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Epithelial cell-derived CD83 restores immune tolerance in the airway mucosa by inducing regulatory T cell differentiation

Running title: CD83 restores immune tolerance

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Abbreviations

Regulatory T cells (Treg); ovalbumin (OVA); CD83 and OVA/MHC II carrying exosomes (83Oexo); CD40 ligand (CD40L); transforming growth factor (TGF); TGF- β -inducible early gene 1 (TIEG1); airway epithelial cell (AEC); epithelial cell adhesion molecule (EpCAM).

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Abstract

The mechanism of generation of regulatory T cells (Treg) remains incompletely understood. Recent studies show that CD83 has immune regulatory functions. This study aims to investigate the role of epithelial cell-derived CD83 in the restoration of immune tolerance in the airway mucosa by inducing the Treg differentiation. In this study, CD83 and ovalbumin (OVA)-carrying exosomes were generated from airway epithelial cells. An airway allergy mouse model was developed to test the role of CD83/OVA-carrying exosomes in the suppression of airway allergy by inducing Treg generation. We observed that mouse airway epithelial cells expressed CD83, that could be up regulated by CD40 ligand. The CD83-deficiency in epithelial cells retarded the Treg generation in the airway mucosa. CD83 up-regulated transforming growth factor- β -inducible early gene 1 expression in CD4⁺ T cells to promote Foxp3 expression. Exposure of primed CD4⁺ T cells to CD83/OVA-carrying exosomes promoted antigen-specific Treg generation. Administration of CD83/OVA-carrying exosomes inhibited experimental airway allergic response. In summary, airway epithelial cells express CD83, that is required in the Treg differentiation in the airway mucosa. Administration of CD83/OVA-carrying exosomes can inhibit airway allergy, that has the translation potential in the treatment of airway allergic disorders.

Keywords: Airway mucosa; epithelial cell; mucosal immunology; CD83; immune tolerance.

Introduction

Regulatory T cells (Treg) are a fraction of T cells with immune regulatory functions, including several subtypes, CD4⁺ CD25⁺ Foxp3⁺ Tregs, type I Tregs, CD8⁺ Tregs, etc ¹. The major function of Tregs is to suppress other immune cell activities, that plays an important role in the maintenance of the homeostasis in the body. Reduction of Tregs may result in autoimmune diseases or allergic diseases, such as rheumatoid arthritis, systemic lupus erythematosus, allergic asthma, and diabetes, etc. ². By adoptive transplantation of Foxp3⁺ Tregs to subjects with immune diseases, researchers found that this is a promising remedy for the solution of immune diseases ³.

However, because of the xenograft rejection, heterogeneous Tregs may be eliminated by the hosts soon after the transplantation. Thus, to generate Tregs in the hosts should be a better approach to restore Tregs in the body.

The Treg generation has been studied extensively ⁴. Because the loss of Foxp3 results in severe autoimmune disorders, such as the immunodysregulation polyendocrinopathy enteropathy X-linked syndrome ⁵, Foxp3⁺ Tregs have been attracting more attention. The canonical point in the generation of Foxp3⁺ Tregs is to induce the expression of transforming growth factor (TGF)- β , the major regulatory mediator of Foxp3⁺ Tregs. Since Foxp3 is the gene transcription factor of TGF- β , thus, to modulate the Foxp3 expression is also a critical factor to be considered. TGF- β -inducible early gene 1 (TIEG1) is a transcription factor and plays an important role in the Foxp3 expression ⁶. Yet, factors regulating TIEG1 expression need to be investigated.

The major immune regulatory mediators of Foxp3⁺ Tregs is TGF- β ⁷, that plays an important role in the maintenance of airway homeostasis ⁸. TGF- β is a non-specific cytokine and released by Tregs upon activation, that can suppress any other activating immune cells ⁹. Thus, the inducible Tregs need to be equipped with targeting ability. The antigen-specific Tregs meet this requirement. Upon exposure to specific antigens, both antigen-specific effector T cells and antigen-specific Tregs are activated. The released TGF- β thus gets the opportunity to target on the activating antigen-specific effector T cells ¹⁰. Our recent publications report an engineered exosome that carry an antigen/MHC II complex. This exosome only recognizes antigen-specific effector cells to modulate target cell activities ¹¹. This strategy can be exploited in antigen-specific Treg generation.

The breakdown of immune tolerance is associated with the pathogenesis of many immune diseases ¹². Epithelial cells contribute to the immune tolerance establishment ¹³. Our previous work showed that epithelial cell-derived integrin α v β 6 played a role in the establishment of immune tolerance ¹⁴. Chemokines derived from epithelial cells also contribute to the immune tolerance development ¹⁵. Epithelial cell-derived nuclear factor- κ B can impair immune tolerance in the airway mucosa. The above information suggests that epithelial cell-derived factors may

involve immune tolerance establishment by Treg generation in the airway mucosa. Our recent work shows that CD83 can suppress antigen-specific Th2 response in the nasal mucosa ¹⁶. Others found that CD83 attenuates autoimmune disorders ¹⁷. CD83 is a member of the immunoglobulin family; it can be produced by B cells and dendritic cells and usually used as a marker of dendritic cells ¹⁸. CD83 can be released to the microenvironment to be soluble CD83 that regulates other immune cell activities ¹⁹. Based on the information above, we hypothesize that CD83 may be associated with the immune regulatory activities in the body. In this study, we observed the role of CD83 in the restoration of immune tolerance by inducing Treg generation in the airway mucosa.

Materials and methods

Reagents

Antibodies for FACS, including CD83 (Alexa Fluor® 488), CD25 (Alexa Fluor® 594), Foxp3 (Alexa Fluor® 488), CD4 (Alexa Fluor® 647), CD11c (Alexa Fluor® 546) and siglec F (Alexa Fluor® 647), MBP (F-6, Alexa Fluor® 488), CRISPR kits of TIEG1, MD-2, TCR α and TLR4, antibodies of CD83 (Clone#: F-5), CTLA-4 (F-8), IL-10 (A-2), neuropilin (A-12), CD63 (MX129.49.5), CD81 (B-11), CD9 (C-4), OVA (2D11), MHC II (7-1H), TCR (H-1), TIEG1 (95-D), TLR4 (25), Foxp3 (F-9), were purchased from Santa Cruz Biotech (Santa Cruz, CA). Antibody of TGF- β (ab190503), EpCAM (ab71916) with or without labeled with PE was purchased from abcam (Cambridge, MA). Antibody of MD-2 was purchased from Biorbyt (Cambridge, UK). ELISA kits of OVA-specific IgE, IL-4, IL-5 and IL-13 were purchased from Dakewe BioMart (Beijing, China). Recombinant CD83 and CD40L protein and Monoclonal anti-CD25 antibody was provided by Sangon Biotech (Shanghai, China). FITC-labeling kit, and OVA were purchased from Sigma Aldrich (St. Louis., MO). Reagents and materials for RT-qPCR and Western blotting were purchased from Invitrogen (Carlsbad, CA).

Mice

BALB/c and C57BL/6 mice were purchased from the Guangdong Experimental Animal Center. DO11.10 mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice with CD83-deficient epithelial cells (CD83d mice) were constructed by the Animal Institute of Beijing Agricultural University (Beijing, China; the developing procedures of this mouse strain are presented in supplemental materials; Fig. S1). Mice were maintained in an SPF facility with accessing water and food freely. The experimental procedures were approved by the Animal Ethical Committee at Shenzhen University.

Lung single cell preparation

After the sacrifice, the lungs were excised, cut into small pieces, and incubated with collagenase IV (1 mg/ml) for 30 min at 37 °C with mild agitation. Single cells were filtered through a cell strainer, harvested by centrifugation (1000 g, 5 min).

Airway epithelial cell (AEC) and immune cell purification

Lung single cells were stained with fluorescence-labeled antibodies of interest (e.g., EpCAM antibody was used for epithelial cell purification). Epithelial cells or other immune cells were purified from the single cells by FACS. Cell purity was checked by FACS. If purity was below 95%, the purification procedures were repeated.

Immunohistochemistry

After the sacrifice, the mouse lungs were excised and snap frozen in liquid nitrogen. Frozen sections were cut in a cryostat, dried overnight at room temperature. The sections were blocked by incubating with 1% bovine serum albumin for 30 min to stop non-specific binding, incubated with the first antibody of CD83 (diluted in 1:100 in PBS) at 4 °C overnight, washed with PBS 3 times, incubated with FITC-labeled secondary antibody (diluted to 1:300) for 2 h at room temperature, washed with PBS 3 times, followed by staining with propidium iodide (5 µg/ml) for 5 min to illustrate the nuclei. Sections were mounted with coverslips and observed with a fluorescence microscope. Positively stained cells were counted in 20 randomly selected micro windows per sample. Sections were coded. The observers were not aware of the code to avoid the observer bias.

Exosome preparation

MLE12 cells (A mouse AEC cell line, purchased from ATCC; Manassas, VA) were cultured in DMEM (Dulbecco's Modified Eagle Medium) supplemented with fetal bovine serum, penicillin, streptomycin, and glutamine. OVA (1 mg/ml) and CD40L-737 (CD40L, in short; 200 nM) were added to the culture to generate the 83Oexosomes (83Oexo, in short). Control exosomes, including 83exo (only treated with CD40L), Oexo (only added OVA), Eexo (medium alone), Bexo (added BSA and CD40L), were also prepared. 16 h later, supernatant was collected, transferred to Ultra-Clear tubes (Beckman Coulter, Indianapolis, IN). The samples were firstly centrifuged at 100,000 g for 12 min, removed half of the supernatant, followed by centrifugation at 100,000 g for 70 min; this procedure was repeated 3 times. Supernatant was removed. Pellets were resuspended in PBS; exosomes were purified by magnetic anti-CD9 antibody system following

previous report²⁰. To verify exosomes, proteins were extracted from exosomes and assessed exosome markers, including CD9, CD63 and CD81, by Western blotting.

