregs, and administration of mExosomes suppressed FA response by facilitating the Treg differentiation.

As IL-2 can be produced by several cells in the intestine, we evaluated the total IL-2 levels in the intestinal tissue extracts. The data show low IL-2 levels in the FA intestinal tissues. Our data are in line with previous studies. IL-2 is an essential cytokine for CD4⁺ T-cell maturation¹⁹ and can drive Treg precursor differentiation into Foxp3⁺ T cells as well as promote Treg proliferation and survival.²⁰ The present data provide further information that IL-2 is negatively correlated with Th2 polarization status and positively correlated with Treg frequency in the FA mouse intestine. The data suggest that insufficient IL-2 can be one of the causative factors in the dysfunctional immune regulatory system and Th2 polarization in the intestine.

We found that PPAR γ was significantly increased in the FA mouse intestine. The levels of PPAR γ were negatively correlated with the IL-2 levels in intestinal protein extracts. The results suggest that PPAR γ may inhibit IL-2 in FA mice. The inference was supported by

further experimental data: administration of a PPAR γ agonist, rosiglitazone, inhibited IL-2 levels in the mouse intestine, while treating mice with a PPAR γ antagonist, GW9662, in the course of sensitization maintained the IL-2 levels and the Treg frequency in the mouse intestine. These data demonstrate that PPAR γ can suppress IL-2 in the FA intestinal tissues. Consistent with our data, Yang *et al.*²¹ also found that IL-4 suppresses IL-2 through macrophage-derived PPAR γ . In addition, an endogenous arachidonic acid derivative, 2-arachidonol glycerol, can increase PPAR γ to suppress IL-2 in T cells.²² In our study, the Th2 polarization in the FA mouse intestine can be a causative factor to increase PPAR γ levels, and the latter further suppresses IL-2 production in the intestine.

Tregs are one of the major cellular components of the immune regulatory system. The immune regulatory system plays a critical role in the maintenance of the immune tolerance in the body. Immune intolerance to food Ags is one of the canonical pathological features of FA. Our data show low Treg frequency in the FA mouse intestinal tissues, and this is in line with published data. Noval Rivas *et al.*²³ found low Treg frequency in the FA mouse intestine and proposed that Tregs were converted to Th2 cells in an FA environment. Our previous work also showed that Treg frequency was lower in the FA

