



Integrin $\alpha\beta6$ cooperates with resiquimod to restore antigen-specific immune tolerance in airway allergy

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ABSTRACT

Background: Integrin $\alpha\beta6$ can convert the transforming growth factor (TGF)- β precursor to the mature form. Resiquimod (R848) can generate TGF- β -producing regulatory T cells (Treg). Thus, to concurrent administration of specific antigen and R848 may generate antigen-specific Tregs, that is expected to restore immune tolerance in subjects with airway allergic diseases (AAD).

Methods: A bio-nanoparticle, designated Rexo, containing an antigen/MHC II complex and R848, was naturally assembled in dendritic cells, that was released as an exosome. An AAD mouse model was developed used to test the effects of Rexo on restoring the immune tolerance in the airways.

Results: Exposure to R848 failed to induce Tregs in the $\beta6$ -deficient mouse airway tissues, that were successfully induced in wild type mice. The results were validated *in vitro* experiments. R848 activated the TLR7/MyD88/p38 signal pathway to increase the $\alpha\beta6$ levels in CD4⁺ T cells, the $\alpha\beta6$ then converted the TGF- β precursor to its mature form, and thus, induced Treg generation. Administration of Rexo restored the antigen-specific immune tolerance in the airways manifesting efficiently suppressing experimental AAD by inducing antigen-specific Tregs in the airways and inhibiting antigen-specific Th2 response.

Conclusions: Rexos can inhibit experimental AAD via inducing antigen-specific Tregs to restore immune tolerance in the airway tissues, suggesting that Rexos have the translational potential to be used in the treatment of AAD.

1. Introduction

Airway allergic diseases (AAD) are the adverse responses to airborne antigens (Ag) by the dysfunctional airway immune system, including allergic rhinitis and allergic asthma. The clinical symptoms of AAD include, in allergic rhinitis, sneezing, profound nasal secretions and nasal congestion. Some allergic rhinitis may complicate nasal polyposis or/and chronic rhinosinusitis [1]. In allergic asthma, patients have breathing difficulty, cough, profound airway secretions. If medical intervention is not carried out properly, complications may occur, such as chronic obstructive pulmonary diseases and pulmonary cardiovascular diseases [2]. The therapeutic efficacy of AAD needs to be improved [3]. In fact, AAD has been a large negative impact on human health and

social economy [4]. It is necessary to further investigate the pathogenesis of AAD and invent novel, safe and effective therapies for AAD.

Th2 polarization plays a critical role in the pathogenesis of AAD [5]. Th2 polarization is a condition that the local tissues are over populated by Th2 cells and the tissues were over saturated with Th2 cytokines, including interleukin (IL)-4, IL-5 and IL-13. The Th2 cytokines, mainly IL-4, induce the IgE production [6]. IgE sensitizes mast cells by binding the high affinity IgE receptors. Re-exposure to specific Ags activates the sensitized mast cells, the mast cells then release allergic mediators, e.g., histamine, leukotrienes and serotonin, to induce AAD attacks [7]. Yet, remedies used to regulate the Th2 polarization are currently limited.

In general, the immune response in the body is tightly regulated by the immune regulatory system [8]. The immune regulatory system

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includes immune regulatory cells (e.g., regulatory T cells, Treg; regulatory B cells, Breg) and immune regulatory mediators (e.g., transforming growth factor (TGF)- β and IL-10). Upon activation, immune regulatory cells release immune regulatory mediators to suppress other immune cell activities to restrict the immune responses within given extent to avoid the unnecessary tissue injuries [8]. Organs with fully functional immune regulatory system are well tolerant to some foreign Ags [9]. In AAD, the established immune tolerance is disrupted [10]. The Th2 polarization in the airway tissues indicates that the immune regulatory system in the airways is dysfunctional. However, factors and mechanism of immune regulatory dysfunction induction are not fully understood yet. More effective remedies are needed using to restore the dysfunctional immune regulatory system.

Previous studies show that R848 (Resiquimod), one of the Toll-like receptor (TLR)7 agonists, can induce Tregs and up regulate Th1 response in the tissues [11,12], which may counteract the Th2 polarization in AAD. Tregs have to be activated to fulfill the immune regulatory function; how the R848-induced Tregs are activated in the local tissues remains to be elucidated. Our previous work showed that one of the integrins, $\alpha\beta6$, played an important role in the generation of Ag-specific Tregs [13]. Thus, we hypothesize that R848 may up regulate the $\alpha\beta6$ expression in CD4⁺ T cells to facilitate the Ag-specific Treg generation; the latter can be activated by specific Ag and consequently, suppress the Ag-specific Th2 polarization and AAD. To test the hypothesis, we generated a bio-nanoparticle that containing R848, specific Ag and MHC II, which was designated Rexo. Administration of Rexo induced Ag-specific Tregs in the airways and efficiently suppressed experimental AAD response.

2. Materials and methods

2.1. Reagents

R848 (Resiquimod; water soluble) was purchased from InvivoGen (San Diego, CA). Antibodies of CD11c (Alexa Fluor® 488), CD4 (Alexa Fluor® 488), CD3 (Alexa Fluor® 546), CD25 (Alexa Fluor® 647), Foxp3 (Alexa Fluor® 546), TGF- β (Alexa Fluor® 594), CD154 (Alexa Fluor® 647), CD9, CD63, CD81, $\beta6$, MyD88, MAPK, TGF- β , LAP, OVA and MHC II were purchased from Santa Cruz Biotech (Santa Cruz, CA). Anti-CD25 mAb (PC61) and TLR7 Ab were purchased from abcam (Cambridge, MA). Phospho-MyD88 Ab, phosphor-MAPK Ab, ELISA kits of MBP, OVA-specific IgE, IL-4, IL-5 and IL-13 was purchased from Dakewe BioMart (Beijing, China). Exosome purification kit was purchased from Miltenyi Biotech (San Diego, CA). Immunoprecipitation kit, ovalbumin (OVA), Alum, IL-4, Granulocyte-macrophage colony stimulating factor (GM-CSF), bovine serum albumin (BSA), collagenase IV, SB202190 (MAPK p38 inhibitor) and Limulus Amebocyte Lysate kit were purchased from Sigma Aldrich (St. Louis, MO). T5910047 (an inhibitor of MyD88) was purchased from Dongcang Biotech (Shanghai, China). Reagents and materials for RT-qPCR and Western blotting were purchased from Invitrogen (Carlsbad, CA). LPS contamination was tested for each reagent, that was below 1 pg/mL (tested with an LPS detecting kit following the manufacturer's protocol).

2.2. Mice

BALB/c mice were purchased from the Guangdong Experimental Animal Center (Guangzhou, China). $\beta6$ -deficient ($\beta6d$) mice, DO11.10 mice and the littermates were purchased from Jackson Laboratories (Bar Harbor, ME). The mice were maintained in a specific pathogen free facility at Shenzhen University and allowed to access food and water freely. The experimental procedures were approved by the Animal Ethical Committee at Shenzhen University (Approve#: SZUAE20180012).

2.3. Cell culture

Cells (10^6 cells/mL) were cultured in complete RPMI1640 medium containing 10 % fetal calf serum, 2 mM glutamine, 0.1 mg/mL streptomycin and 100 U/mL penicillin. The medium was changed in 2–3 days. The cell viability was more than 99 % as assessed by Trypan blue exclusion assay.

2.4. Generation of bone marrow-derived dendritic cells (BMDC)

The femur bones were excised from naive BALB/c mice. The BMs were flushed out the bones with saline. Red blood cells were lysed with a red blood cell lysis buffer. BM cells were cultured (10^6 cells/mL) in complete RPMI1640 medium supplemented with IL-4 (20 ng/mL) and GM-CSF (20 ng/mL). On day 9, cells were harvested; CD11c⁺ DCs were purified by flow cytometry cell sorting (FCS) (Supplementary Fig. S1). The purity of isolated cells was 98 % as assessed by flow cytometry.

2.5. Flow cytometry

Cells were collected from relevant experiments. On the surface staining, cells were stained with fluorescence labeled antibodies (indicated in figure legends; diluted to 1:100) or isotype IgG for 30 min at 4 °C. After washing with phosphate buffered saline (PBS) 3 times, 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 enhance the cell membrane permeability) for 1 h. After washing with PBS 3 times, cells were processed with the procedures of surface staining. The data were processed with software package Flowjo (Tree Star Inc., Ashland, OR). Data obtained from isotype IgG staining were used as the gating references. In the analysis, plots at the low-left corner were gated out as that usually were dead cells and cell debris.