Depletion of TCR, TLR4, TIEG1 and MD-2 expression in CD4⁺ T cells

CD4⁺ T cells were isolated from the naive mouse spleen by FACS sorting. The purity of the cells was greater than 95%. Expression of TCR, or TLR4, or TIEG1, or MD-2, was depleted from CD4⁺ T cells with commercial CRISPR (clustered regularly interspaced short palindromic repeats) reagent kits following the manufacturer's instruction. The effects of gene depletion were assessed by Western blotting 48 h later.

Generation of Tregs by 83Oexo

In vitro study: CD4⁺ CD62L⁺ T cells were isolated from the DO11.10 mouse spleen and cultured in the presence of 83Oexo or control exosomes (including Oexo, 83exo, Bexo and Eexo) at 20 µg proteins/ml and IL-2 (20 ng/ml) for 5 days. On the other hand, CD4⁺ CD62L⁺ T cells were isolated from the BALB/c mouse spleen and cultured in the presence of 83Oexo and IL-2 for 5 days. On day 6, cells were harvested and analyzed by FACS to determine the frequency of CD25⁺ Foxp3⁺ Tregs.

In vivo study: DO11.10 mice received nasal instillation containing 83Oexo (50 µl per nostril; 1 mg 83Oexo protein/ml) or control exosomes (including Oexo, 83exo, Bexo and Eexo) daily for 1 week. Mice were sacrificed 2 days after the last injection. Lungs were excised; single cells were prepared with the lung tissues and analyzed by FACS to assess the frequency of Foxp3⁺ Tregs. To differentiate the Tregs were central or peripheral Tregs, CD4⁺ CD25⁺ CD127⁻ Tregs were purified from the lungs; proteins were extracted from the Tregs and analyzed the neuropilin levels by Western blotting.

Assessment of immune suppressive effects of 83Oexo-generated Tregs on CD4⁺ T cell proliferation

CD4⁺ CD25⁻ T cells (Teff) and DCs were isolated from the DO11.10 mouse spleen. Teff cells were labeled with carboxyfluorescein succinimidyl ester (CFSE). Teff cells were cultured with Tregs at

a ratio of 1:1 in the presence of OVA (10 µg/ml) and DC (DC:T cell = 1:5) and IL-2 for 3 days. Cells were then analyzed by FACS, the CFSE-dilution assay.

Airway allergy mouse model development

BALB/c mice were subcutaneously injected with OVA (100 µg/mouse) mixed in 0.1 ml alum on the back skin on day 0 and day 7, respectively. From day 15 to day 21, mice were challenged daily by receiving nasal instillation 50 µl OVA (1 mg/ml, in saline) per nostril. On day 22, mice were challenged with a large dose of antigen (50 µl per nostril; 5 mg OVA/ml). A schematic diagram showing the sensitization procedures is presented in Fig. S2 in supplemental materials. The airway allergic response was observed and recorded as described below.

Assessment of airway resistance

AHR tests were performed 24 h after the last OVA challenge of the mice. Mice were anesthetized by intraperitoneally injecting with 5% chloral hydrate at 400 mg/kg. Tracheal intubation was performed, mice were fixed in a closed body drawing box and mechanically ventilated. Mice were nebulized with methacholine at gradient dosages (0, 6.25, 12.5, 25, and 50 µg/ml at 0.1 ml). Then, data from 5 seconds to 1 minute after inhalation were collected, and the lung resistance was calculated and presented as fold change.

Assessment of OVA-specific IgE and Th2 cytokines in BALF

Levels of OVA-specific IgE and Th2 cytokines in BALF were determined by enzyme-linked immunosorbent assay (ELISA) with commercial reagent kits following the manufacturer's instructions.

Administration of AEC-derived exosomes in mice with airway allergy

Two days after the last antigen challenge, sensitized mice received nasal instillation containing 83Oexo (50 µl per nostril; 1 mg 83Oexo protein/ml) or control exosomes (including Oexo, 83exo, Bexo and Eexo) daily for 1 week; or treated with anti-CD25 Ab (αCD25, 0.25 mg/mouse, i.p., one time; control mice were treated with isotype IgG) 1 week before the 83Oexo treatment.

Administration of CD40L/OVA in sensitized mice

Since the 83Oexo was released by AECs after exposing to CD40L/OVA in the culture, it is necessary to test if directly applying CD40L-OVA has inhibitory effects on airway allergic response. Wild type mice and CD83d mice (mice with CD83-deficient epithelial cells; this mouse strain is described in supplemental materials) were sensitized to OVA as described above. WT mice and CD83d mice were treated with CD40L (10 mg/ml)/OVA (1 mg/ml) at 50 μ l per nostril daily for 1 week.

Statistical analysis

The data are presented as means $\pm$ SEM. Normality of distribution was assessed by the Kolmogorov-Smirnov test plus one-way parametric ANOVA followed by a Bonferroni or Dunnett's multiple comparison post hoc test. Dose-response curves of the AHR assessment were analyzed using two-way parametric ANOVA plus a Bonferroni multiple comparison post hoc test. $P < 0.05$ was set as a significant criterion.

Some experimental procedures are presented in supplemental materials.

Results

CD40 ligand (CD40L) increases CD83 expression in mouse airway epithelial cells (AEC)

MLE12 cells (a mouse AEC cell line) were cultured in the presence of CD40L at gradient concentrations for 48 h. We found that MLE12 cells expressed CD83; exposure to CD40L increased the CD83 expression in MLE12 cells in a dose-dependent manner (Fig. 1A-D). To verify the data, naive mice were treated with CD40L or saline by nasal instillation daily for 3 days. After the sacrifice, the lungs were excised; single cells were prepared with the lung tissues and analyzed by flow cytometry (FACS). With EpCAM as the epithelial cell marker, CD83⁺ AECs were observed in the lung tissues, which were markedly increased after CD40L-treatment (Fig. 1E-F). We then isolated EpCAM⁺ epithelial cells by FACS. RNA and proteins were extracted from the epithelial cells and analyzed by RT-qPCR and Western blotting. The results showed that the CD83 expression was detectable in lung epithelial cells at both mRNA and protein levels in saline-treated mice, which were significantly increased after CD40L-treatment (Fig. 1G-H). The data were verified by immunohistochemistry data, in which CD83⁺ AECs were visualized in lung AECs; exposure to CD40L significantly increased the number of CD83⁺ AECs (Fig. 1I-J). The results demonstrate that airway epithelial cells express CD83, that can be up regulated by exposure to CD40L. Similar phenomenon was also observed in mouse intestinal epithelial cells (Fig. S3).

Mice with CD83-deficient epithelial cells fail to induce regulatory T cells (Treg) in the lung

Since CD83 may be involved in the immune tolerance development²¹, we next search the probability that CD83 may be required in the Treg generation. To this end, wild type (WT) mice and mice with CD83-deficient epithelial cells (CD83d mice, in short) were treated with ovalbumin (OVA)²² or/and CD40L, or saline (used as a control) through nasal instillation daily for 7 days. Mice were sacrificed on day 8. The lungs were excised; single cells were prepared and analyzed by FACS. The results showed that treatment with OVA markedly increased Treg counts in the WT mouse lungs as compared to the saline group, while the Treg counts were not apparently altered in the lung of CD83d mice. Treatment with both OVA and CD40L further increased Treg generation in WT mice, but not in CD83d mice. Treatment with CD40L alone only slightly increased Treg counts ($p>0.05$) (Fig. 2A-B). As no neuropilin was detected, these OVA-induced

Tregs are regarded as inducible Tregs²³. Other immune tolerant markers, CTLA-4, Foxp3 and TGF- β were also detected, while IL-10 was below the detectable levels (Fig. 2C-F). The results indicate that epithelial cell-derived CD83 is required in the generation of Tregs in the airway tissues.

AEC-derived exosomes carry CD83 and OVA/MHC II complexes

Data of Fig. 1-2 indicate that after exposure to CD40L, the CD83 expression in AECs was up regulated, that facilitated the Treg differentiation in the airway tissues in the presence of specific antigens. Thus, it is of significance to elucidate the underlying mechanisms and substances mediating the effects of AECs on Treg differentiation. Since CD83 is a transmembrane protein, it may leave the bearing cells in the form of exosome. Thus, after exposing MLE12 cells to CD40L and OVA in the culture for 2 days, exosomes were isolated from the supernatant. We found that the exosomes contained CD83 as well as the markers of exosomes, including CD9, CD63 and CD81 (Fig. 3A). Similar phenomenon was also found in exosomes isolated from the primary AEC culture (Fig. S4). Besides, complexes of MHC II/OVA were also found in the exosomes (Fig. 3B). The exosomes specifically bound to OVA-specific CD4⁺ T cells, which was abolished by depleting the TCR (Fig. S5) in CD4⁺ T cells (Fig. 3C-D). Since CD83 has the immune tolerance-inducing potential, MHC II/OVA complexes intend to interact with OVA-specific CD4⁺ T cells, the exosomes may have immune tolerogenic properties.