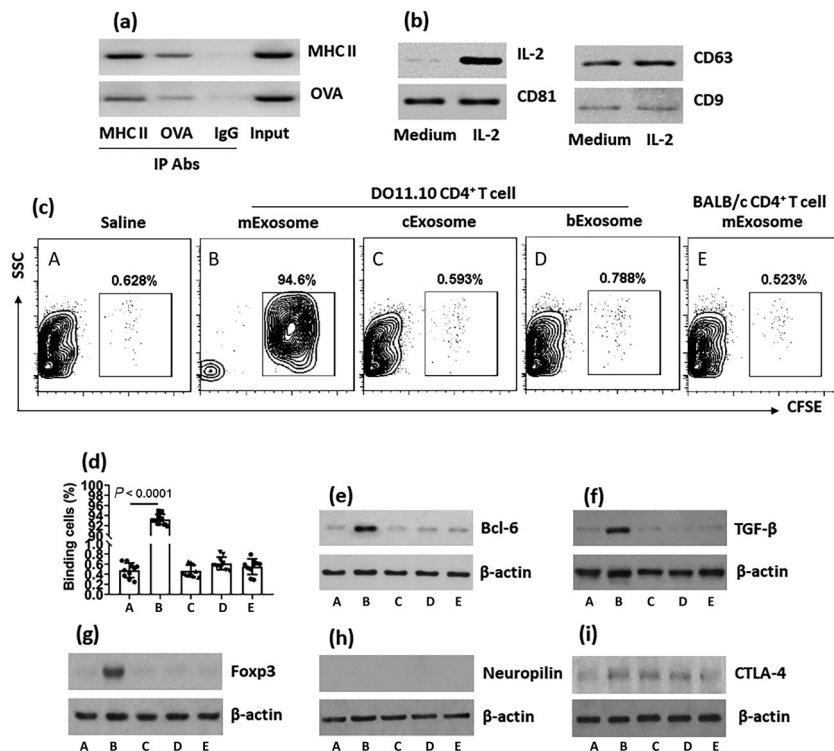


Figure 5. mExosomes bind Ag-specific CD4⁺ T cells to expand regulatory T cell (Treg) population. **(a, b)** proteins were extracted from mExosomes and analyzed by coimmunoprecipitation. **(a)** Immunoblots show a complex of ovalbumin (OVA) and major histocompatibility complex class II (MHC II) in the protein extracts. **(b)** Immunoblots show that the protein extracts contain interleukin (IL)-2, CD81, CD63 and CD9. **(c)** mExosomes and control exosomes were labeled with carboxyfluorescein succinimidyl ester (CFSE) and added to DO11.10 CD4⁺ T cell (or BALB/c CD4⁺ T cells, control) culture at conditions denoted above each subpanel; 30 min later, the cells were harvested and analyzed by flow cytometry. Gated flow cytometry plots show frequency of exosome-bound CD4⁺ T cells. **(d)** Bars show summarized data of **(c)**. **(e–i)** CD4⁺ T cells were treated with the procedures of **(c)**; cells were harvested 48 h later and analyzed by western blotting. Immunoblots show **(e)** Bcl-6, **(f)** transforming growth factor-β (TGF-β), **(g)** Foxp3, **(h)** neuropilin and **(i)** CTLA-4 levels in the CD4⁺ T cells. Statistics of **(d)**: ANOVA + the Bonferroni test. Each group consists of 10 mice. Each sample was analyzed in triplicate. Each dot in bars represents data obtained from one sample. bExosome, bovine serum albumin (BSA) and IL-2-carrying exosome; cExosomes, control exosome (i.e. exosomes that do not carry IL-2 and OVA); CFSE, carboxyfluorescein succinimidyl ester; Ig, immunoglobulin; SSC, side scatter.

mouse intestine.²⁴ The present data also show that Treg frequency in the FA intestine is negatively correlated with FA response, including diarrhea, core temperature drop and increase in intestinal epithelial barrier permeability. This phenomenon was also found in the previous studies.²⁴ Together, the reduced Treg frequency in the intestinal tissues can be an important causative factor in FA pathogenesis.

Restoring Treg generation can be an optimal approach to restore the homeostasis in the intestine with FA.²⁴ Treg generation has been employed in the studies of FA treatment.^{24,25} In addition, generating Tregs is one of the major goals in Ag-specific immunotherapy.²⁶ The present study added IL-2 into the mExosomes. This therapeutic device specifically contacted Ag-specific CD4⁺ T cells and provided IL-2, which induced the target cells to express Bcl-6 and develop into Tregs. A previous study also

indicated that low doses of IL-2 can generate Tregs in FA subjects.²⁷ Our previous work showed that Bcl-6 inhibited the expression of GATA-3 in Th2 cells; subsequently, Foxp3 was increased, and Th2 cytokines were reduced in the cells.²⁸ As the nonspecific Tregs suppress other immune cell activities in a nonspecific manner, subjects with allergic diseases may not benefit from the nonspecific Tregs. This is a weakness of employing the nonspecific Tregs to modulate the abnormal immune responses. The mExosomes can avoid this weakness by the specific structure, the OVA–MHC II complexes and the carrying IL-2. The present data show that this structure ensures the mExosomes can only bind to OVA-specific CD4⁺ T cells to facilitate the Treg expansion.