2.6. Real-time quantitative RT-PCR (RT-qPCR)

Total RNA was extracted from cells collected from relevant experiments and converted to cDNA with a reverse transcription kit following the manufacturer's instructions. The samples were amplified in a qPCR device (CFX96 Touch Real-Time PCR Detection System) with SYBR Green Master Mix in the presence of relevant primers of $\beta6$ (aaaaccctgtctccgcata and tgatggtgttgaggctgca), TGF- β (tcgctgtgtacacagcacc and actgcttcccgaatgtctga) and TLR7 (atatcccagaggcccatgtg and acacacattggcttggacc). The results were processed with the $2^{-\Delta\Delta Ct}$ method and presented as relative quantification (RQ).

2.7. Protein extracts

Cells were lysed with a lysis buffer (protein extracting buffer (1.5 mM MgCl₂; 10 mM HEPES; 10 mM KCl; 1 mM EDTA; 0.5 mM DTT; 0.05 % NP40) and centrifuged at 13,000 g for 10 min. The supernatant was collected to be the cytosolic protein extracts. The pellets were resuspended in a nuclear lysis buffer (5 mM HEPES; 1.5 mM MgCl₂SO₄; 4.6 M NaCl; 0.5 mM DTT; 0.2 mM EDTA; 26 % glycerol) for 30 min and centrifuged at 13,000 g for 10 min. The supernatant was collected to be the nuclear protein extracts. All the procedures were carried out at 4 °C.

2.8. Western blotting

Proteins were fractioned by SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) and transferred onto a PVDF membrane. After incubating with 5% skim milk for 30 min to block the non-specific binding, the membrane was incubated with primary antibodies (see figures in detail; diluted to 1:500) overnight at 4 °C, washed

with TBST (Tris-buffered saline containing 0.05 % triton X-100) 3 times, incubated with secondary antibodies (labeled with peroxidase; diluted to 1:5000) for 2 h at ambient temperature, washed with TBST 3 times. Immunoblots on the membrane were developed with the enhanced chemiluminescence and photographed in an imaging device. Integrated density of immunoblots was calculated with software package ImageJ.

2.9. Immunoprecipitation (IP)

Proteins were precleared by incubating with protein G sepharose for 2 h. Samples were centrifuged at 5000 g for 10 min. Supernatant was collected and incubated with antibodies of interest (see figure for detail) or isotype IgG overnight to form immune complexes. The immune complexes were precipitated by incubating with protein G sepharose for 2 h and centrifuged for 10 min at 5000 g. The sepharose beads were collected and washed with PBS for 3 times. Proteins on the beads were eluted with an elution buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) and analyzed by Western blotting.

2.10. Development of an AAD mouse model

Wild type and $\beta 6$ -deficient ($\beta 6d$) mice were immunized with subcutaneously injecting ovalbumin (OVA, a specific Ag; 0.1 mg/mouse) in 0.1 mL alum (50 μ g/100 μ L aluminum hydroxide; Thermo Scientific) in the back skin on day 0 and day 3, respectively. Mice were challenged with OVA (5 mg/mL)-containing nasal instillation (50 μ L per nostril) daily from day 8 to day 14. On day 15, mice were challenged with OVA (10 mg/mL)-containing nasal instillation (50 μ L per nostril) and the AAD responses were assessed.

2.11. Inflammatory scores of lung histology

Referring to published procedures [14], the inflammation in the lung tissues was scored with a 5-point (0–4) scoring system, that 0, no inflammation; 1, occasional inflammatory cells in the lung tissues; 2, apparent accumulations of inflammatory cells around the bronchioles or/and blood vessels; 3, multifocal and even greater inflammation around bronchioles or/and blood vessels than that observed in grade 2; 4, severe multifocal inflammatory cell infiltration.

2.12. Airway resistance measurements

On day 15, mouse airway resistance was measured using a Buxco Elan RC mouse volume-controlled ventilator (Harvard Bioscience Inc. St. Paul, MN). Mice were under general anesthetization by i.p. injection of ketamine (25–40 mg/kg) and xylazine (5.6–10 mg/kg). Tracheostomy was performed; a 20 G flexible catheter was fixed in the trachea. The mice were then given gradient concentrations of methacholine with an in-line nebulizer. Airway resistance was measured over 3-min intervals after each dose.

2.13. Collection of bronchoalveolar lavage fluids (BALF)

Mice were under general anesthetization. BALF was collected (0.8 mL saline, two times) by tracheal intubation.

2.14. Lung histology

Mice were sacrificed by cervical dislocation. The lung tissues were excised upon the sacrifice and fixed in 4% formalin overnight. Paraffin sections were prepared and stained with eosin and hematoxylin. Sections were observed with a light microscope.

2.15. Enzyme-linked immunosorbent assay (ELISA)

Cytokines and IgE in the serum or BALF were measured by ELISA

with commercial reagent kits following the manufacturer's instruction.

2.16. Assessment of Treg's immune suppressive function

By FCS, $CD4^+ CD25^-$ T cells (Teff) and DC were isolated from the DO11.10 mouse spleen; $CD4^+ CD25^+ CD127^-$ Tregs were isolated from the lung tissues of mice treated with Rexo or control exosomes. Cell purity was greater than 95 % as assessed by FCS. Teffs were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and cultured with Tregs and DCs (Teff:Treg:DC = 10:2:1) in the presence or absence of OVA (10 μ g/mL) for 3 days. The Teff proliferation were analyzed with FCS, the CFSE-dilution assay.

2.17. Depletion of tregs in mice

Three days before the Rexo therapy, mice were peritoneally injected with anti-CD25 Ab (0.25 mg/mouse) to deplete Tregs.

2.18. Generation of rexos

BMDCs were cultured in the presence of R848 (0.5 mg/mL) and OVA (0.5 mg/mL) overnight. Supernatant was collected and processed for exosome purification with combining the established ultracentrifugation and the magnetic antibody purification [13,15]. 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. Exosomes were pelleted at 100,000 g for 1 h and resuspended in PBS. Exosomes were then further processed by magnetic antibody purification following published procedures [16]. The protein in exosomes was quantified using a Bradford assay. Exosome markers, CD9, CD63 and CD81, were detected in the exosome extracts by Western blotting. A complex of the OVA and MHC II was detected in the exosomes. The exosomes also contained R848, that was detected by the high-efficient liquid chromatograph (HPLC). These exosomes were designated Rexo. Control exosomes were also prepared, including OVA-exo (exosomes carrying OVA alone) and R848-exo (exosomes carrying R848 alone).

2.19. Determination of R848 concentration in exosomes by HPLC analysis

R848-containing exosomes were prepared as described above and sent to the HPLC Facility at Shenzhen University. The HPLC analysis was carried out by professional staff in the facility. Briefly, exosomes were resuspended in cold trichloroacetic acid (5%), sonicated (Branson Sonifier 250; Branson Ultrasonics, Danbury, CT, USA) 3 times (30 s each, on ice), followed by centrifuging at 15,000 g for 20 min. Samples were then analyzed by HPLC chromatography. R848 was quantified against the standard curve obtained by commercial R848 in a series of double dilution.

2.20. Preparation of lung mononuclear cells (LMC)

The lungs were excised upon the sacrifice, cut into small pieces and cultured in the presence of collagenase IV (1 mg/mL) at 37 °C with mild agitation. Single cells were filtered through a cell strainer (70 μ m first, then 40 μ m) and collected by centrifugation (1000 g, 10 min). LMCs were further isolated by the Percoll gradient density centrifugation.

2.21. Immune cell isolation

Single cells were prepared from the mouse spleen or lung tissues by enzyme-digestion approach, labeled with fluorescence-labeled antibodies and purified by flow cytometry (FCS). Cell purity of isolated cells was assessed by FCS. If purity did not reach 95 %, cells were reprocessed with FCS (Supplementary Fig. S2).

2.22. Generation of Treg in the airway tissues by R848

Mice were treated with nasal instillation (50 μ L/nostril) containing R848 (0.1 mg/mL saline; control mice were treated with control exosomes) daily for one week. Control mice were received saline alone. The mice were then sacrificed one day after the last injection. The lungs were excised. Lung mononuclear cells (LMCs) were prepared with the lung tissues and analyzed by FCS.

2.23. β 6 reconstitution

β 6-expressing plasmids were constructed and provided by Shanghai Sangon Biotech (Shanghai, China). Briefly, following the manufacturer's instruction, the β 6-expressing plasmids or control plasmids (empty plasmids) were transfected to β 6-deficient (β 6d)-CD4⁺ T cells with the aid of electroporation using a Bio-Rad Gene Pulse set at 950 μ F and 280 V. The transfer effects were assessed by Western blotting 48 h after the transfection.

