

Adoptive cell therapy of tolerogenic dendritic cells as inducer of regulatory T cells in allergic rhinitis

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Background: Regulatory T cells (Tregs) have become promising candidates for immunotherapy in allergic rhinitis (AR). The contributing role of tolerogenic dendritic cells (tDCs) in the augmentation of Tregs in AR remains to be determined.

Methods: The properties of tDCs in expanding Tregs and their potential to ameliorate AR were evaluated in vitro and in vivo.

Results: Monocyte-derived tDCs stimulated with *Der-matophagoides pteronyssinus* allergen 1 favored the generation of activated Tregs (aTregs) and suppressed effector T-cell responses in a transforming growth factor-beta/interleukin-10 (TGF- β /IL-10)-dependent manner in vitro. The adoptive transfer of tDCs inhibited allergic air-

way inflammation in the mouse model, whereas depletion of CD25⁺ cells or blocking TGF- β /IL-10 signaling eliminated the inhibitory effect, indicating that Tregs were involved in the anti-inflammatory activity of tDCs.

Conclusion: Our data show that tDCs are a potential therapeutic target in AR. © 2018 ARS-AAOA, LLC.

Key Words:

allergic rhinitis; tolerogenic dendritic cells; regulatory T cells; adoptive transfer

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Allergic rhinitis (AR) and asthma are chronic allergic airway diseases (AADs) that have become significant global health concerns, affecting up to 40% of the population.^{1,2} AADs are characterized by chronic allergic

airway inflammation, preferential T-helper 2 (Th2) immune responses, and enhanced allergen-specific immunoglobulin E (IgE) production.^{3,4}

Recently, cellular immunotherapy for AADs has attracted much attention, and regulatory T cells (Tregs), as the master regulators of all immune responses, are the most promising candidates. Natural Tregs (nTregs) are primarily derived from the thymus, and induced Tregs (iTregs) are differentiated from Foxp3⁺ CD25⁺ CD4⁺ T cells in the periphery or in vitro. Both Tregs maintain immunosuppressive activity in the inflammatory microenvironment and may prevent AADs.⁵⁻⁷ However, induction of endogenous Tregs by allergen-specific immunotherapy (SIT)⁸ and the adoptive transfer of exogenous Tregs⁹ have a number of limitations and fatal risks, such as functional insufficiency and severe systemic side effects.¹⁰

Dendritic cells (DCs) are a heterogeneous group of potent antigen-presenting cells (APCs) that direct host immune responses toward either immunity or tolerance, and are called inflammatory dendritic cells (infDCs) or tolerogenic dendritic cells (tDCs), respectively. tDCs, conceivably corresponding to functional subtypes rather than to unique DC lineages, have been reported to potentially induce tolerance via promoting Treg-cell conversion from naive T cells and/or expanding pre-existing Treg cells.¹¹⁻¹³ To date,

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although phase I trials using tDCs as inducer of Tregs have been conducted in autoimmune disease,^{14,15} such as type I diabetes and rheumatoid arthritis, it remains far from certain whether tDCs could be used as cellular immunotherapy candidates in restoring Tregs of the patients with AR.

In this study, we found that monocyte-derived tDCs stimulated by *Dermatophagoides pteronyssinus* allergen 1 (Derp1) favored the differentiation and expansion of CD45RA⁺Foxp3^{high}-activated Tregs (aTregs), a functionally suppressive subset of Tregs first described by Sakaguchi et al,¹⁶ and suppressed effector T-cell responses in a transforming growth factor-beta/interleukin-10 (TGF- β /IL-10)-dependent manner in vitro. In vivo study showed that the adoptive transfer of bone marrow-derived tDCs from normal mice significantly reduced the allergic airway inflammation by inducing Tregs in allergic model mice. Collectively, our results reveal tDCs as a potential therapeutic target to restore impaired respiratory immunity in AR.

Materials and methods

DC differentiation and stimulation

This study was approved by the ethics committee of The First Affiliated Hospital, Sun Yat-sen University, and informed consent was obtained from each healthy donor (No. 2016–196). CD14⁺ monocytes were isolated from human peripheral blood mononuclear cells (PBMCs) of healthy donors by positive selection using anti-CD14-conjugated magnetic microbeads (MACS; Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's instructions. tDCs were differentiated by culturing CD14⁺ cells with human granulocyte-macrophage colony-stimulating factor (GM-CSF; 50 ng/mL; R&D Systems, Minneapolis, MN), IL-4 (10 ng/mL; R&D), TGF- β (40 ng/mL; R&D), and IL-10 (20 ng/mL; R&D) for 7 days in RPMI 1640 medium containing 100 U/mL penicillin/streptomycin (Sigma-Aldrich, St Louis, MO) and 10% fetal bovine serum (FBS; Gibco/Thermo Fisher, Waltham, MA). During the last 2 days, DCs were stimulated by adding extractive purified endotoxin-free Derp1 (1 μ g/mL; Indoor Biotechnology, Charlottesville, VA). tDCs and tDCs stimulated by Derp1 (Derp1-tDCs) were further purified by flow cytometry sorting, gating on TGF- β ⁺ cells. In parallel, immature DCs (imDCs) were differentiated by culturing CD14⁺ cells with GM-CSF (50 ng/mL) and IL-4 (10 ng/mL) for 7 days. During the last 2 days, mature DCs (mDCs) were obtained from imDCs matured with lipopolysaccharide (LPS; 1 μ g/mL; Sigma-Aldrich).