In summary, we prepared mExosomes that could specifically bind to Ag-specific CD4⁺ T cells and induce

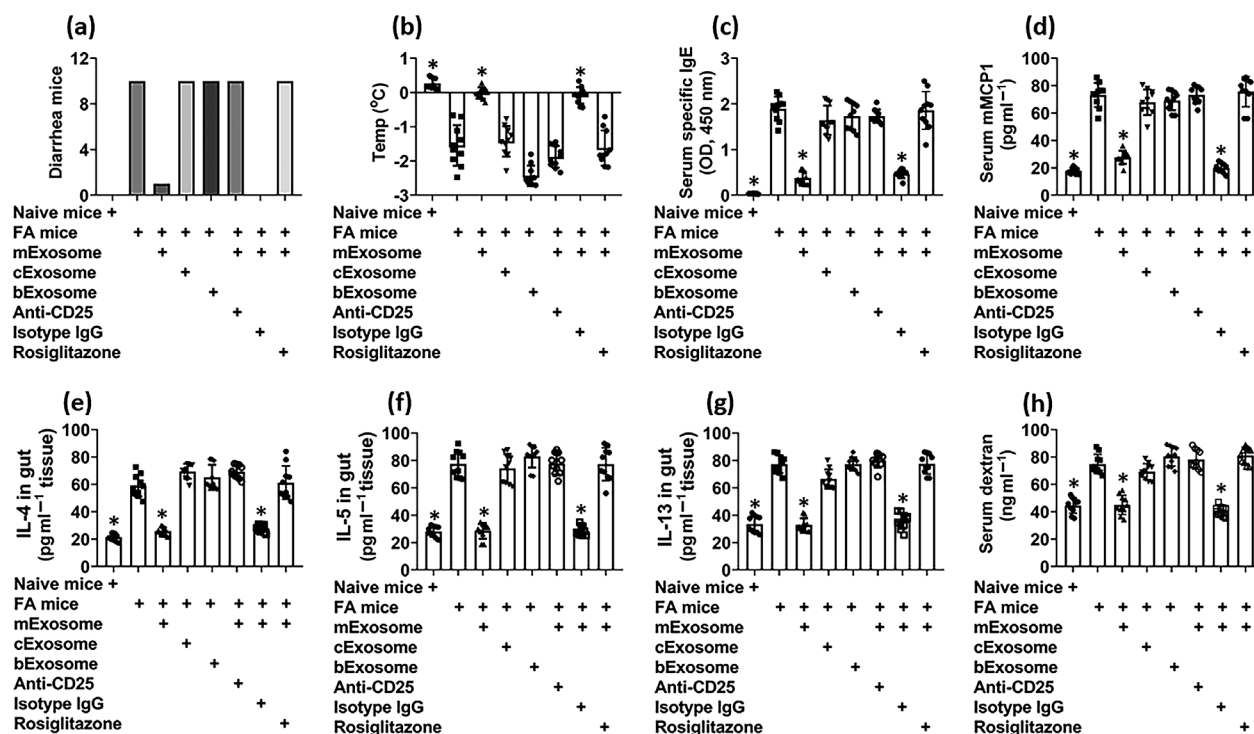


Figure 6. Administration of mExosomes suppresses experimental food allergy (FA) via generating regulatory T cells (Tregs). (a–h) FA mice were prepared and treated with the procedures denoted on the x axis of bar graphs. Bars show FA responses in mice after challenging with (a, b) specific Ag, ovalbumin (OVA), including diarrhea and drop of core temperature (Temp), (c, d) serum levels of specific immunoglobulin E (IgE) and mouse mast cell protease-1 (mMCP1), (e–g) T helper-2 (Th2) cytokines in intestinal tissue extracts, (h) serum FITC-dextran levels to demonstrate the intestinal epithelial barrier function. ANOVA + Dunnett's test was used for statistical analysis. * $P < 0.0001$, compared with the FA-OVA group. Each group consists of 10 mice. Each sample was analyzed in triplicate. Each dot in bars represents data obtained from one sample. Ab, antibody. bExosome, bovine serum albumin (BSA) and IL-2-carrying exosome; cExosome, control exosome (exosomes do not carry IL-2 and OVA); FITC, fluorescein isothiocyanate; mExosome, IL-2-modified exosomes; mExosome–Ab (or cAb), mice were injected with anti-CD25 Ab (or control Ab, isotype IgG) 3 days prior to administration of mExosomes; OVA, FA mice were challenged with OVA alone; Rosiglitazone, a peroxisome proliferator-activated receptor- γ (PPAR γ) agonist (10 mg kg⁻¹ in 0.1 mL saline) gavage fed every other day for three times.