CD83 promotes Foxp3 expression in CD4⁺ T cells by inducing TIEG1

As shown by Fig.2, CD83 is involved in the Foxp3⁺ Treg generation in the mouse airway tissues. The data imply that CD83 is associated with the Foxp3 expression in CD4⁺ T cells. Since TIEG1 regulates the Foxp3 expression⁶, CD83 may be capable of inducing TIEG1 expression. To this end, CD4⁺ CD62L⁺ T cells were isolated from the naive mouse spleen and stimulated with CD83 in the culture for 3 days. We found that CD83 significantly increased the TIEG1 expression in CD4⁺ T cells (Fig. 4A-B). Since CD83 binds Toll-like receptor-4 (TLR4) and myeloid differentiation factor-2 (MD-2) complex to elicit the immune suppressive effects²⁴, we inferred that TLR4/MD-2 complexes may be involved in the TIEG1 induction by CD83. To test this, CD4⁺ T cells were depleted the expression of TLR4 or MD-2 (Fig. S6, Fig. S7) and then exposed to CD83 in the

culture. Indeed, depletion of either TLR4 or MD-2 abolished the CD83-induced TIEG1 expression (Fig. 4A-B). The data implicate that CD83 induces Foxp3 expression in CD4⁺ T cells via increasing the TIEG1 expression. To verify the data, TIEG1-deficient CD4⁺ T cells were prepared (Fig. S8) and then stimulated by CD83 in the culture for 3 days. The Foxp3 expression in CD4⁺ T cells induced by CD83 was abolished (Fig. 4C-D). The results suggest that TIEG1 mediates the CD83-induced Treg differentiation by inducing Foxp3 expression.

Exosomes carrying CD83 and OVA/MHC II complexes generate OVA-specific Tregs

DO11.10 CD4⁺ T cells were prepared and exposed to exosomes carrying CD83 and OVA/MHC II complexes (83Oexo, in short), or control exosomes, including exosomes carrying CD83 alone (83exo), BSA (Bexo), OVA alone (Oexo) and empty exosomes (Eexo) in the culture for 5 days. The cells were analyzed by FACS. The results showed that exposure to 83Oexo, but not 83exo, or Bexo, or Oexo, or Eexo, markedly induced Treg differentiation. Exposure of BALB/c CD4⁺ T cells to 83Oexo did not have appreciable effects on the Treg differentiation (Fig. 5A-B). The induced Tregs showed immune suppressive function on OVA-specific CD4⁺ T cell proliferation (Fig. 5C-D), but not suppressed peanut-specific CD4⁺ T cell proliferation (not shown). In addition, DO11.10 mice and BALB/c mice were treated with 83Oexo through nasal instillation daily for 7 days. Mice were sacrificed on day 9. We found Foxp3⁺ Tregs were generated in the lungs. To differentiate the Tregs are central or peripheral Tregs, proteins were extracted from isolated Tregs and analyzed by Western blotting. The results showed that only trifling neuropilin levels were detected in the Tregs (Fig. 5E-F, Fig. S9). The data demonstrate that 83Oexo can generate OVA-specific Tregs.

Administration of 83Oexo suppresses airway allergic response

Next, we assessed the effects of 83Oexo on airway allergic responses. An airway allergy mouse model was developed using OVA as a specific antigen. The mice showed airway hyperreactivity, increases in eosinophil infiltration, specific IgE and Th2 cytokines in bronchoalveolar lavage fluid (BALF). Treatment with 83Oexo efficiently suppressed the airway allergic response, while treating allergic mice with Eexo or Bexo or 83exo, did not have apparent suppressive effects on the airway allergic response. Depletion of Tregs with anti-CD25 antibody injection also blocked the

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suppressive effects of 83Oexo in airway allergic response. In addition, mice with CD83d-epithelial cells showed allergic response in the airways (not shown); treating sensitized CD83d mice with a mixture of OVA and CD40L showed no suppressive effects on airway allergic response (Fig. 6). By assessing Tregs in BALF, both 83Oexo and 83exo could induce Tregs; however, only 83Oexo could induce the OVA-specific Tregs while 83exo could not (Fig. 7).

Discussion

In this study, we found that mouse airway epithelial cells expressed CD83 that could be up regulated by CD40L. This is in line with previous studies indicate that CD40L increased the CD83 expression in B cells ²⁵. Mice with CD83d epithelial cells failed to induce Tregs in the lung tissues, indicating that epithelial cell-derived CD83 is required in Treg differentiation in the airway tissues. Mechanistically, we found that CD83 left epithelial cells in the form of exosomes. CD83 induced TIEG1 in CD4⁺ T cells that facilitated the Treg development ⁶. Further experiments revealed that concomitantly exposure of airway epithelial cells to CD40L and OVA in the culture resulted in the epithelial cell-derived exosomes that contained CD83 and OVA/MHC II complexes (83Oexo, in short). We observed that administration of 83Oexo induced antigen-specific Tregs in the lung and efficiently suppressed airway allergic response.

It is the consensus that Tregs play a central role in immune tolerance ²⁶. The Treg dysfunction may result in many diseases such as allergic diseases, immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome, rheumatoid arthritis, type 1 diabetes, or cancer ²⁷. Thus, it is of significance to understand the factors and mechanisms in the induction of Tregs. The present data show that the AEC-derived CD83 can induce Tregs. This is in line with previous reports; Doebbler et al reported that intrinsic CD83 was essential for Treg differentiation. Depletion of CD83 resulted in loss of the downstream Treg-specific markers and induced inflammation ²⁸. Kreiser et al reported that Tregs expressed CD83 and CD83 was associated with Treg's immune suppressive function ²⁹. Our data show a novel aspect of CD83 that CD83 can be expressed by AECs. The CD83 leaves epithelial cells in a form of exosomes, that can reach and interact with remote immune cells and thus, participate immune regulatory activities ³⁰. This finding enriches the understanding of studies about epithelial cells and immune tolerance since epithelial cells are well known by their immune tolerogenic properties ³¹.

The data show that the Treg generation in the airway tissues by OVA-administration requires the presence of CD83 in epithelial cells. This finding suggests that the epithelial cell-derived CD83 is an important factor in the maintenance of the homeostasis in the airway mucosa because Tregs

have the immune regulatory ability on suppressing other immune cell activities; proper immune activities are essential for the homeostasis of the body. Epithelial cells are the first line of the airway tissues to interact with the exterior environment. Multiple airborne substances, including competent antigens may be inhaled into the airway and absorbed by epithelial cells and transferred into the deep regions in the mucosa ³². The absorbed antigens may elicit immune activities, one of which is to cooperate with CD83, as shown by the present data, to induce antigen-specific Treg differentiation that contributes to immune tolerance ³¹.

To generate Tregs has been employed to inhibit immune disorders, both in laboratory and in clinics ²⁷, in which to equip the inducible Tregs to target specific immune cells is a canonical requirement. The present data demonstrate an optimal strategy to induce Tregs to meet this goal. After concomitantly exposing to OVA and CD40L in the culture, exosomes were released from AECs. These exosomes contain CD83 and OVA/MHC II complexes (83Oexo). This confers the 83Oexo targeting ability; the OVA/MHC II complexes have high avidity to bind the OVA-specific TCR on OVA-specific T cells. As shown by the present data, only OVA-specific T cells can be recognized by 83Oexo; 83Oexo then induce targeted OVA-specific T cells to be OVA-specific Tregs. This meets the study goal. The induced Tregs suppress OVA-specific T cell proliferation, but did not suppress peanut-specific T cell proliferation. This guaranty the induced Tregs only target on designed immune cells.

We found that CD4⁺ T cells also had the TLR4/MD-2 complexes that could be activated by CD83. This is in line with previous reports; Horvatinovich et al indicate that CD83 can bind CD44 to activate the TLR4/MD-2 pathways on monocytes ²⁴, in line with which, the data show that exposure to CD83 induces the TIEG1 expression in CD4⁺ T cells; this was the TLR4/MD-2-dependent, that was verified by depletion of either TLR4 or MD-2 to abolish the CD83-induced TIEG1 expression in CD4⁺ T cells. TIEG1 is a transcription factor that cooperates with Foxp3 to trigger Treg differentiation ⁶. Our data expand this knowledge slot by showing that TIEG1 mediates the effects of CD83 on Treg differentiation by promoting Foxp3 expression in CD4⁺ T cells.

The data show that administration of 83Oexo efficiently suppresses airway allergic response. Th2 dominant immune response plays a central role in the pathogenesis of airway allergy³³. Although the present data do not show that 83Oexo has direct effects on suppressing Th2 response in the airway tissues, 83Oexo can induce antigen-specific Treg differentiation, the latter is capable of inhibiting antigen-specific CD4⁺ T cell proliferation. Thus, 83Oexo-induced Tregs can inhibit the allergic response in the airway tissues since Th2 polarization plays a cardinal role in the pathogenesis of airway allergy³⁴. This is verified by that depletion of Tregs in mice with anti-CD25 Ab abolished the 83Oexo-induced suppression of airway allergy. Currently, the therapeutic effects of allergic asthma are not satisfactory³⁵. The present data shed a light on this area. 83Oexo may be developed into a remedy to be used in the treatment of airway allergic disorders.

It should be realized that EpCAM is not only expressed by AECs, all epithelial cells in the body, including Langerhans cells³⁶, also express EpCAM. Thus, the EpCAM Cre mice are not AEC specific. The CD83-depletion is not only induced in AECs, but in all epithelial cells and Langerhans cells of EpCAM Cre mice. Whether the current experimental approaches also alter the Treg-induction in other organs, such as in the intestine, may need to be further investigated. Additionally, the Treg frequency in the airway tissues of EpCAM Cre mice is equal to that of WT mice, indicating that the epithelial cell-derived CD83 is not essential to Treg development, but can facilitate the antigen-specific Treg development with the presence of the antigens. On the other hand, we found that, although CD83 can induce Treg development, administration of the 83exo could not alleviate experimental airway allergy, while the 83Oexo could. The fact indicates that exposure to either CD83 or CD83/OVA can induce Treg development; Tregs induced by CD83/OVA can be activated in an OVA-allergic environment to alleviate allergic responses, while Tregs induced by CD83 alone cannot be activated in such an environment and thus, no therapeutic effects can be elicited.

In the Western blotting experiments, we observed a single band of CD83 staining at 32 kDa. Another band of 45-60 kDa (corresponding to glycosylated CD83) has been reported by others³⁷, using a different antibody.

In summary, the present data show that AECs express CD83. AEC-derived 83Oexo can induce antigen-specific Tregs via the CD83/TLR4/MD-2/Foxp3 pathway. Administration of 83Oexo can efficiently suppress experimental airway allergic response, suggesting that 83Oexo has translational potential to be used in the treatment of airway allergic disorders.