2.24. Rexo therapy

One day after completing the sensitization, mice were treated with nasal instillation (50 μ L/nostril; 50 mg protein/mL) containing Rexos or control exosomes (R848-carrying exosomes or OVA-carrying exosomes) daily for 7 consecutive days. To deplete Tregs, mice were peritoneally injected with anti-CD25 Ab (0.25 mg/mouse) once 3 days prior to the beginning of the Rexo therapy.

2.25. Statistics

Each experiment was repeated at least 3 times. Each experimental group consists of 10 mice. Each sample was tested in triplicate with the

average of 3 readouts as one datum. The data are presented as mean $\pm$ SEM. The difference between two groups was determined by the Student *t*-test. Multiple comparisons were performed with ANOVA followed by the Bonferroni test or the Dunnett's test. *P* < 0.05 was considered statistical significance.

3. Results

3.1. Mice with α v β 6-deficient (α v β 6-d) fail to induce Tregs by R848

We and others reported previously that integrin α v β 6 played a critical role in the Treg induction [13,17]. Treating CD4⁺ T cells with R848, a TLR7 agonist, can induce Treg development [11,18]. After making sure that mouse CD4⁺ T cells expressed TLR7 (Fig. S3), we treated mice with R848 to observe the role of α v β 6 in the Treg generation by exposing to R848. α v β 6-deficient (β 6d) mice and wild type (WT) mice were treated with R848 through nasal instillation daily for one week. We found that administration of R848 induced Tregs in the airway tissues of WT mice, but not in β 6d mice (Fig. 1A–B). Then, we expanded the findings of animal model study with a cell culture experiment. We isolated CD4⁺ T cells from the naive mouse spleen. The CD4⁺ T cells were treated with an established Treg-generation approach [11,18] by culturing CD4⁺ T cells in the presence or absence of R848 for 3 days. We found that exposure to R848 significantly increased the β 6 expression in CD4⁺ T cells in an R848 concentration-dependent manner (Fig. 1C–E). The results indicate that α v β 6 is required in the Treg induction by R848.

3.2. The TLR7/MyD88/MAPK signal transduction pathway is involved in the R848-induced β 6 expression in CD4⁺ T cells

Published data indicate that MAPK phosphorylation is associated with the activities of TLR7 [19]; R848 is a TLR7 agonist. We then

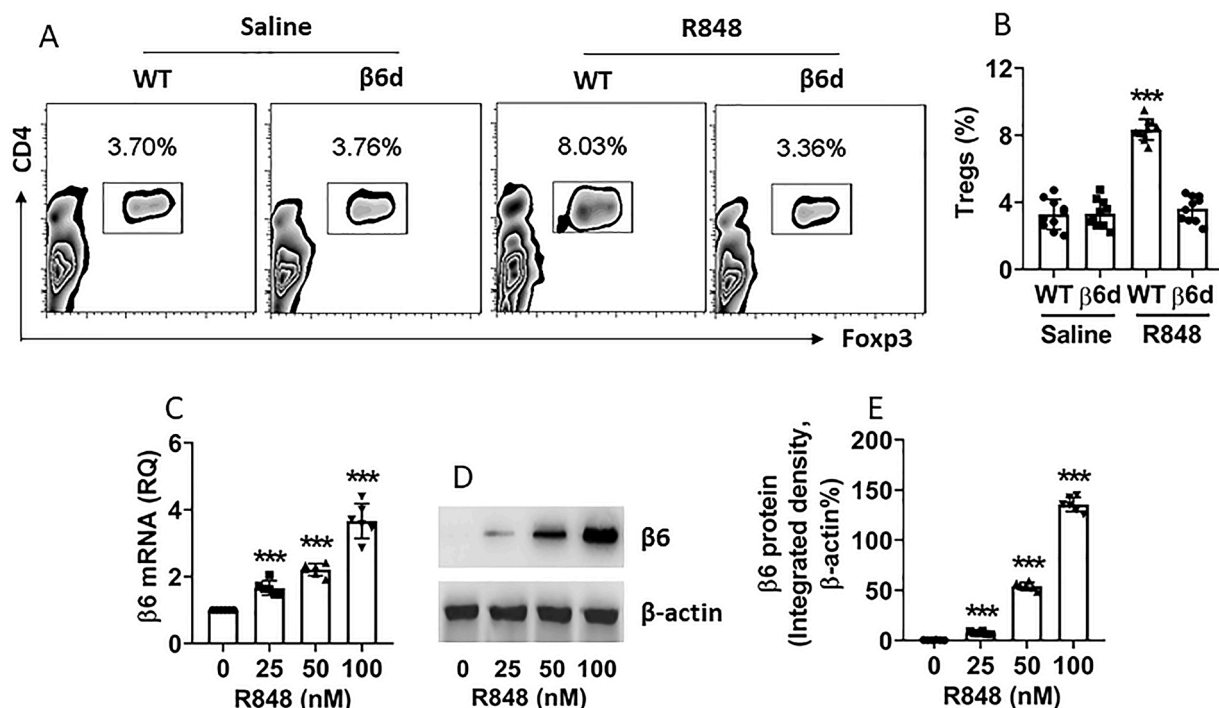


Fig. 1. α v β 6 is required in R848-inducing Tregs in the airway tissues. A–B, wild type (WT) mice and β 6-deficient (β 6d) mice were treated with saline or R848-containing (0.1 mg/mL) nasal instillation (50 μ L/nostril) daily for 7 days. Mice were sacrificed on day 8. The lungs were excised. Lung mononuclear cells (LMC) were isolated and analyzed by FCS. A, gated FCS (FCS) plots show Treg frequency in LMCs (The CD4⁺ Foxp3⁺ Tregs also CD25⁺; data not shown). B, bars show mean $\pm$ SEM of Treg counts in LMCs from 10 mice per group. C–E, CD4⁺ CD25⁺ T cells were isolated from the naive mouse spleen and exposed to R848 in the culture at indicated concentrations on the X axis for 3 days. The cells were analyzed by RT-qPCR and Western blotting. C, bars show mean $\pm$ SEM of β 6 mRNA levels in CD4⁺ T cells. D, immunoblots show β 6 protein levels in CD4⁺ T cells. E, bars show mean $\pm$ SEM of the integrated density of β 6 immunoblots in panel D. Each group consists of 6 mice. ****p* < 0.001 (ANOVA followed by the Dunnett's test), compared with the WT/saline group (B) or group 0 (C, E).

assessed the phosphorylation of MAPK and its upstream factor MyD88 of TLR7 in CD4⁺ T cells with or without exposing to R848 in the culture. The results showed that exposure to R848 significantly increased the phosphorylation of MyD88, p38, ERK1/2 and I κ B α as well as increase in TRAF3 and TRAF6 in CD4⁺ T cells (Fig. 2A–L). Inhibition of either p38 or MyD88 abolished the R848-induced β 6 expression in CD4⁺ T cells (Fig. 2M–O). The results demonstrate that MyD88 and p38 are involved in the R848-induced β 6 expression in CD4⁺ T cells.

3.3. α v β 6 mediates the effects of R848 on increasing TGF- β in CD4⁺ T cells by transferring latent TGF- β (LTGF β) to its mature form

We found that, after exposure to R848, the TGF- β mRNA levels were

increased in both β 6d (Fig. S4) and WT CD4⁺ T cells (Fig. 3A), but the mature form of TGF- β protein was only detected in WT CD4⁺ T cells, not detected in β 6d CD4⁺ T cells (Fig. 3B), in which the LTGF β was detected instead (Fig. 3C). Reconstitution of β 6 (Fig. 3D) recovered the induction of active TGF- β in β 6d CD4⁺ T cells by R848 (Fig. 3E). The R848-induced TGF- β was also detected in culture supernatant (Fig. 3F). The results demonstrate that R848 increases TGF- β expression in CD4⁺ T cells requires α v β 6 to convert LTGF β to its mature form. The induced TGF- β can be released into the microenvironment.