differentiation into Tregs. Administration of mExosomes efficiently suppressed experimental FA, which suggests that the mExosomes have translational potential for the treatment of FA.

METHODS

Reagents

Antibodies of MHC II, OVA, IL-2, IL-10, CTLA-4, CD81, CD63, CD9, Foxp3 and TGF- β were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). IL-2 protein, CD25 antibody, ELISA kits of IL-4, IL-5, IL-13, TGF- β and PPAR γ were purchased from Dakewe Biomart (Beijing, China). Antibodies of CD11c (FITC), CD4 (FITC), CD25 (PE) and Foxp3 (APC) were purchased from BD Biosciences (Franklin Lakes, NJ, USA). The neuropilin antibody was purchased from Abcam (Cambridge, MA, USA). OVA, bovine serum albumin, granulocyte-macrophage colony-stimulating factor, Rosiglitazone, GW9662, FITC-dextran, lipopolysaccharide and

immunoprecipitation kit were purchased from Sigma Aldrich (St. Louis, MO, USA). Reagents and materials for western blotting were purchased from Invitrogen (Carlsbad, CA, USA).

Mice

BALB/c mice were purchased from the Guangzhou Experimental Animal Center (Guangzhou, China). DO11.10 mice and IL-2-deficient (IL-2d) mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Mice were maintained in a specific pathogen-free facility with free access to food and water. The experimental procedures were approved by the Animal Ethical Committee at Shenzhen University.

Development of an FA mouse model

Following established procedures, mice were subcutaneously injected with OVA (0.1 mg per mouse; mixed in 0.1 mL alum) on the back skin on days 0 and 7, respectively. The mice were challenged with OVA (1 mg per mouse in 0.1 mL