Conflicts of interest: None to declare.

Data availability: All the data are included in this paper and the supplemental materials.

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

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Figure legends

Figure 1. CD40L increases CD83 expression in airway epithelial cells (AEC). A-D, MLE12 cells (a mouse AEC cell line) were exposed to CD40 ligand (CD40L) in the culture at indicated concentrations for 2 days. A, gated flow cytometry plots show CD83⁺ MLE12 cell counts. B, bars show summarized MLE12 cell counts. C, bars show CD83 mRNA levels in MLE12 cells. D, immunoblots show CD83 protein levels in MLE12 cells. E-J, naive BALB/c mice (6 mice per group) were treated with CD40L (0.1 ml/nostril; 1 mg/ml) or saline daily for 3 days. Mice were sacrificed; the lungs were excised; single cells were prepared and analyzed by flow cytometry, and immunohistochemistry. E, gated cells are CD83⁺ AECs (EpCAM is an epithelial cell marker). F, bars show summarized CD83⁺ AEC counts. G-H, EpCAM⁺ AECs were purified by flow cytometry and analyzed by RT-qPCR and Western blotting. Bars show CD83 mRNA levels and immunoblots show CD83 protein levels. I-J, the lung tissues were processed for immunohistochemistry. Representative images show that many epithelial cells were stained CD83⁺ (in green; the nuclei were stained in red by propidium iodide staining to show tissue structure) (original magnification: $\times 100$). Bars show CD83⁺ cell counts (20 micro-windows were randomly selected from each sample; average of the 20 counts was used as one datum). Each experiment was repeated at least 3 times. Data of bars are presented as means $\pm$ SEM. Each sample was analyzed in triplicate. Statistics: ANOVA followed by the Bonferroni test.

Figure 2. CD83-depletion retards Treg differentiation in the airway tissues. WT mice and CD83d mice were treated with OVA (10 mg/ml), or saline, or CD40L (50 μ g/ml), or both CD40L and OVA, through nasal instillation (100 μ l per nostril) daily for 7 consecutive days. A, single cells were prepared with the mouse lung tissues (or thymus, control) and analyzed by flow cytometry. Gated plots in the first-row show Treg populations; the second-row show neuropilin⁺ Tregs; the third-row show CTLA-4⁺ Tregs. B-D, bars show Treg counts (B), neuropilin⁺ Treg counts (C) and CTLA-4⁺ Treg counts (D). The data of bars are presented as means $\pm$ SEM of 6 mice per group. * and #, $p < 0.001$ (ANOVA followed by the Dunnett's test), compared with the WT group treated with saline (*), or compared with the CD83d/OVA group (#). E-H, Tregs (CD4⁺ CD25⁺ CD127⁻) were isolated from the lung tissues (or thymus) by flow cytometry cell sorting. Proteins were

extracted from isolated Tregs (or LPS-primed B cells, used as the IL-10 positive control, indicated by symbol &) and analyzed by Western blotting. Immunoblots show the protein levels of TGF- β (E), CTLA-4 (F), neuropilin (G), and IL-10 (H). Samples of individual mice were analyzed separately. Each sample was analyzed in triplicate. Group labels of bar groups and immunoblots are the same as panel A.

Figure 3. AEC-derived exosomes bind CD4⁺ T cells in an Ag-specific manner. AEC-derived exosomes were purified from culture supernatant and analyzed. A, exosome protein extracts were analyzed by Western blotting. Immunoblots show CD83 and exosome-relative markers (CD9, CD63 and CD81) in the protein extracts. B, exosome protein extracts were analyzed by co-immunoprecipitation (co-IP). Immunoblots show a complex of OVA/MHC II in exosome extracts. C-D, FITC-labeled exosomes were added to BALB/c CD4⁺ T cells or DO11.10 CD4⁺ T cells at 20 μ g protein/ml; cells were harvested 3 h later; the rate of CD4⁺ T cell binding rate of FITC-exosome was determined by flow cytometry. C, gated flow cytometry plots show binding rate; bars show means $\pm$ SEM of binding rate from 3 independent experiments. TCRd: TCR-deficient CD4⁺ T cells (achieved by TCR CRISPR approach). Control: DO11.10 CD4⁺ T cells were treated with control CRISPR reagents. Each experiment was repeated 3 times. Each sample was analyzed in triplicate. #, exosomes carry BSA instead of OVA. Statistics: ANOVA followed by the Bonferroni test.

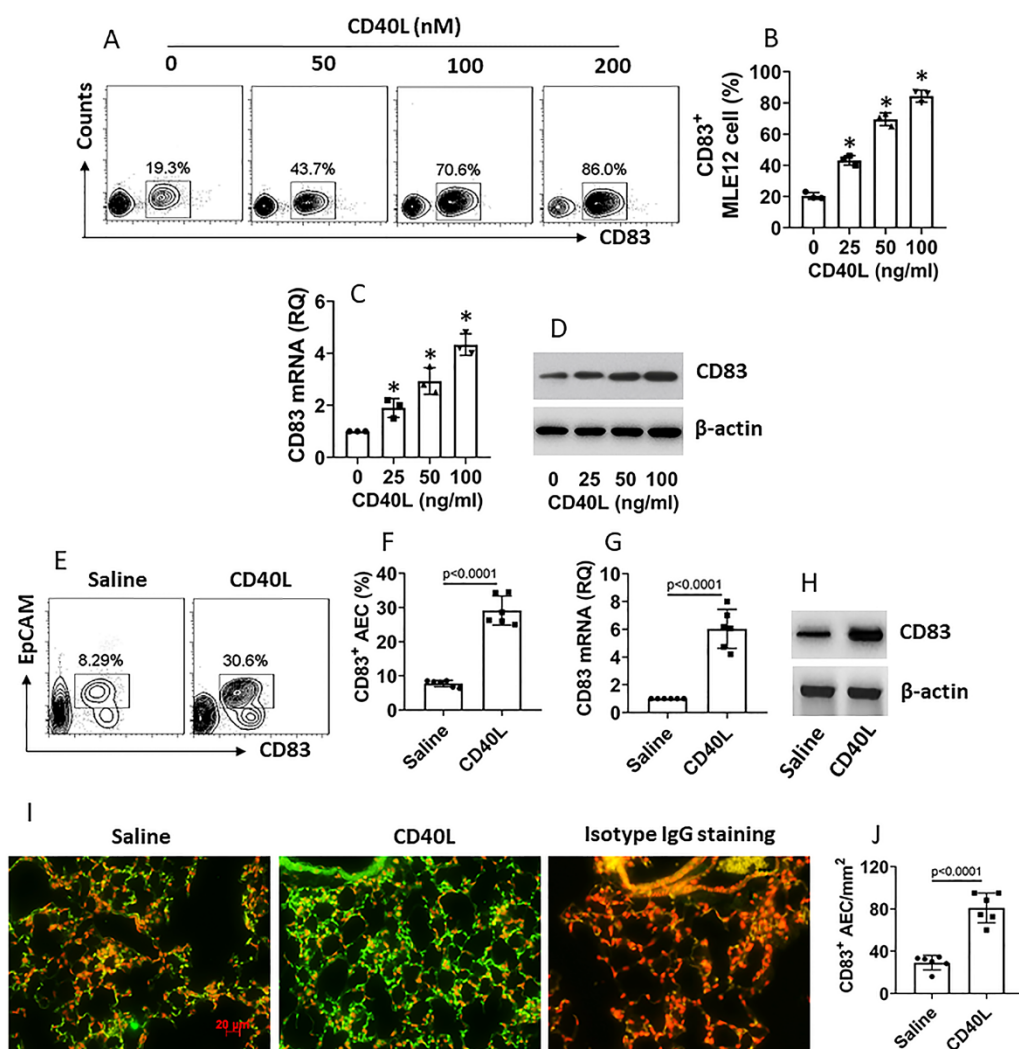
Figure 4. CD83 modulates TIEG1 expression to increase Foxp3 expression in CD4⁺ T cells. A-D, CD4⁺ CD62L⁺ T cells were isolated from the naive mouse spleen and stimulated with CD83 (1 μ g/ml) in the culture for 3 days. Cells were analyzed by RT-qPCR and Western blotting. A-B, bars show TIEG1 mRNA levels and immunoblots show TIEG1 proteins levels in CD4⁺ T cells. TLR4d: TLR4-deficient CD4⁺ T cells. MD-2d: MD-2-deficient CD4⁺ T cells. Control: CD4⁺ T cells were treated with control CRISPR reagents. C-D, bars show Foxp3 mRNA and immunoblots show Foxp3 protein levels in CD4⁺ T cells. The data of bars are presented as mean $\pm$ SEM. Each sample was analyzed in triplicate. Each experiment was repeated 3 times. Statistics: ANOVA followed by the Bonferroni test.

Figure 5. AEC-derived, CD83 and Ag/MHC II complex-carrying exosomes (83Oexo) induce Treg differentiation. A-B, DO11.10 or BALB/c CD4⁺ CD62L⁺ T cells (Teff) were cultured in the presence of 83Oexo or control exosomes at 20 µg protein/ml for 5 days. The cells were then analyzed by flow cytometry. A, gated flow cytometry plots show Treg counts. B, bars show mean ± SEM of 3 independent experiments. C-D, DO11.10 CD4⁺ T cells (labeled with CFSE) were cultured in the presence of OVA (the specific Ag; used to activate T cells) with or without the presence of Treg or naive CD4⁺ T cell (nTC) at a ratio of 1:5 (Treg:Teff); DCs were present at 1:5 (DC:T cell). Three days later, cells were analyzed by flow cytometry. C, gated histograms show proliferating cell counts. D, bars show mean ± SEM of proliferating Treg counts of 3 independent experiments. E-G, DO11.10 mice and BALB/c mice were treated with 83Oexo (via nasal instillation; 50 µl per nostril; 1 mg 83Oexo protein/ml) daily for 1 week. Mice were sacrificed 2 days after the last instillation. Lungs were excised; single cells were prepared with the lung tissues and analyzed by flow cytometry. E, gated plots show Treg counts. F, bars show mean ± SEM of Treg counts from 6 mice per group. *p<0.01, compared with group a (ANOVA followed by the Dunnett's test). Each sample was analyzed in triplicate. Group labels below bar graph x axis and immunoblots are the same as flow cytometry plot subpanels on the left side.