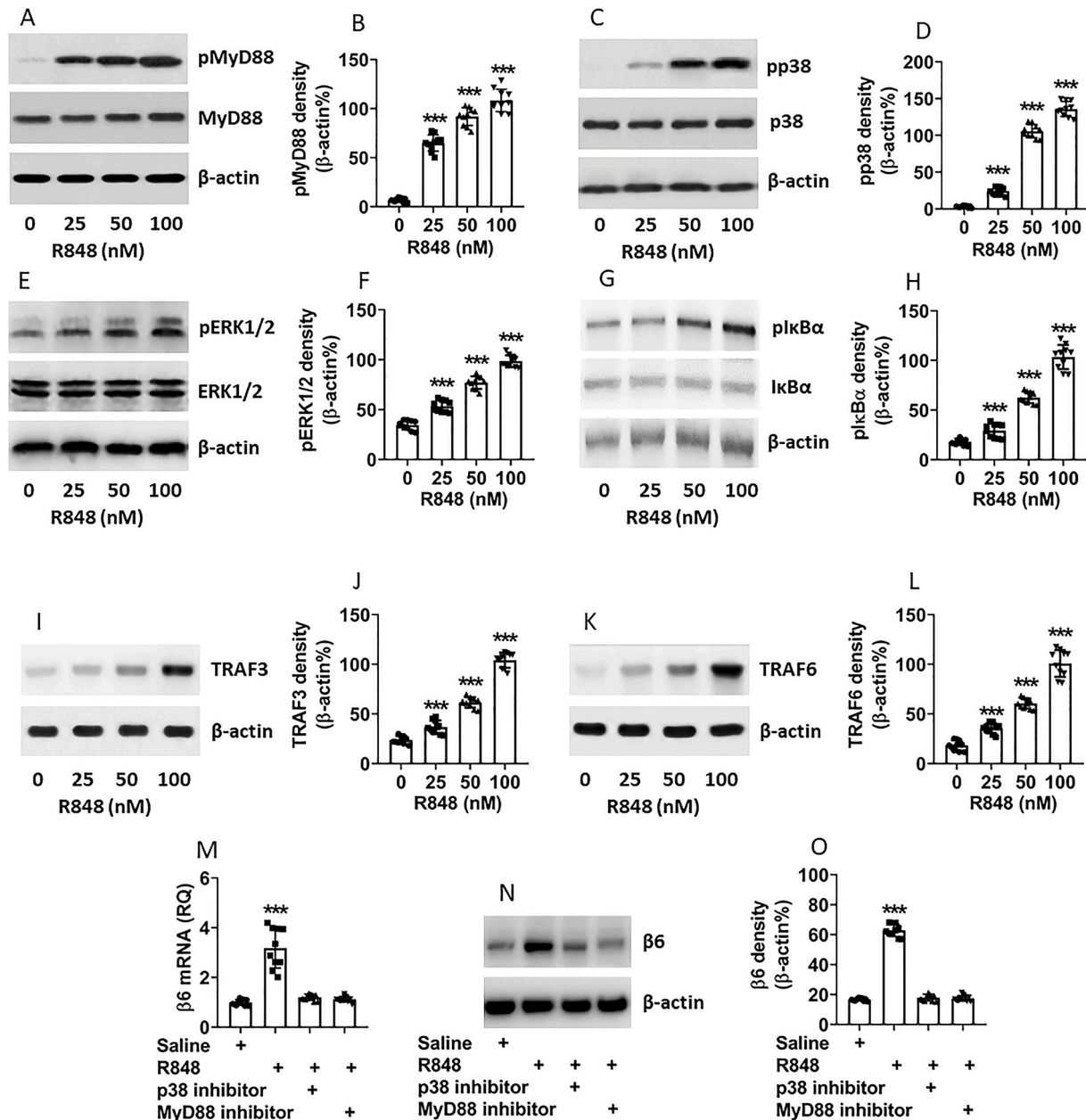


Fig. 2. MyD88/MAPK p38 pathway is involved in R848-induced β 6 expression in CD4⁺ T cells. A–L, naive CD4⁺ T cells were exposed to R848 in the culture at indicated concentrations on the X axis overnight. Immunoblots show phosphorylated MyD88 (pMyD88) (A), pp38 (C), pERK1/2 (E), pI κ B α (G), TRAF3 (I) and TRAF6 (K) in CD4⁺ T cell protein extracts. Bars indicate the integrated density of immunoblots (B, D, F, H, J). M–O, naive CD4⁺ T cells were treated with the procedures denoted below the X axis of panel M for 48 h. M, bars show β 6 mRNA levels in CD4⁺ T cells. N, immunoblots show β 6 protein levels in CD4⁺ T cells. O, bars show the integrated density of β 6 immunoblots in panel N. The data of bars are presented as mean $\pm$ SEM. ***, $p < 0.001$ (ANOVA followed by the Dunnett's test), compared with group 0 (B, D, F, H, J) or the saline group (M, O). MyD88 inhibitor, T5910047 (10 μ M); p38 inhibitor, SB202190 (1 μ M).

3.4. Preparation and characterization of R848/Ag-carrying exosomes (Rexo)

By exposing dendritic cells to OVA and R848 in the culture overnight, bio-nanoparticles, the Rexos, were generated. Rexos carried OVA (the specific Ag)/MHC II complexes and R848 (Fig. 4A-C). The Rexos could specifically bind to Ag-specific CD4⁺ T cells (Fig. 4D-E); it indicates that the complexes of OVA peptides and MHC II on the surface of the exosomes. We also found that Rexo, but not the BSA-carrying exosomes, could induce TGF- β expression in Ag-specific CD4⁺ T cells (Fig. 4F-G). The results indicate that Rexo can convert Ag-specific CD4⁺ T cells to Tregs. In addition, the IL-10 expression was not apparently altered in the CD4⁺ Tregs (Fig. S5).

3.5. Administration of Rexo attenuates experimental AAD

Based on the data reported in Figs. 1–4, we inferred that this Rexo might inhibit AAD by generating Ag-specific Tregs in the airway tissues. Thus, an AAD mouse model was developed with OVA as the specific Ag (Fig. S6). After completing the sensitization, mice were treated with Rexos or control exosomes daily for 7 days. We found that AAD mice showed AAD-like response, including inflammation in the lung tissues (Fig. 5A-B), airway hyper responsiveness (Fig. 5C), increase in serum specific IgE (Fig. 5D), high levels of inflammatory cytokines (MBP and Th2 cytokines) were detected in bronchoalveolar lavage fluids (BALF) (Fig. 5E-H), and lower IFN- γ levels in BALF (Fig. 5I). The AAD-like response was significantly attenuated in mice received the Rexo-therapy (The dosage of Rexo-therapy was tested in a preliminary experiment; Fig. S7), which did not occur in β 6d mice. Removal of either R848 or OVA from Rexos abolished the suppressive effects. The Rexo-

therapy slightly increased the IFN- γ levels in BALF, but not reach the significant criterion (Fig. 5). In addition, we treated AAD mice with exosomes alone (no R848, no OVA), that did not alter the AAD response in mice (data not shown).

3.6. Administration of Rexo suppresses Ag-specific Th2 cells by activating Ag-specific Tregs

Furthermore, we observed that administration of Rexos markedly increased the Treg frequency in the lung. R848-carrying exosomes (no OVA) also increased Treg numbers in the lung while OVA-carrying exosomes (no R848) did not increase Treg numbers in the lungs of AAD mice (Fig. 6A-C). Exposure to Rexos *in vivo* activated Tregs in the AAD mouse lungs as the CD154 (an activation marker of T cells [20]) expression was significantly up regulated in Tregs. Although exposure to R848-carrying exosomes also increased Treg number in the lung, however, the Tregs were seldomly activated, nor in those of AAD mice received OVA-carrying exosomes (Fig. 6D-F). To test if Rexos alter Treg functions, Tregs were isolated from the lungs of mice received Rexos or from mice received R848-carrying exosomes, and analyzed by the CFSE-dilution assay. We observed that Rexo-induced Tregs significantly suppressed effector T cell proliferation while no apparent immune suppressive effects were observed in R848-carrying exosome-induced Tregs (Fig. 6G-H). In addition, no CD4⁺ Foxp3⁺ Tregs were detected in mice received the anti-CD25 Ab treatment in the lung (Fig. S8). The results demonstrate that Rexo can induce Ag-specific Tregs that have immune suppressive functions.