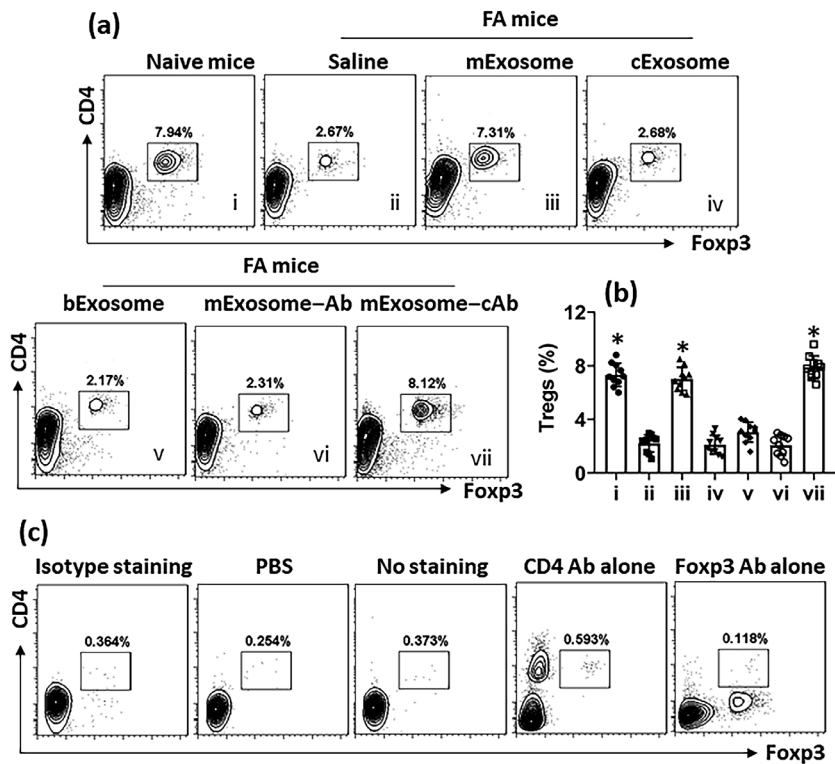


Figure 7. mExosomes regulate regulatory T cell (Treg) frequencies in food allergy (FA) mouse intestine. Lamina propria mononuclear cells (LPMCs) were isolated from mice described in Figure 6 and analyzed by flow cytometry to assess the Treg frequency. **(a)** Gated flow cytometry plots show Treg frequency in LPMCs. These gated cells were also CD25⁺ (not shown). **(b)** Bars show summarized Treg frequency data of 10 samples per group. Statistics used were as follows: ANOVA + Dunnett's test. * $P < 0.0001$, compared with the FA-OVA group. **(c)** Flow cytometry plots show staining control results of the "fluorescence minus one" method. Phosphate-buffered saline buffer was used to dilute staining antibodies. Each group consists of 10 mice. Each sample was analyzed in triplicate. Each dot in bars represents data obtained from one sample. Ab, antibody; bExosome, bovine serum albumin (BSA) and IL-2-carrying exosome; cExosome, control exosome (exosomes do not carry IL-2 and OVA); mExosome, IL-2-modified exosomes; OVA, ovalbumin; PBS, phosphate-buffered saline.

saline) through gavage feeding on days 9, 11 and 13, respectively. On day 15, mice were challenged by gavage feeding with OVA (5 mg per mouse in 0.1 mL saline). FA response (core body temperature and diarrhea) was observed and recorded within 30 min after the challenge. Blood samples were collected after killing mice by cervical dislocation. Small intestinal segments were excised and processed for further experiments (Supplementary figure 1a).

Immunohistochemistry

Small intestinal segments were excised upon killing and snap frozen in liquid nitrogen. Cryosections were cut (5 μ m thick) and dried in room temperature overnight. After being fixed with cold acetone for 20 min, sections were blocked by incubating with 1% bovine serum albumin for 30 min and stained by the established procedures of immunohistochemistry of our laboratory.²⁹ The sections were stained with anti-Foxp3 antibody (clone number 6H3C5H3; Santa Cruz Biotech) at 1:200 or isotype IgG overnight at 4°C, washed with phosphate-buffered saline (PBS) three times, incubated with peroxidase-labeled secondary antibody for 2 h at room temperature and again

washed with PBS three times. Positive staining was revealed by the 3,3'-diaminobenzidine system. Foxp3⁺ cells were regarded as Tregs in the intestinal tissues. Sections were observed under a light microscope. Positive stained cells were counted in 20 randomly selected fields from each sample; the average of 20 counts was used as one datum. All sections were coded. The observer was not aware of the code to avoid observer bias.

Administration of mExosomes in FA mice

FA mice were treated with mExosomes (0.1 mg per mouse in saline, intraperitoneally) or control exosomes or saline alone daily from day 18 to day 25. A group of FA mice was treated with anti-CD25 antibody (0.25 mg per mouse in 0.1 mL saline, intraperitoneally) on day 15 to deplete Tregs (control mice received isotype IgG at the same dosage; Supplementary figure 1b).

Isolation of lamina propria mononuclear cells

Jejunal segments were excised from mice. The tissues were cut into small pieces ($2 \times 2 \times 2$ mm³) and incubated with collagenase IV at 1 mg mL⁻¹ at 37°C for 30 min with mild

agitation. Single cells were passed through a cell strainer (70 μm first, then 40 μm) and centrifuged at 1000g for 5 min. Lamina propria mononuclear cells were purified from single cells with Percoll gradient-density centrifugation. The cell viability was greater than 98% as assessed by Trypan blue exclusion assay.