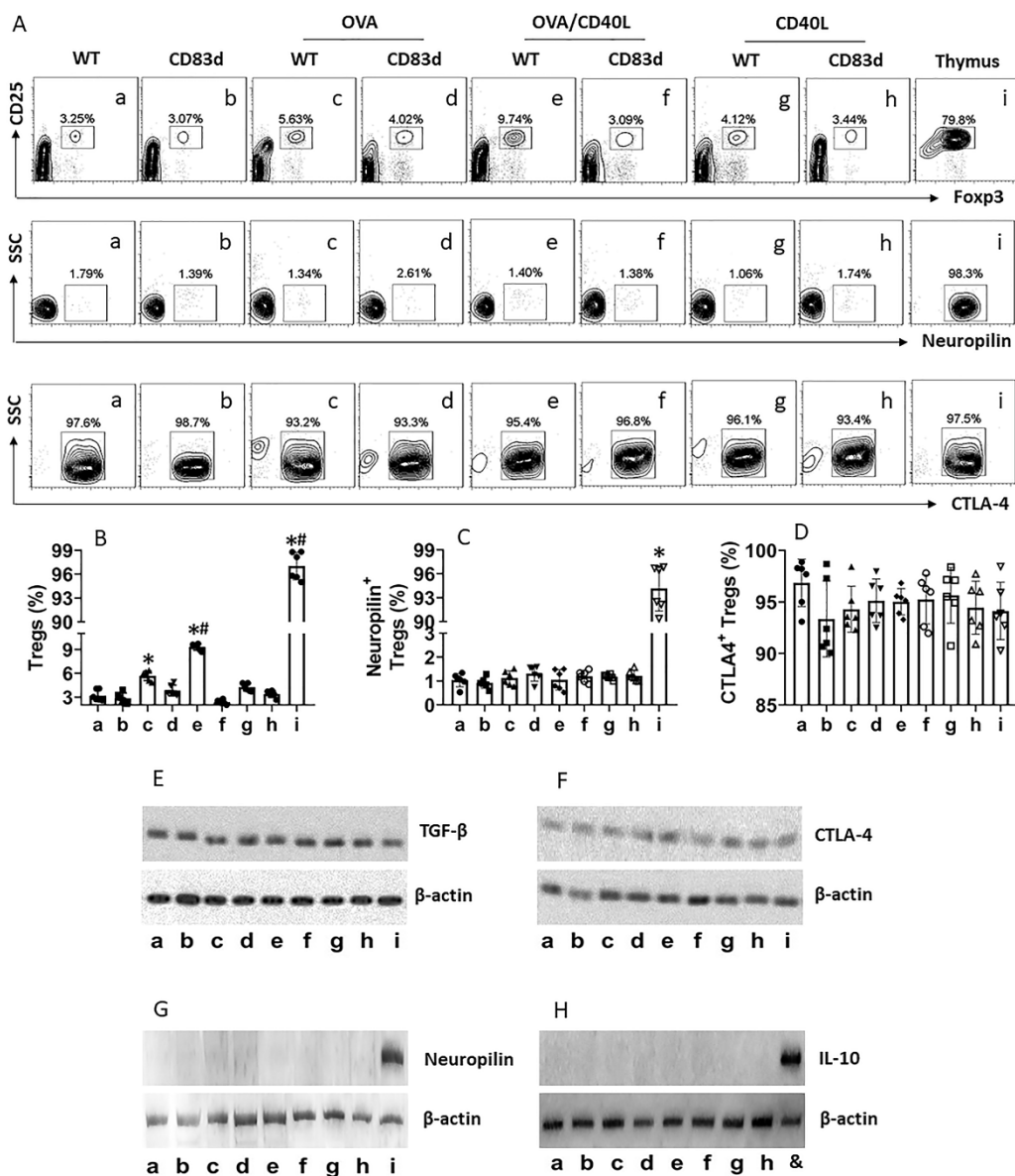
Figure 6. 83Oexo suppresses allergic response in the airway tissues. An airway allergy mouse model was developed. Upon completing the sensitization, sensitized (sens, in short) mice were treated with 83Oexo (via nasal instillation; 50 µl per nostril; 1 mg 83Oexo protein/ml) daily for 1 week, or control exosomes; or treated with anti-CD25 Ab (αCD25) before the 83Oexo treatment. A, airway resistance records of mice in response to the methacholine challenge. B, specific IgE (sIgE) levels in BALF (bronchoalveolar lavage fluid). C, eosinophil counts in BALF. D, gated flow cytometry plots show Eo counts in BALF. E-G, Th2 cytokine levels in BALF. Each group consists of 6 mice. Data of bars are presented as mean ± SEM of 6 mice per group. Each sample was analyzed in triplicate. *p<0.0001 (ANOVA followed by the Bonferroni test), compared with the "sens" group mice. 83Oexo: exosomes contain CD83 and OVA/MHC II complexes. 83exo: exosomes contain CD83. Oexo: exosomes contain OVA. Eexo: empty exosomes. Bexo: exosomes contain BSA instead of OVA (used as a control Ag). CD83d: mice with CD83-deficient epithelial cells. CD40Lo: mice were treated with a mixture containing CD40L (10 mg/ml)/OVA (1 mg/ml) at

50 μ l per nostril daily for 1 week. α CD25 (cAb): sens mice were treated with anti-CD25 Ab (or isotype IgG) prior to administration with 83Oexo.

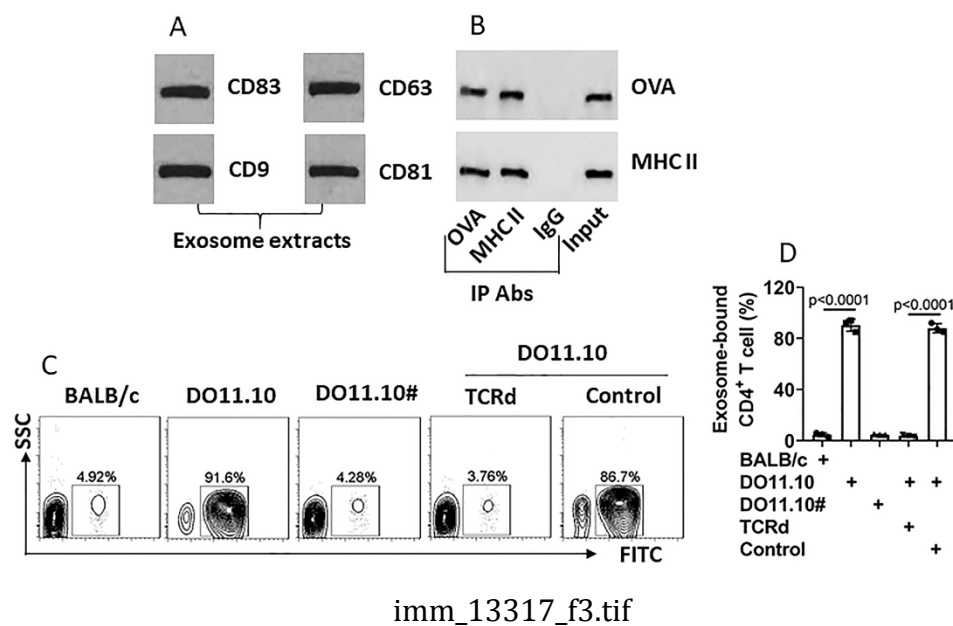
Figure 7. 83Oexo induces antigen-specific Tregs in the airways. Sensitized mice (Sens) were treated with 83exo or 83Oexo as described in figure 6. BALF was collected from mice upon the sacrifice. Single cells were isolated from BALF, cultured in the presence of OVA (5 μ g/ml; the specific antigen) or BSA (5 μ g/ml; an irrelevant antigen) overnight and analyzed by flow cytometry. A, gated plots show Treg frequency in BALF-isolated cells. B, bars show summarized Treg counts in BALF. C, gated histograms show CD154⁺ (CD154 is a marker of T cell activation) Treg frequency. D, bars show summarized CD154⁺ Treg counts. Each group consists of 6 mice. *, $p < 0.05$ (ANOVA followed by the Dunnett's post hoc test), compared with group b.