Flow cytometry

DCs were identified by surface marker expression of CD83, CD86, CD80, CD40, and HLA-DR, using fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, phycoerythrin-cyanin-7 (PE-Cy7)-, allophycocyanin (APC)-, and eFluor450-conjugated antibodies (eBioscience, San Diego, CA). Moreover, DCs were stained

with peridinin-chlorophyll-protein complex-cyanin-5.5 (PerCP-Cy5.5)-, APC-, PE-, Brilliant Violet 421-, and PE-Cy7-conjugated antibodies against human immunoglobulin-like transcript 3 (ILT3), immunoglobulin-like transcript 4 (ILT4), inducible T-cell costimulator ligand (ICOS-L), programmed death ligand 1 (PD-L1), and human leukocyte antigen G (HLA-G) (eBioscience). Foxp3⁺CD4⁺ T-cell subsets (CD45RA⁺Foxp3^{low} resting Tregs [rTregs], CD45RA⁺Foxp3^{high} activated Tregs [aTregs], and CD45RA⁺Foxp3^{low}CD4⁺ nonsuppressive T cells [nTregs]), first described by Sakaguchi et al,¹⁶ were identified using anti-CD3-eFluor 605NC, anti-hCD4-FITC, and anti-hCD45RA-eFluor 450 for surface staining; anti-hFoxp3-PE for intracellular staining (eBioscience). Flow cytometry was performed on a cytometer (Cytomics Navios; Beckman Coulter, Mississauga, ON, Canada), and data were analyzed with FlowJo version 10.0 software (Tree Star, Eugene, OR).

Allogeneic mixed-leukocyte reactions

DCs were cultured with allogeneic naive CD4⁺ T cells at a ratio of 1:10, which was normalized for all samples, and the proliferation of CD4⁺ T cells was measured by carboxyfluorescein succinimidyl ester (CFSE). Alternatively, DCs were added to fresh mixed-leukocyte reactions (MLRs) consisting of naive CD4⁺ T cells and APCs (mitomycin C-treated CD3-depleted PBMCs) and analyzed by using enzyme-linked immunosorbent assay (ELISA) and CFSE. In other experiments, tDCs-educated CD4⁺ T cells and culture supernatants (conditioned medium, CM) of tDCs-allogeneic CD4⁺ T-cell cocultures were isolated and used in in vitro suppression assays.

In vitro suppression assays

Naive CD4⁺ T cells were stimulated with soluble anti-CD3/anti-CD28 monoclonal antibodies (mAbs) in the presence or absence of tDCs and/or Transwell culture plates and/or neutralizing antibodies against TGF- β or IL-10R. In parallel, tDCs were added to fresh MLRs in the presence or absence of neutralizing antibodies against TGF- β , IL-10R, ILT3, or ICOS-L. In other experiments, the percentage of aTregs from CD4⁺ T cells stimulated with tDCs and mDCs were analyzed by flow cytometry. Alternatively, tDCs-educated CD4⁺ T cells or CM were isolated from the primary induction cultures and added to fresh MLRs in the presence or absence of neutralizing antibodies against TGF- β (1 μ g/mL; R&D) or IL-10R (1 μ g/mL; R&D).

Cytokine production assays

Cytokines were measured in cell-culture supernatants with commercially available sandwich ELISA kits (R&D Systems) for human interferon-gamma (IFN- γ), IL-12p70, TNF- α , IL-4, IL-13, IL-10, TGF- β and for mice IL-4, IL-5, IL-13, IFN- γ , and ovalbumin (OVA)-specific IgE following the manufacturer's instructions.

Bone marrow-derived tDCs differentiation and mouse model of airway allergic disease

Female BALB/c mice (6 weeks of age) were purchased from the Experimental Animal Center, Sun Yat-sen University (Guangzhou, People's Republic of China), and housed under specific pathogen-free conditions. All procedures were performed according to protocols approved by the institutional animal care and use committee of Sun Yat-sen University (No. IACUC 2016–163XS). Bone marrow-derived tDCs (BM-tDCs) were differentiated by culturing bone marrow cells with murine GM-CSF (40 ng/mL; Peprotech, Rocky Hill, NJ), IL-4 (10 ng/mL; R&D), TGF- β (30 ng/mL; R&D), and IL-10 (20 ng/mL; R&D) for 7 days, and were then purified by flow cytometry sorting.