Cell culture

Cells were cultured in Roswell Park Memorial Institute-1640 medium that was supplemented with antibiotics (penicillin, 100 U mL^{-1} ; streptomycin, 0.1 mg mL^{-1}) and L-glutamine (2 mM). Medium was changed in 2–3 days. The cell viability was greater than 98% as assessed by Trypan blue exclusion assay.

ELISA

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

Preparation of protein extracts

Intestinal tissues were cut into small pieces and homogenized with a homogenizer containing protein extracting buffer. Cells were collected from relevant experiments and lysed with a lysis buffer {10 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], 1.5 mM MgCl_2 , 10 mM KCl, 0.5 mM dithiothreitol, 1 mM ethylenediaminetetraacetic acid and 0.05% NP40}. Lysates were centrifuged at 13 000g for 10 min. Supernatant was collected and used as the cytosolic proteins. Pellets were resuspended and incubated in a nuclear lysis buffer {5 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], 1.5 mM MgCl_2SO_4 , 4.6 M NaCl, 0.2 mM ethylenediaminetetraacetic acid, 0.5 mM dithiothreitol and 26% glycerol} for 30 min. Lysates were centrifuged at 13 000g for 10 min. Supernatant was collected and used as the nuclear proteins. The procedures were performed at 4°C.

Western blotting

Proteins were fractioned by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane. The membrane was treated with the western blotting procedures, including blocking with 5% skim milk in PBS for 30 min, incubated with primary antibodies of interest (1:500; see figures for antibody types) at 4°C overnight, washed with Tris-buffered saline containing 0.1% Triton X-100 three times, incubated with peroxidase-labeled secondary antibodies for 2 h at room temperature and washed with Tris-buffered saline containing 0.1% Triton X-100 three times. Immunoblots on the membrane were developed by the enhanced chemiluminescence method and photographed in an imaging device (UVP System, Cambridge, UK).

Immunoprecipitation

Proteins were precleared by incubating with protein G agarose beads for 2 h. The beads were removed by centrifugation at 5000g for 10 min. Supernatant was collected and incubated

with antibodies of interest (refer to the figures for detail) or isotype IgG overnight to form immune complexes. The immune complexes were precipitated by incubating the samples with protein G agarose beads for 2 h. The beads were collected by centrifugation at 5000g for 10 min. Proteins on the beads were eluted with an eluting buffer and analyzed by western blotting. All procedures were carried out at 4°C.

Flow cytometry

On the surface staining, cells were stained with antibodies of interest (see figures in detail; labeled with fluorescence) for 30 min on ice. The cells were washed with PBS three times and analyzed with a flow cytometer (FACSCanto II). In the intracellular staining, cells were fixed with 1% paraformaldehyde (containing 0.1% Triton X-100) for 1 h, washed with PBS three times and processed with the surface staining procedures. The results were analyzed by FlowJo (v7.6; Tree Star Inc., Ashland, OR, USA). The isotype IgG staining data were used as gating references.

Generation of bone marrow-derived dendritic cells

The bone marrows were flushed out from the femurs of naïve BALB/c mice and treated with the red blood cell lysis buffer to remove red blood cells. Bone marrow cells were cultured with complete Roswell Park Memorial Institute-1640 medium supplemented with granulocyte–macrophage colony-stimulating factor (25 ng mL^{-1}). On day 9, cells were harvested, and the CD11c^+ cells were purified by flow cytometry cell sorting. Cell purity was greater than 98% as assessed by flow cytometry.

Exosome purification

Bone marrow-derived dendritic cells were cultured in the presence of lipopolysaccharide (100 ng mL^{-1}) and IL-2 (50 $\mu\text{g mL}^{-1}$) overnight. Culture supernatant was collected and subjected to established procedures¹⁶ for exosome purification. In brief, the supernatants were collected and centrifuged at 300g (10 min), 1200g (20 min) and 10 000g (30 min), respectively, to remove cell debris. Exosomes were pelleted at 100 000g for 1 h and resuspended in PBS for further experiments. The protein in exosomes was quantified using a Bradford assay.