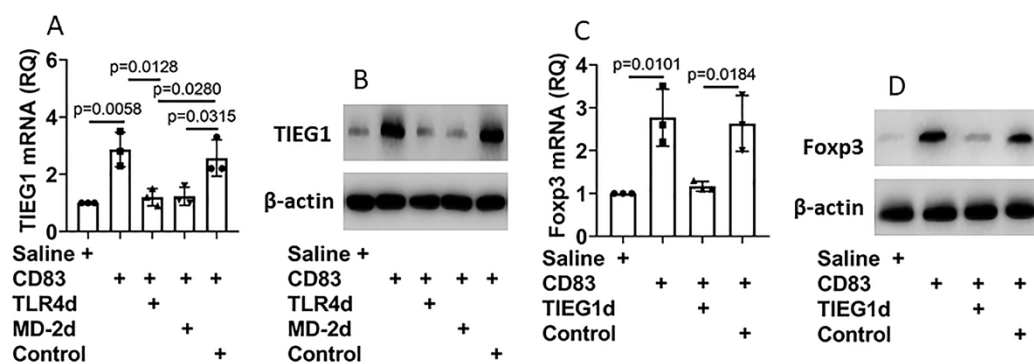


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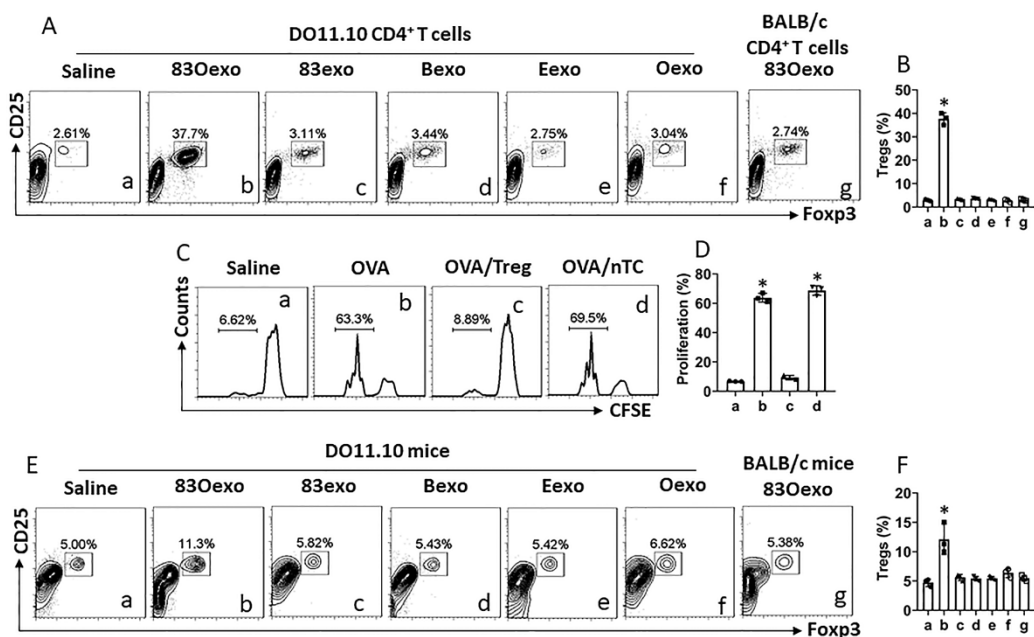


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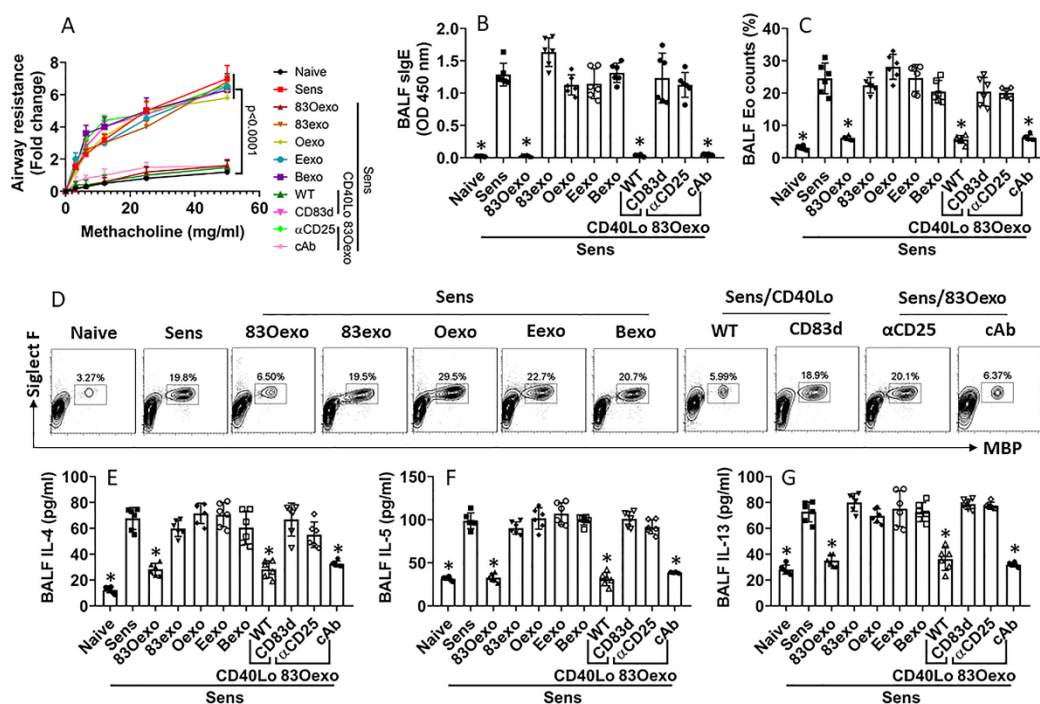




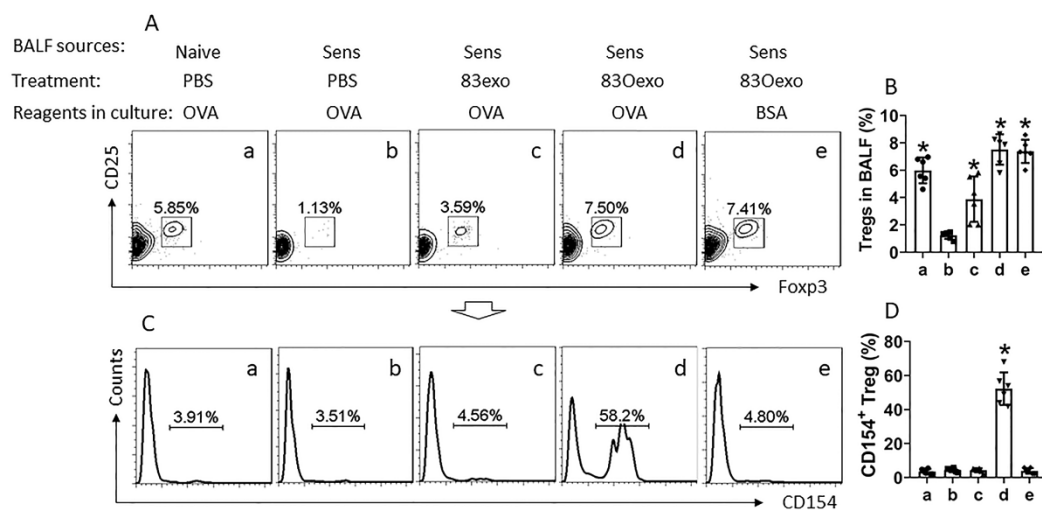
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Supplemental materials

Cell culture

Cells were cultured in RPMI1640 medium supplemented with penicillin (100 U/ml), streptomycin (0.1 mg/ml), fetal bovine serum (10%) and glutamine (2 mM). Cell viability was greater than 99% as assessed by Trypan blue exclusion assay.

Flow cytometry

In surface staining, cells (10^6 cells/sample) were stained with fluorescence-labeled antibodies of interest (diluted to 1:100) or isotype IgG for 30 min on ice. In intracellular staining, cells were fixed with 1% paraformaldehyde (containing 0.05% Triton X-100 to increase the membrane permeability) for 1 h at room temperature; washed with phosphate-buffered saline (PBS), and then followed the procedures of surface staining. The cells were analyzed with a flow cytometer (FACSCanto II, BD Bioscience). Data were analyzed with software package FlowJo (TreeStar, Oakland, OR). Data obtained from isotype IgG staining were used as gating reference.

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 reagent kit following the manufacturer's instruction. Samples were then amplified in a qPCR device (CFX96 Touch System; Bio-Rad) with SYBR Green Master Mix and relevant primers, including CD83 (gctctcctatgcagtgctct and actctgtagcttcttgggg), TIEG1 (catccgtcacacagctgatg and tgtctctgaggaaggcacag) and Foxp3 (cacccaggaaagacagcaac and ctgcaccacttctcttggga). The results were calculated with the $2^{-\Delta\Delta C_t}$ method and presented as relative quantification (RQ).

Protein extraction

Cells were harvested from relevant experiments and lysed with a lysis buffer (10 mM HEPES; 1.5 mM MgCl₂; 10 mM KCl; 0.5 mM DTT; 1 mM EDTA; 0.05% NP40 and protease inhibitor cocktail). Lysates were centrifuged at 13000 g for 10 min. Supernatant was collected and used as the cytosolic proteins. Pellets were resuspended in a nuclear lysis buffer (5 mM HEPES; 1.5 mM MgCl₂SO₄; 4.6 M NaCl; 0.2 mM EDTA; 0.5 mM DTT; 26% glycerol) and incubated for 30 min. Lysates were centrifuged at 13000 g for 10 min. Supernatant was collected and used as the nuclear proteins. All procedures were carried out at 4 °C.

Western blotting

Total proteins were extracted from cells collected from relevant experiments, fractioned by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and transferred onto a polyvinylidene fluoride (PVDF) membrane. The membrane was incubated with 5% skim milk for 30 min to block non-specific binding; followed by incubating with primary antibodies of interest (diluted to 1:300) overnight at 4 °C, washed with TBST (Tris-buffered saline containing 0.05% Tween 20) 3 times, incubated with peroxidase-labeled secondary antibodies (diluted to 1:5000) for 2 h at room temperature. Immunoblots on the membrane were developed by the enhanced chemiluminescence and recorded with an imaging device.

Immunoprecipitation (IP)

All procedures were carried out at 4 °C. Proteins were precleared by incubating with protein G agarose beads for 2 h. The beads were removed from the samples by centrifugation at 5,000 g for 10 min. Antibodies of interest were added to the supernatant and incubated overnight. Immune complexes in the samples were precipitated by incubating with protein G agarose beads for 2 h. The beads were collected by centrifugation at 5,000 g for 10 min. Proteins on beads were eluted with an eluting buffer and analyzed by Western blotting.