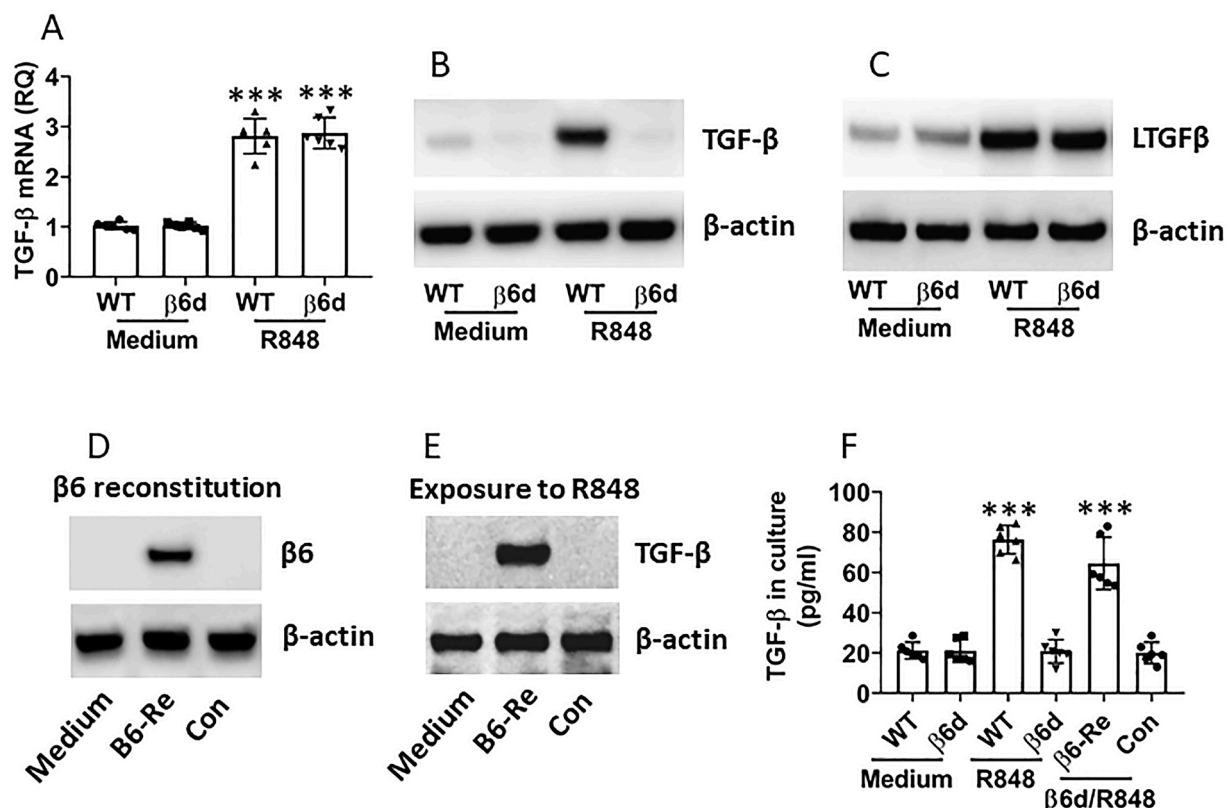


Fig. 3. α v β 6 mediates the effects of R848 on inducing TGF- β in CD4⁺ T cells. A-C, WT and β 6d CD4⁺ T cells were exposed to R848 (100 nM) in the culture for 48 h. Bars show mean $\pm$ SEM of TGF- β mRNA (A); immunoblots show TGF- β protein (B) or LTGF β (the precursor of TGF- β) (C) in CD4⁺ T cells. D, immunoblots show results of β 6 reconstitution in β 6d CD4⁺ T cells. E, immunoblots show TGF- β protein in β 6d CD4⁺ T cells with β 6 reconstitution after exposing to R848 in the culture for 48 h. F, bars show mean $\pm$ SEM of TGF- β levels in the culture supernatant. ***, $p < 0.001$ (ANOVA followed by the Dunnett's test), compared with the WT/medium group. β 6-Re: β 6 reconstitution. Con: β 6d-CD4⁺ T cells were treated with control plasmids.

4. Discussion

In this study, we generated a bio-nanoparticle, the Rexos, that carried a complex of MHC II and specific Ag as well as R848. The complex of MHC II/Ag could specifically bind to and activate Ag-specific CD4⁺ T cells. The R848 can interact with TLR7 in CD4⁺ T cells and activate the cells. Therefore, the Rexos carry two signals to activate CD4⁺ T cells (two signals are required to activate T cells [21]). The results showed that administration of Rexos efficiently suppressed the experimental AAD by generating Ag-specific Tregs and suppressing Ag-specific Th2 polarization.

It is a consensus that the major function of Treg is to regulate other immune cells' activities. Thus, to generate Tregs has been considered an optimal therapy for immune diseases [10]. The major pathogenic feature of AAD is the Th2 polarization in the airway tissues [22]. Therefore, Tregs are expected to suppress the Th2 cell activities to restore the Th1/Th2 balance, restore the immune tolerance, and suppress AAD response. Therefore, therapeutic application of Tregs would ideally suppress Th2 activities to restore the Th1/Th2 balance, restore immune tolerance, and suppress AAD. This is best exemplified by administration of Ag-specific immunotherapy, which can induced Tregs as well as reduce clinical symptoms of asthma patients. Bohm et al. found that OVA-specific immunotherapy induced Foxp3⁺ Tregs in asthmatic mice [23]. However, allergen immunotherapy in asthmatics is limited to patients with controlled asthma and without high-risk comorbidities or medications; even after completing 3–5 years of therapy some patients are unable to maintain clinical benefit after therapy is stopped [23–25].

The Rexos also carry R848, a TLR7 agonist. The data show that administration of R848 induces Tregs in the mouse lungs. This is in line with previous studies. Gaignage et al. found that R848 could increase Treg generation to protect organ transduction [26]. Van et al. found that R848 could suppress experimental asthma [11]. Our recent work also showed that encapsulating R848 and Ag epitopes into nanoparticles inhibited experimental food allergy [18]. Based on those pioneer studies, the present study further provided mechanistic evidence that exposure to R848 induced the expression of $\alpha\beta6$ in CD4⁺ T cells via activating the MyD88/p38/ERK1/2/I κ B α pathway. The data also show that TRAF3 and TRAF6 were increased in CD4⁺ T cells upon exposure to R848 in the culture. As TRAF6 is a ubiquitin E3 ligase, that speeds up protein ubiquitination [27]. Both TRAF6 and TRAF3 is associated with TGF- β expression [28]. Our previous work showed that $\alpha\beta6$ converted TGF- β precursor (LTGF β) to its mature form in DCs [13]. The mechanism of this event is that $\alpha\beta6$ binds to the prodomain, apply force, and release the TGF- β [29]. The present data show that R848 can induce the $\alpha\beta6$ expression in CD4⁺ T cells and convert LTGF β to its mature forms. These TGF- β ⁺ CD4⁺ T cells showed immune regulatory activities, indicating that these cells are Tregs.

The data show that Rexo administration efficiently suppressed the experimental AAD, the airway allergy. Currently, there are many remedies available for the treatment of airway allergy, e.g., corticosteroid sprays and allergen specific immunotherapy [30]. Although application of corticosteroids or histamine antagonists have good effects on the symptom control of airway allergy, the therapeutic effects only last for a short time. Allergen specific immunotherapy is a specific remedy for airway allergy, it requires too long time, 3-year treatment is required; its