Assessment of mExosomes binding Ag-specific CD4^+ T cells

CD4^+ T cells were isolated from the DO11.10 mouse spleen by flow cytometry cell sorting. The purity of isolated cells was more than 98% as assessed by flow cytometry. The cells were incubated with mExosomes (labeled with carboxyfluorescein succinimidyl ester) for 30 min and assessed by flow cytometry.

Depletion of Tregs in mice

Three days prior to the administration of mExosomes, mice were peritoneally injected with anti-CD25 antibody

(0.25 mg per mouse). One week later, sample mice were killed; lamina propria mononuclear cells were prepared and analyzed by flow cytometry. No CD25⁺ T cells remained in the intestine (data not shown).

Statistics

The data are presented as mean $\pm$ s.e.m. The difference between two groups was determined by the Student's *t*-test. Multiple comparisons were performed with ANOVA and Dunnett's test or a Bonferroni test ($P < 0.05$). The correlation between the two groups was determined by Pearson's correlation assay.

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AUTHOR CONTRIBUTIONS

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CONFLICT OF INTEREST

None to declare.

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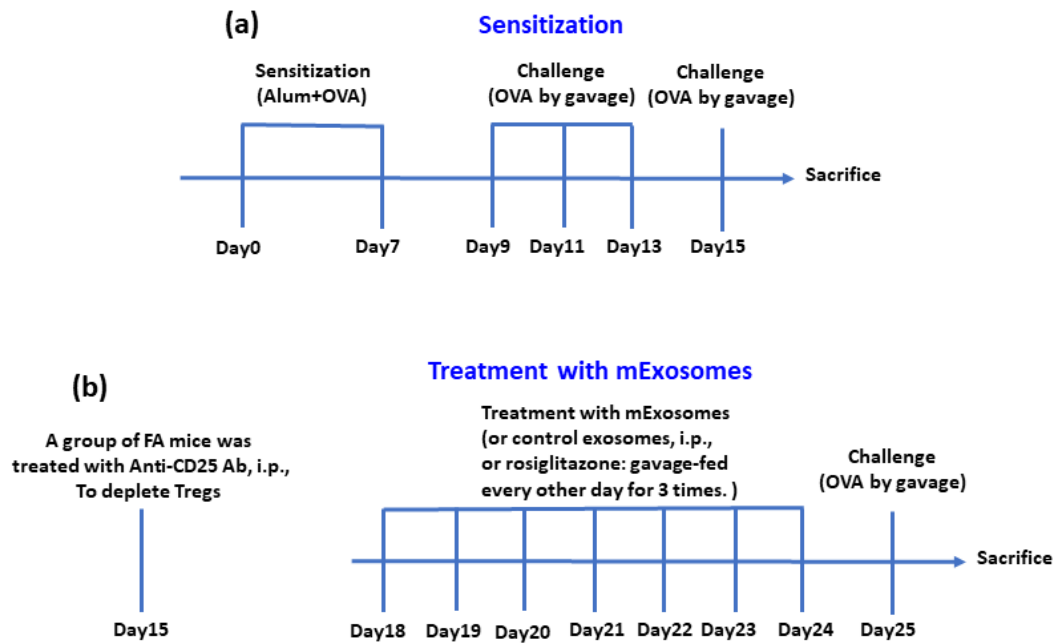
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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Supplemental figure 1