Generation of mice with *CD83*-deficient epithelial cells (*CD83d* mice)

The *CD83* gene in epithelial cells were specifically knocked out in C57BL/6 mice through genetic engineering approach. To avoid affecting the role of *CD83* in other cells, such as immune cells, the *CD83* gene was specifically depleted from epithelial cells with EpCAM as the specific cell marker. Following published protocols (Elife 2014; 3: e01949; J Clin Invest 1996; 98: 600-3), we firstly developed mice with loxP-flanked *CD83* gene (flox). A gene targeting vector containing 3 loxP sites was constructed, of which 2 of them flanking the neomycin resistance gene. The genomic locus was modified between vector and the *CD83* gene in embryo stem (ES) cells by homologous recombination approach. The loxP-flanked *CD83* gene was targeted by EpCAM-Cre expression in ES cells (EpCAM is specifically expressed in epithelial cells, including airway epithelial cells; Hasegawa K, et al. Fraction of MHCII and EpCAM expression characterizes distal lung epithelial cells for alveolar type 2 cell isolation. Respir Res. 2017; 18: 150). Using the modified ES cells, a mouse line containing loxp-*CD83* was generated. Then, the mouse strain harboring two loxP sites in the *CD83* gene was crossed with another mouse strain expressing EpCAM-Cre recombinase. Thus, only in cells expressing EpCAM-Cre the *CD83* gene becomes inactivated, the *CD83* gene remained active in other cells of the body. To keep the *CD83* gene active until the experimental period, the Cre gene was modified to be functional only in the ribosomes, but could not penetrate the nuclear membrane. Thus, the loxp-*CD83* gene was remained intact in the mice. One week prior to the experiments, the mice were fed with tamoxifen citrate salt (Sigma Aldrich, St. Louis, MO; 0.3 g/kg in 0.2 ml 10% Etoh in corn oil) daily for 5 consecutive days. Control mice received oral gavage of 0.2 ml 10% EtoH in oil. Tamoxifen increases the permeability of the nuclear membrane to allow the EpCAM-Cre penetrate the nuclei to cleave the flox-*CD83* gene. As assessed by RT-qPCR and Western blotting, all EpCAM⁺ epithelial cells in the airway tissues did not express *CD83* (Fig. S1).

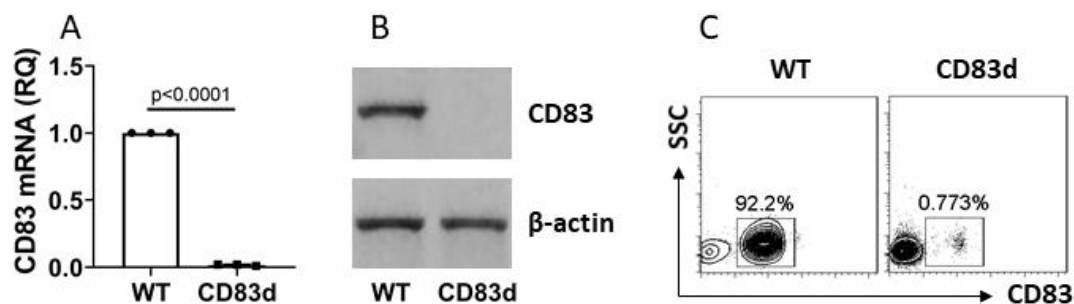


Figure S1. Assessment of *CD83* expression in airway epithelial cells. Airway epithelial cells were isolated from WT mice and *CD83d* mice by flow cytometry with EpCAM as the cell marker.

The cells were analyzed by RT-qPCR, Western blotting and flow cytometry. A, bars show CD83 mRNA levels. B, immunoblots show CD83 protein levels. C, gated flow cytometry plots show CD83⁺ epithelial cell frequency. The data represent 3 independent experiments.

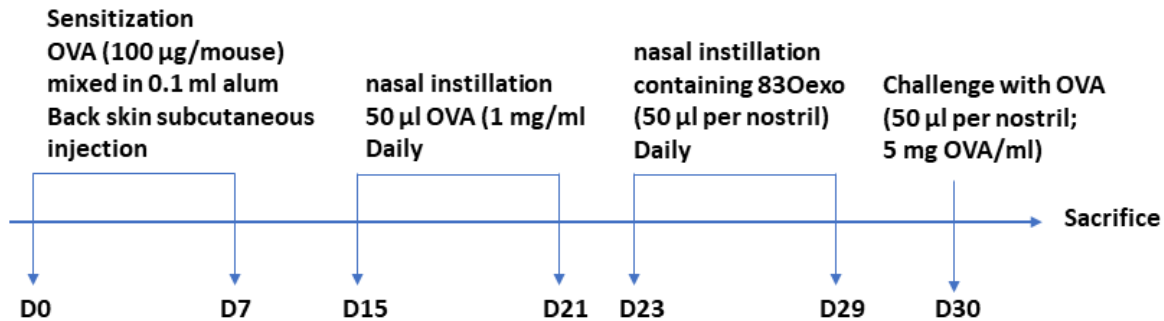


Figure S2. A schematic diagram of mouse sensitization and treatment.

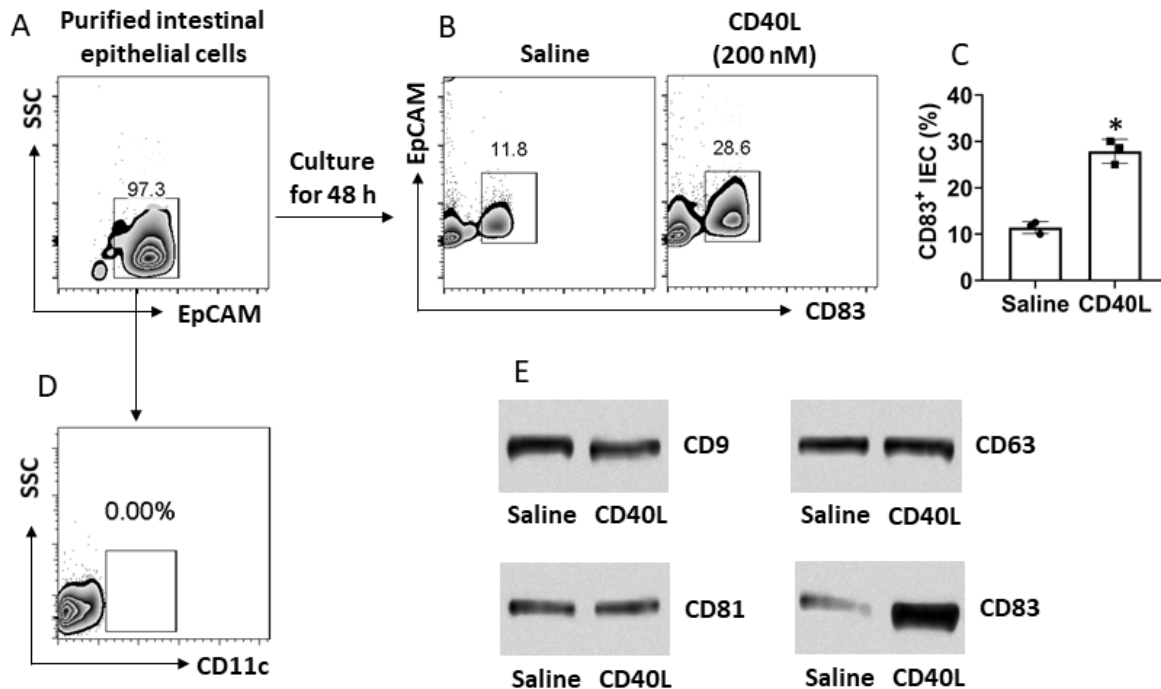


Figure S3. CD83 expression in intestinal epithelial cells (IEC). Primary intestinal epithelial cells were isolated from the naïve BALB/c mouse intestine and purified by FCCS with the EpCAM as a marker of epithelial cells. A, gated plots show purified IEC. B, purified IECs were cultured in Transwells in the presence of CD40L in the culture (RPMI1640 medium) for 48 h. Gated plots show CD83⁺ IECs. C, bars show mean $\pm$ SEM of CD83⁺ IECs from 3 independent experiments. *, $p < 0.001$, compared with the saline group. D, the isolated IECs were also stained with CD11c Ab to see if DCs were mixed in these cells. The results show that no CD11c⁺ DCs were mixed in the isolated epithelial cells. E, culture supernatant was processed for exosome purification as described in the text. Proteins were extracted from the exosomes and analyzed by Western blotting. Immunoblots show exosome markers (CD9, CD63 and CD81) and CD83.