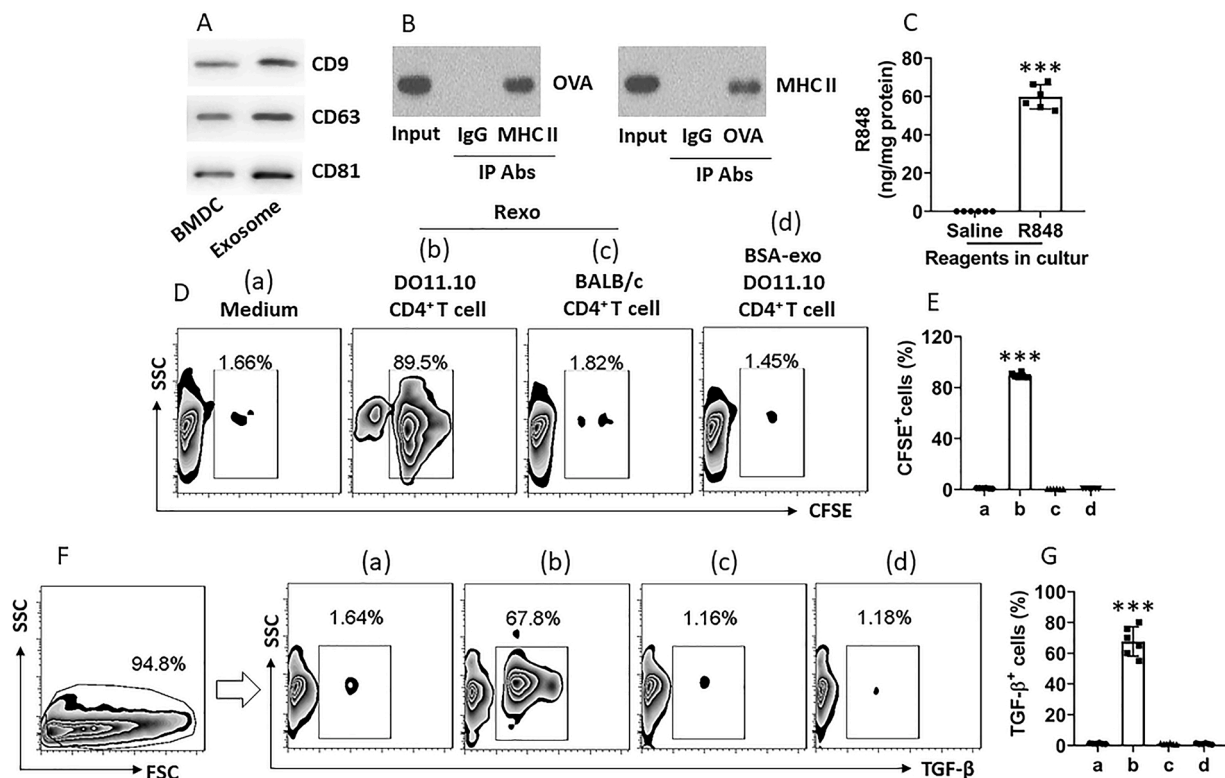


Fig. 4. Characterization of Rexo. BMDcs were exposed to R848 (0.5 mg/mL) and OVA (0.5 mg/mL) in the culture overnight. Exosomes were purified from the culture supernatant as described in the text. A, immunoblots show CD9, CD63 and CD81 in Rexo extracts and BMDc protein extracts (used as controls). B, immunoblots show a complex of MHC II and OVA in Rexo extracts. C, bars show mean $\pm$ SEM of R848 levels in Rexo extracts (by HPLC). D-E, CD4⁺ T cells were isolated from the spleen of DO11.10 mice and BALB/c mice and cultured in the presence of CFSE-labeled Rexos (BSA-carrying exosomes; exo) for 30 min. The cells were analyzed by FCS. Gated FCS plots show Rexo- or BSA-exo-bound CD4⁺ T cells. Bars show mean $\pm$ SEM of Rexo-bound CD4⁺ T cell counts. F-G, CD4⁺ CD25⁺ T cells were isolated from DO11.10 mouse and BALB/c mouse spleen; the cells were cultured in the presence of Rexo (5 μ g/mL; or BSA) and IL-2 (10 ng/mL) for 5 days. The cells were analyzed by FCS. Gated FCS plots show induced Tregs. Bars show mean $\pm$ SEM of Treg counts. ***, $p < 0.001$, compared with the saline group (C; the Student *t*-test) or with group a (E and G; ANOVA followed by the Dunnett's test). Group labels of E and G are the same as FCS plots on the left side.

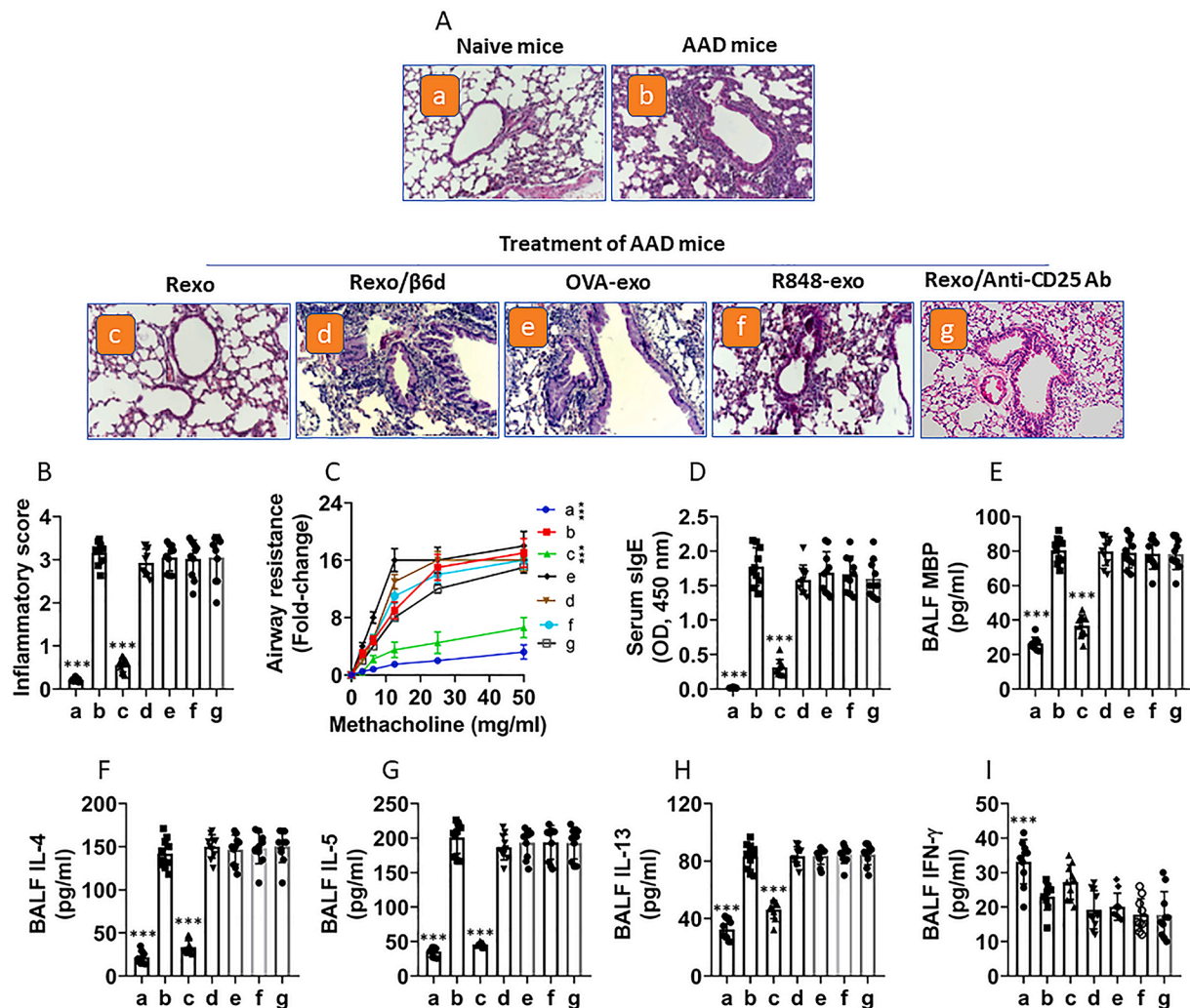


Fig. 5. Administration of Rexo inhibits experimental AAD. An AAD mouse model was developed with WT mice or β 6d mice. AAD mice were treated with the procedures denoted above each image of panel A. A, formalin-fixed lung tissue sections were stained with H&E. Representative histology images show lung tissue structure. B, inflammatory scores in the lung tissue histology. C, airway resistance changes in mice upon challenging with methacholine. D, serum specific IgE levels. E–I, cytokines levels of MBP, IL-4, IL-5, IL-13 and IFN- γ in BALF. The data of bars are presented as mean $\pm$ SEM. ***, $p < 0.001$, compared with group b (ANOVA followed by the Dunnett's test). Each group consists of 10 mice. Rexo (OVA-exo: exosomes carrying OVA alone; R848-exo: exosomes carrying R848 alone): After completing sensitization, AAD mice were treated with Rexo (or OVA-exo, or R848-exo)-containing nasal instillation (50 μ L/nostril; 0.1 mg protein/mL) daily for 7 days. Anti-CD25 Ab: Mice were peritoneally injected with anti-CD25 Ab (0.25 mg/mouse) once 3 days prior to the beginning of the Rexo therapy.

therapeutic effects are also to be improved [30,31]. The present data show that the R848-carrying Rexos may be an effective remedy for the treatment of airway allergy as Rexos induce Ag-specific Tregs that can inhibit Ag-specific Th2 cells. As Ag-specific Th2 cells play fundamental role in the pathogenesis of airway allergy [22], Rexos may be a novel agent to be used in the treatment of airway allergy.