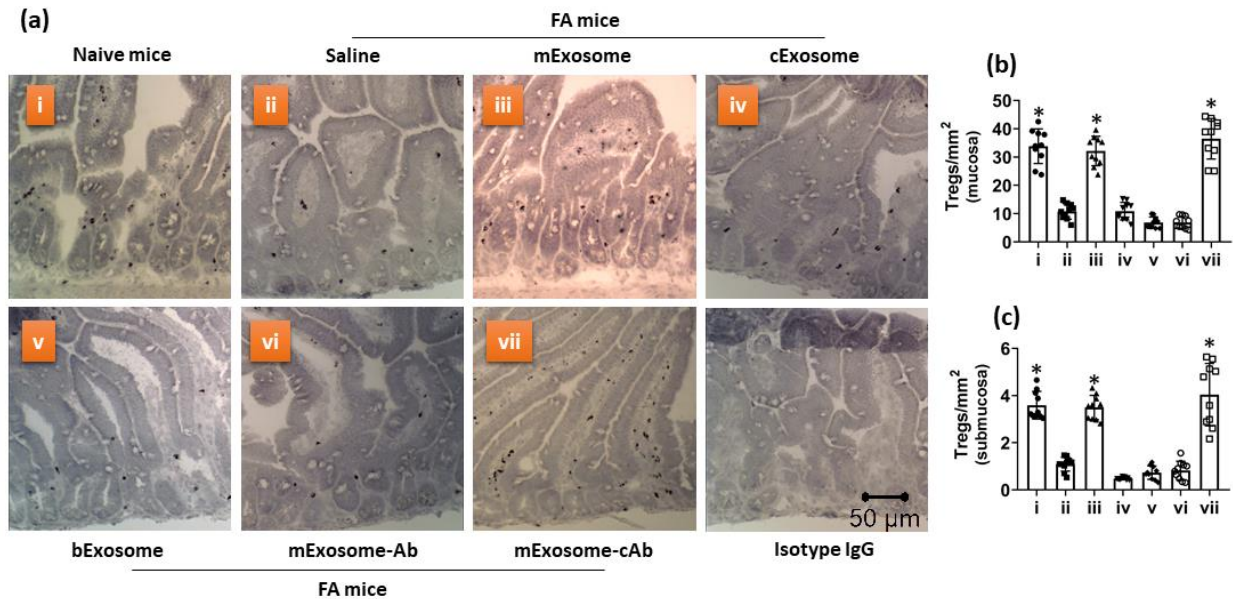


A schematic diagram of FA mouse model development and treatment with mExosomes.

(a) Mice were subcutaneously injected with ovalbumin (OVA; 0.1 mg/mouse; mixed in 0.1 ml alum) on the back skin on day 0 and day 7, respectively. The mice were challenged with OVA (1 mg/mouse in 0.1 ml saline) through gavage-feeding on day 9, 11 and 13, respectively. On day 15, mice were challenged by gavage-feeding with OVA (5 mg/mouse in 0.1 ml saline), and sacrificed to assess the FA response.

(b) FA mice were treated with mExosomes (0.1 mg/mouse; mixed in 0.1 ml saline; or control exosomes or saline) daily from day 18 to day 24. The mice were challenged with OVA on day 25 and assessed the FA response. On the other hand, a group of FA was i.p. injected with anti-CD25 Ab (0.25 mg/mouse) to deplete Tregs; or rosiglitazone (a PPAR γ agonist; 10 mg/kg, in 0.1 ml saline) gavage-fed every other day for 3 times to increase PPAR γ .

Supplemental figure 2



mExosomes expand Tregs in the mouse FA intestinal tissues. Intestinal segments were excised from each mouse described in Figure 6. Cryosections were prepared from each sample and processed for immunohistochemistry. (a) Representative images show Foxp3⁺ Tregs (in dark brown) were counted under a light microscope in 20 fields randomly selected from each sample; the average of 20 counts was used as one datum. (b) Bars show means $\pm$ SEM of Tregs counts in intestinal mucosa of 10 mice per group. (c) Bars show means $\pm$ SEM of Tregs counts in intestinal submucosa of 10 mice per group. * $p < 0.01$, compared with group ii (ANOVA+Dunnett's test). Each sample was analyzed in triplicate. Each dot in bars presents data obtained from one sample.