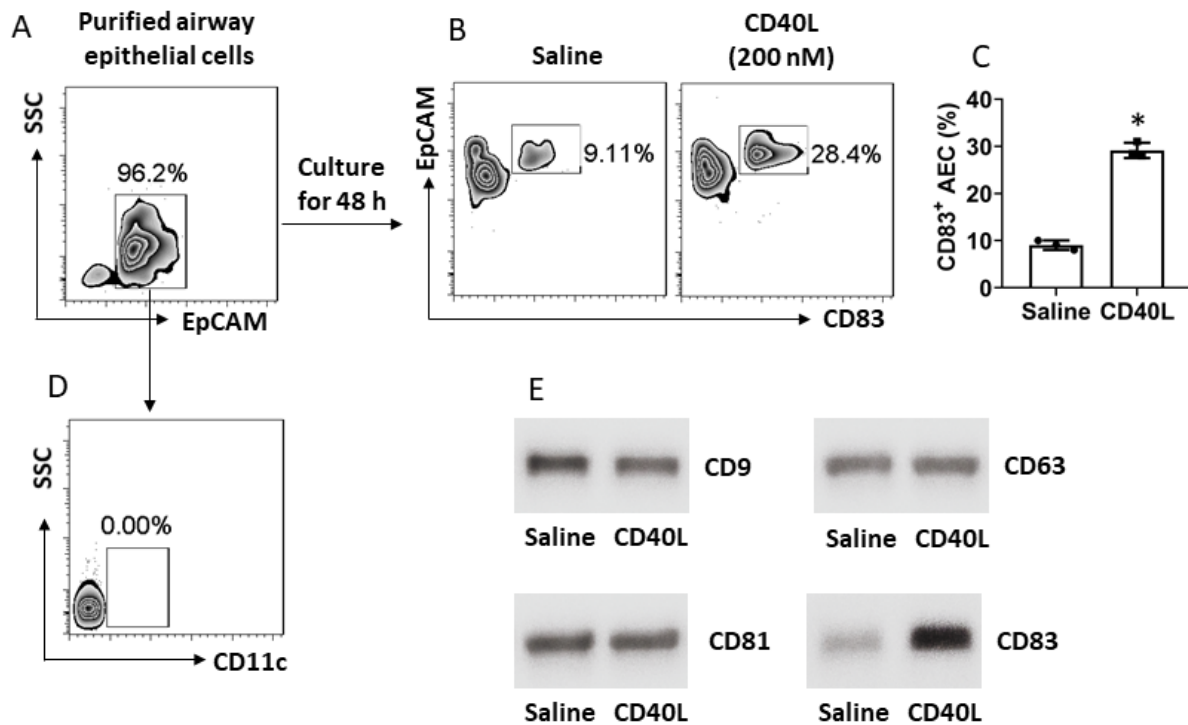


Figure S4. CD40L increases CD83 expression in primary AECs. Primary airway epithelial cells were isolated from the naïve BALB/c mouse airway tissues and purified by FCCS with the EpCAM as a marker of epithelial cells. A, gated plots show purified AEC. B, purified AECs were cultured in Transwells in the presence of CD40L in the culture (RPMI1640 medium) for 48 h. Gated plots show CD83⁺ AECs. C, bars show mean $\pm$ SEM of CD83⁺ AECs from 3 independent experiments. *, $p < 0.001$, compared with the saline group. D, the isolated AECs were also stained with CD11c Ab to see if DCs were mixed in these cells. The results show that no CD11c⁺ DCs were mixed in the isolated epithelial cells. E, culture supernatant was processed for exosome purification as described in the text. Proteins were extracted from the exosomes and analyzed by Western blotting. Immunoblots show exosome markers (CD9, CD63 and CD81) and CD83.

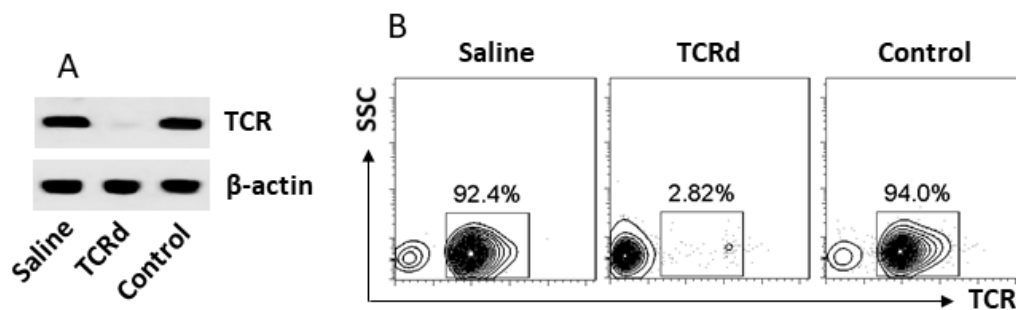


Figure S5. Assessment of TCR levels in CD4⁺ T cells after CRISPR treatment. CD4⁺ CD62L⁺ T cells were isolated from the mouse spleen by flow cytometry cell sorting. The cells were treated with

a commercial TCR CRISPR reagent kit following the manufacturer's instruction. Cells were harvested 48 h after the treatment and analyzed by Western blotting and flow cytometry. A, immunoblots show TCR protein levels. B, gated flow cytometry plots show TCR⁺ cell counts. The data represent 3 independent experiments.

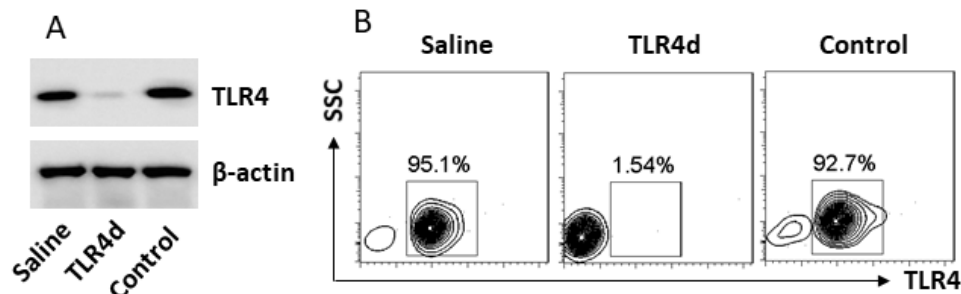


Figure S6. Assessment of TLR4 levels in CD4⁺ T cells after CRISPR treatment. CD4⁺ CD62L⁺ T cells were isolated from the mouse spleen by flow cytometry cell sorting. The cells were treated with a commercial TLR4 CRISPR reagent kit following the manufacturer's instruction. Cells were harvested 48 h after the treatment and analyzed by Western blotting and flow cytometry. A, immunoblots show TLR4 protein levels. B, gated flow cytometry plots show TLR4⁺ cell counts. The data represent 3 independent experiments.

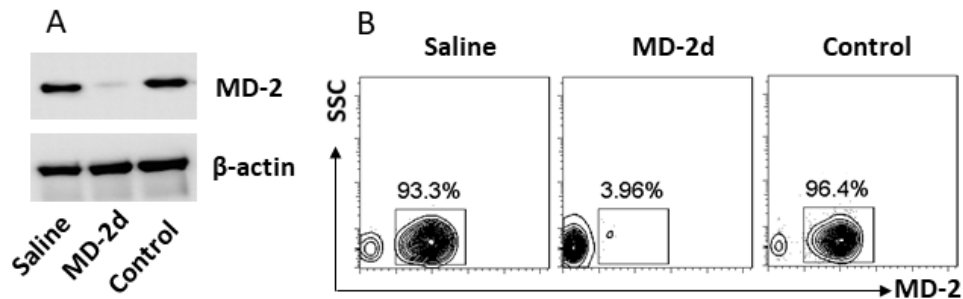


Figure S7. Assessment of MD-2 levels in CD4⁺ T cells after CRISPR treatment. CD4⁺ CD62L⁺ T cells were isolated from the mouse spleen by flow cytometry cell sorting. The cells were treated with a commercial MD-2 CRISPR reagent kit following the manufacturer's instruction. Cells were harvested 48 h after the treatment and analyzed by Western blotting and flow cytometry. A, immunoblots show MD-2 protein levels. B, gated flow cytometry plots show MD-2⁺ cell counts. The data represent 3 independent experiments.

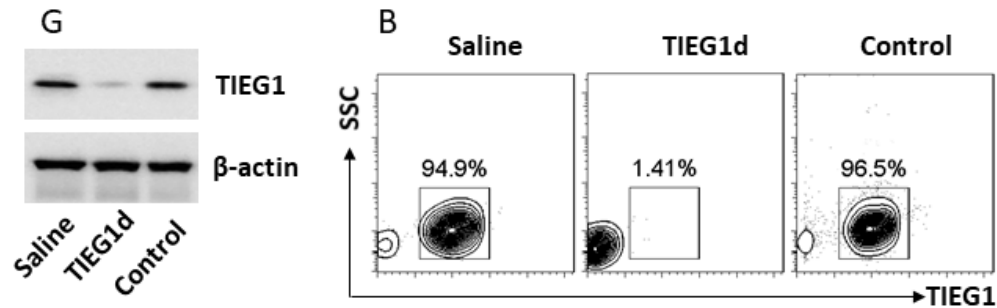


Figure S8. Assessment of TIEG1 levels in CD4⁺ T cells after CRISPR treatment. CD4⁺ CD62L⁺ T cells were isolated from the mouse spleen by flow cytometry cell sorting. The cells were treated with a commercial TIEG1 CRISPR reagent kit following the manufacturer's instruction. Cells were harvested 48 h after the treatment and analyzed by Western blotting and flow cytometry. A, immunoblots show TIEG1 protein levels. B, gated flow cytometry plots show TIEG1⁺ cell counts. The data represent 3 independent experiments.

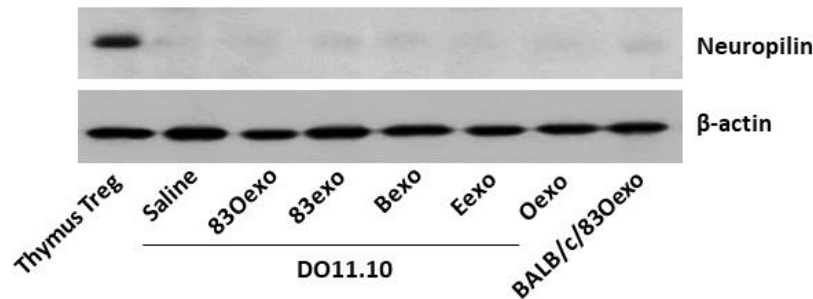


Figure S9. Tregs isolated from the airway mucosa do not express neuropilin. DO11.10 mice and BALB/c mice were treated with exosomes (denoted below the immunoblots). CD4⁺ CD25⁺ CD127⁻ Tregs were isolated from the airway tissues by flow cytometry and analyzed by Western blotting. The immunoblots show neuropilin staining. The results indicate that neuropilin can be detected in Tregs isolated from the thymus; in Tregs isolated from the airway tissues of DO11.10 mice and BALB/c mice, only trifle levels of neuropilin were detected; there is no appreciable difference between samples obtained from DO11.10 mice and BALB/c mice. The results demonstrate that the increased Tregs in the airway tissues are inducible Tregs.