We found that, although administration of R848 alone-carrying exosomes can increase the Treg frequency in the lungs, these Tregs seems incompetent to suppress the Ag-specific AAD as observed in the present study. This phenomenon may be because although R848 can induce the Treg development as shown by the present data, these Tregs are not activated by exposure to specific Ag, OVA, indicating that the induced Tregs are not OVA-specific. Thus, to inhibit Ag-specific AAD, Rexos are required, which carry both R848 and OVA, the specific Ag, that can induce and activate OVA-specific Tregs to suppress AAD in mice.

The present data show that high Th2 cytokine levels were found in BALF of AAD mice, indicating a Th2 polarization status in the airway tissues. In general, the immune responses in the airway tissues are tightly regulated by the immune regulatory system [32]. The Th2

polarization status in the mice of the present study implicate that the immune regulatory system is dysfunctional in the airway tissues, that may be one of the causative factors to contribute to the development of the immune intolerant status [33]. The reasoning is validated by the findings that significantly fewer Treg counts were found in the AAD mouse airway tissues. By administering the Rexos, the Treg counts in the AAD mouse airway tissues were recovered to the naive control mouse levels; the AAD symptoms were also attenuated. The data demonstrate that administration of Rexos can restore the Ag-specific immune tolerance in the AAD mouse airway tissues.

On the other hand, it is reported that R848 can up regulate the Th1 response [12]. We did observe that the IFN- γ levels were lower in BALF of AAD mice, that was up regulated slightly ($p = 0.1033$) after the Rexo-therapy. Therefore, the suppressive effects on AAD are unlikely generated from the Rexo-induced IFN- γ elevation, but from the Ag-specific Treg generation.

In summary, the present study shows that administration of R848/OVA-carrying exosomes, the Rexo, can inhibit experimental AAD by generation and activation of Ag-specific Tregs. The results suggest that the Rexos have translational potential in the treatment of AAD and other

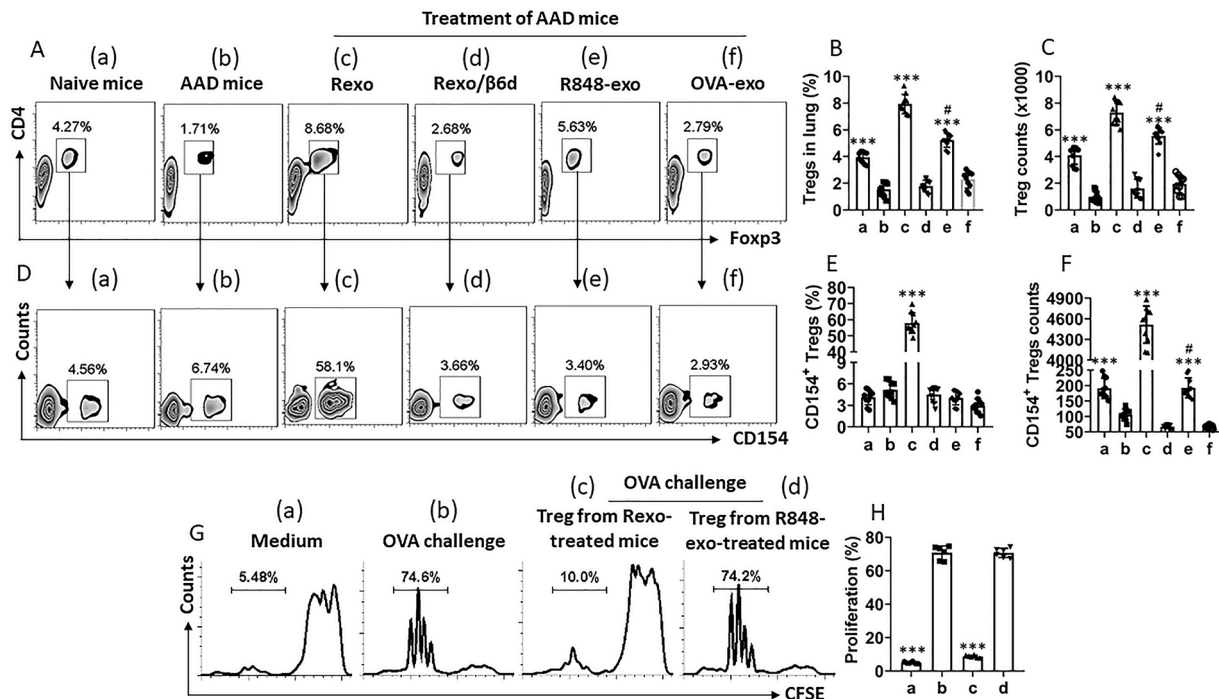


Fig. 6. Exposure to specific Ag activates Ag-specific Tregs to suppress Ag-specific CD4⁺ T cell activities. Mice were treated with the procedures denoted on each subpanel of A. Lung mononuclear cells (LMC) were isolated from the lung tissues and analyzed by FCS. A, gated FCS plots show CD4⁺ Foxp3⁺ Treg (these cells are also CD25⁺, data not shown) frequency in LMCs. B-C, summarized Treg percentage (B) and Treg counts (C) in LMCs. D, gated FCS plots show CD154⁺ (an activation marker of T cells) Treg frequency (the pre-gating was performed on CD4⁺ Foxp3⁺ cells in LMCs). E-F, summarized activated Treg percentage (E) and activated Treg counts (F). G, CD4⁺ CD25⁻ T cells (effector) and DCs were isolated from the DO11.10 mouse spleen. Tregs were isolated from the lungs of mice treated with Rexos or R848-exos. The culture conditions are denoted above each subpanel. Gated histograms show proliferated effector cell frequency. H, bars show summarized proliferating effector cell counts. Data of bars are presented as mean $\pm$ SEM. ***p < 0.001 (ANOVA followed by the Dunnett's test), compared with group b. Each group consists of 10 mice. Group labels of bar graphs are the same as FCS plots on the left side. R848-exo: Exosomes carrying R848 (no OVA). OVA-exo: Exosomes carrying OVA (no R848).

allergic diseases.

Author contributions

FM, YYZ, GY, LHM, DBL and LTY performed experiments, analyzed data and reviewed the manuscript. PCY, YN and ZGL organized the study and supervised experiments. PCY designed the project and prepared the manuscript.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgement

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the

online version, at doi:<https://doi.org/10.1016/j.imlet.2020.12.011>.

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Supplemental materials

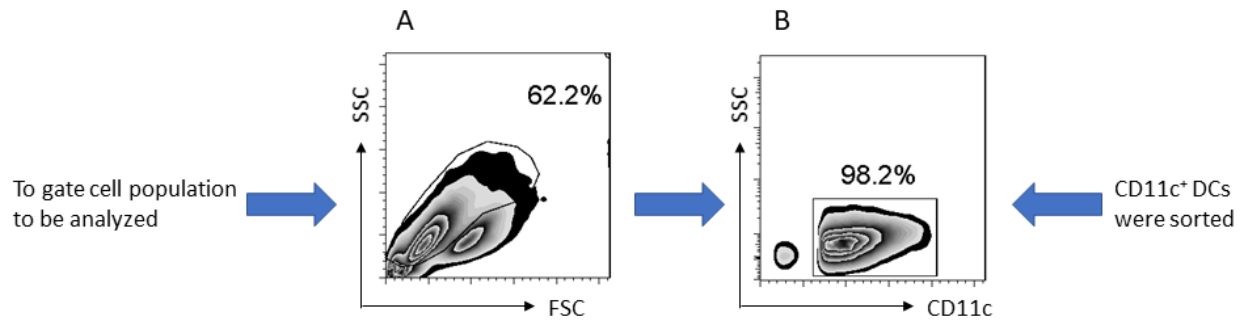


Figure S1. Isolation of DCs. BMDCs were prepared as described in the text. BMDCs were stained with Alexa Fluor[®] 488-CD11c Ab. The positively stained CD11c DCs were sorted by flow cytometry (FCS). A, to gate cells to be analyzed. B, CD11c⁺ DCs were further sorted.

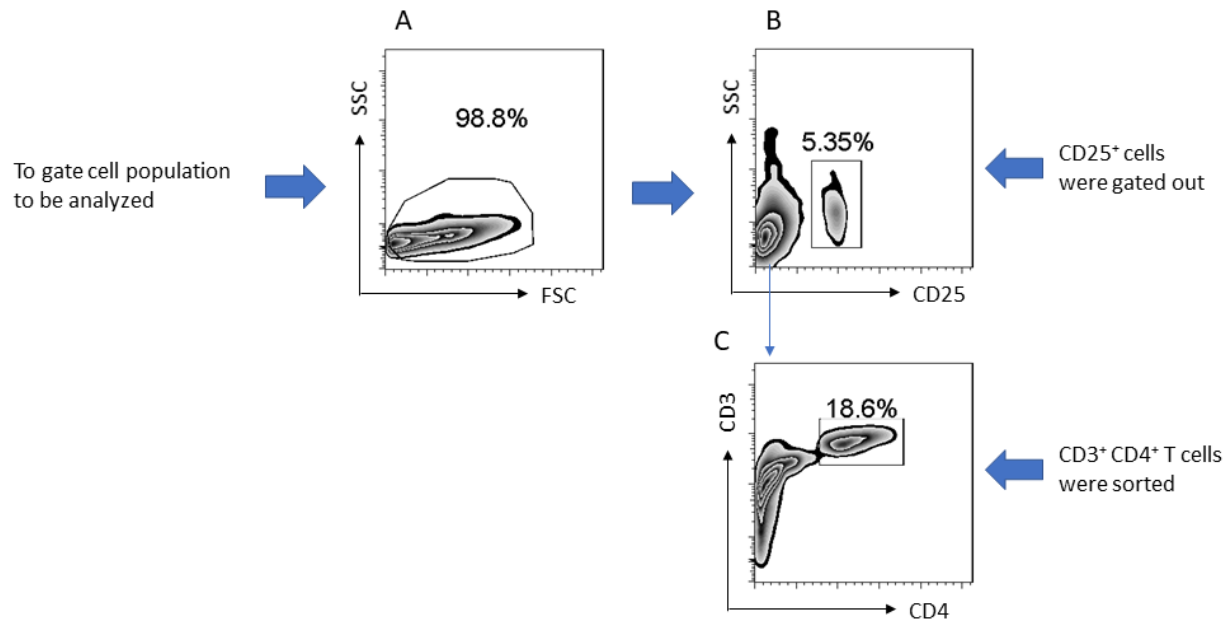


Figure S2. Isolation of CD4⁺ T cells. Mouse spleen cells were prepared and stained with Abs of CD4 (Alexa Fluor[®] 488), CD3 Alexa Fluor[®] 546), CD25 (Alexa Fluor[®] 647) as described in the text. A, cells to be selected were gated first. B, CD25⁺ cells were negatively selected out. C, from those CD25⁻ cells the CD3⁺ CD4⁺ T cells were sorted.

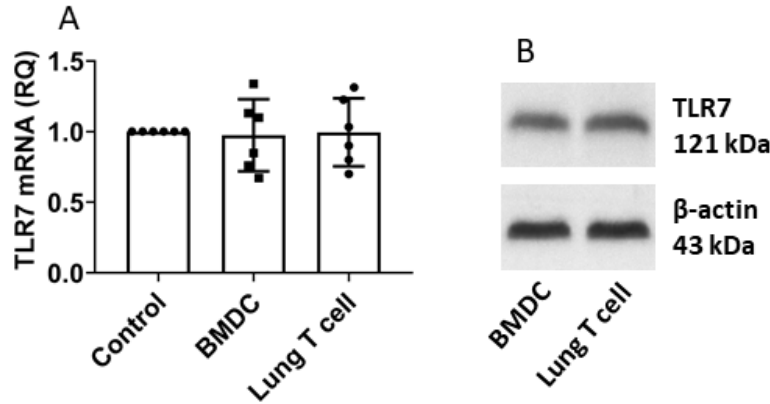


Figure S3. Assessment of the TLR7 expression in BMDC and CD4⁺ T cells. RNA and proteins were extracted from BMDCs and CD4⁺ T cells (isolated from the lung tissues by FCS) and analyzed by RT-qPCR and Western blotting. The bars show TLR7 mRNA (A) and TLR7 proteins (B). The data of panel B are from one experiment that represent 6 independent experiments. The control samples are the mixture of BMDC and lung T cell.

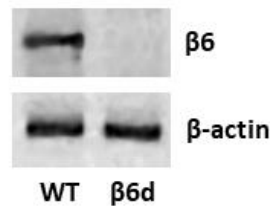


Figure S4. Assessment β6 expression in CD4⁺ T cells. CD4⁺ T cells were isolated from WT mice and β6d mice. Protein extracts were prepared from the cells and analyzed by Western blotting. The immunoblots show β6 protein levels in CD4⁺ T cells.

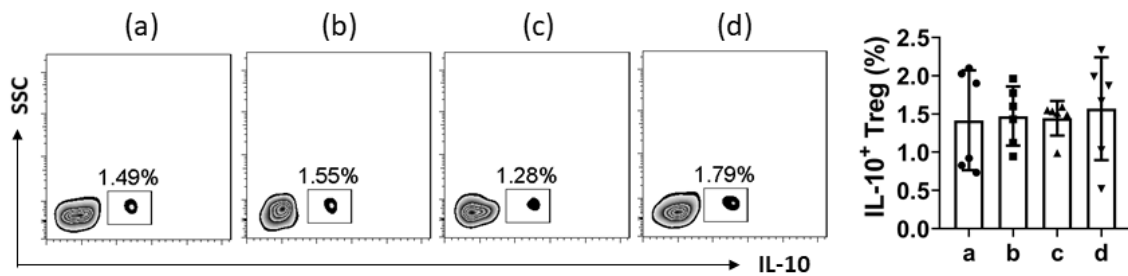


Figure S5. Assessment of IL-10⁺ Tregs. Supplemental data of Fig. 4. The group labels are the same as Fig. 4D. Gated FCS plots show IL-10⁺ Tregs. Bars show mean ± SEM of the IL-10⁺ Treg counts of 6 mice per group.

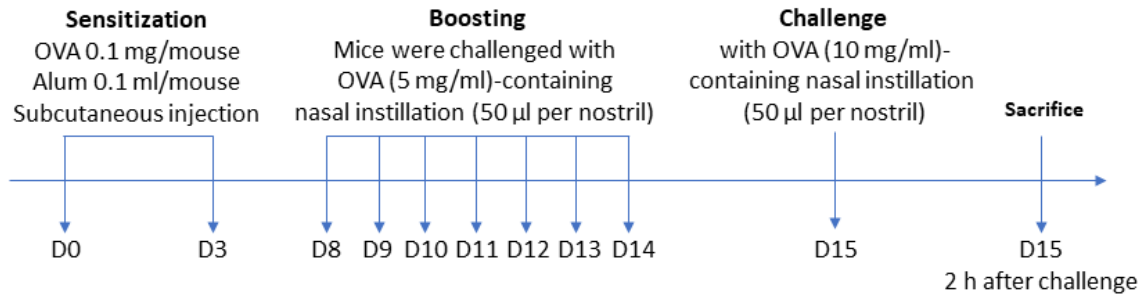


Figure S6. A schematic of AAD development in mice. D = Day.

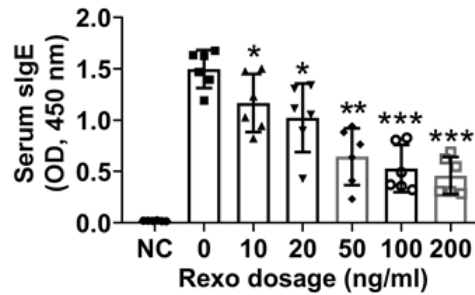


Figure S7. Assessment of specific IgE (sIgE) suppression by administration of Rexo in gradient doses. AAD mice were treated with nasal instillation (containing gradient Rexo concentrations as denoted on the x axis) as described in the text. After the sacrifice, blood samples were collected from each mouse. The serum was isolated from the samples and analyzed by ELISA. Bars show mean $\pm$ SEM of the serum sIgE levels from 6 mice per group. Statistical method: ANOVA followed by the Dunnett's test. *, p<0.05; **, p<0.01; ***, p<0.001 (compared with the dose 0 group).

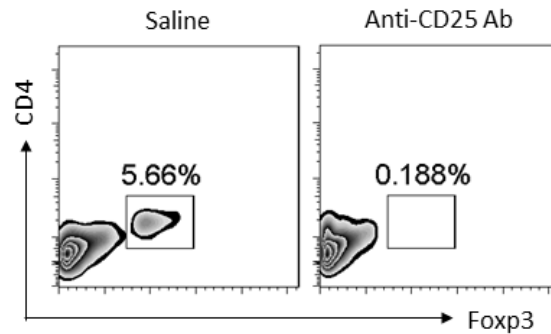


Figure S8. Depletion of Tregs with anti-CD25 Ab. Three days before the Rexo therapy, mice were peritoneally injected with anti-CD25 Ab (0.25 mg/mouse) to deplete Tregs. Gated FCS plots show Treg counts.