Upper airway allergic inflammation was induced as previously reported^{17,18} (see Fig. 5A). Thirty-six female BALB/c mice were equally divided into 6 groups in accordance with the differing sensitization, treatment, and challenge. Briefly, the mice were sensitized with 40 μ g of OVA (Sigma-Aldrich) emulsified with 100 μ L of adjuvant aluminum hydroxide (Sigma-Aldrich) in 100 μ L of phosphate-buffered saline (PBS) on days 1, 5, 9, and 13. On days 14 and 17, mice were injected intraperitoneally with neutralizing antibody anti-CD25 mAb (500 μ g/mL; R&D) or anti-TGF- β mAb (500 μ g/mL; eBioscience)/anti-IL-10 mAb (500 μ g/mL; eBioscience).^{19–21} Mice were then treated by adoptive transfer of BM-tDCs on day 20. From days 21–24, the mice were challenged with aerosolized 5% OVA solution for 30 minutes and instilled intranasally with 20 μ L of OVA (40 mg/mL). Samples were collected on day 25.

Evaluation of nasal symptoms

The frequency of sneezing and nose rubbing that occurred in the 10-minute period after the last challenge was determined, as previously reported.²² Mice were subjected to single-blind observation by examiners who had no knowledge of the experimental groups.

Evaluation of inflammatory cells and cytokines in nasal and bronchoalveolar lavage fluids

On day 25, the nasal lavage fluid (NALF) and bronchoalveolar lavage fluid (BALF) were collected. Briefly, the upper level of the trachea was ligated and a 22-gauge catheter was inserted into the nasopharynx. The nasal cavities were gently perfused with 1 mL cold PBS, and NALF was collected from the nose. BALF was obtained after lavage with 1 mL of cold PBS via a 20-gauge needle inserted through the upper part of the trachea. After centrifugation, the cells present within the NALF and BALF were counted using a hemocytometer and then cytospun onto glass slides and stained with Diff-Quick. A total of 300 cells per slide were evaluated for eosinophils, macrophages, neutrophils, and lymphocytes at $\times 400$ magnification, as previously reported.²² The levels of IL-4, IL-5, IL-13, and IFN- γ

in the supernatants were measured using ELISA analysis following the manufacturer's instructions (R&D).

Lung and nasal histology and inflammation scoring

Lung and nasal tissues were removed after the lavage and fixed in 10% neutral formalin for 36 hours. The lungs were then embedded in paraffin, and nasal tissues were embedded in paraffin after being decalcified. Nasal tissues were prepared in a coronal plane at a distance of 5 mm from the nasal vestibule, and lung sections were prepared at a thickness of 4 μ m. The sections were then stained with hematoxylin and eosin (H&E) for nasal tissues, and for lung tissues they were stained with both H&E and periodic acid-Schiff (PAS). The number of eosinophils was evaluated in the submucosal area of the whole nasal septum by microscopy ($\times 400$ magnification). Goblet cell (PAS-positive cell) counts and inflammation score in the lungs were obtained in a blinded fashion using a reproducible scoring system, as previously described.²² Briefly, for quantifying lung inflammation, 5 sections across the main bronchus of each animal were randomly selected and given scores ranging from 0 to 3, based on the level of peribronchial inflammation and perivascular inflammation. The values were given according to the following inflammatory parameters: 0 when no inflammation was detectable; 1 for occasional cuffing with inflammatory cells; 2 for most bronchi or vessels surrounded by a thin layer (1–5 cells) of inflammatory cells; and 3 when most bronchi or vessels were surrounded by a thick layer (more than 5 cells) of inflammatory cells. For quantifying the goblet cell hyperplasia, the percentage of PAS-positive cells in epithelial areas was examined from 8–10 tissue sections per mouse.

Statistical analysis

GraphPad Prism version 6 (GraphPad, La Jolla, CA) was used for statistical analysis. Multiple comparisons of sampled data were performed using the Kruskal-Wallis test. We used a Kruskal-Wallis rank sum test followed by a Mann-Whitney *U* test for 2-group comparisons of the data with an abnormal distribution. We used a Mann-Whitney *U* test or Wilcoxon matched-pairs test for pairwise comparisons. $p < 0.05$ was considered statistically significant. When cumulative data are shown, the data are derived from independent donors, and each value represents a single donor, whereas, when representative data are depicted, values are shown from replicate experiments by using samples from the same donor.

Results

Derp1-tDCs are hyporesponsive to LPS stimulation in vitro

There was a striking decrease in CD83, CD86, CD80, and HLA-DR expression on Derp1-tDCs and tDCs compared with mature dendritic cells (mDCs) (Fig. 1A and B), whereas CD40 expression was similar among mDCs,

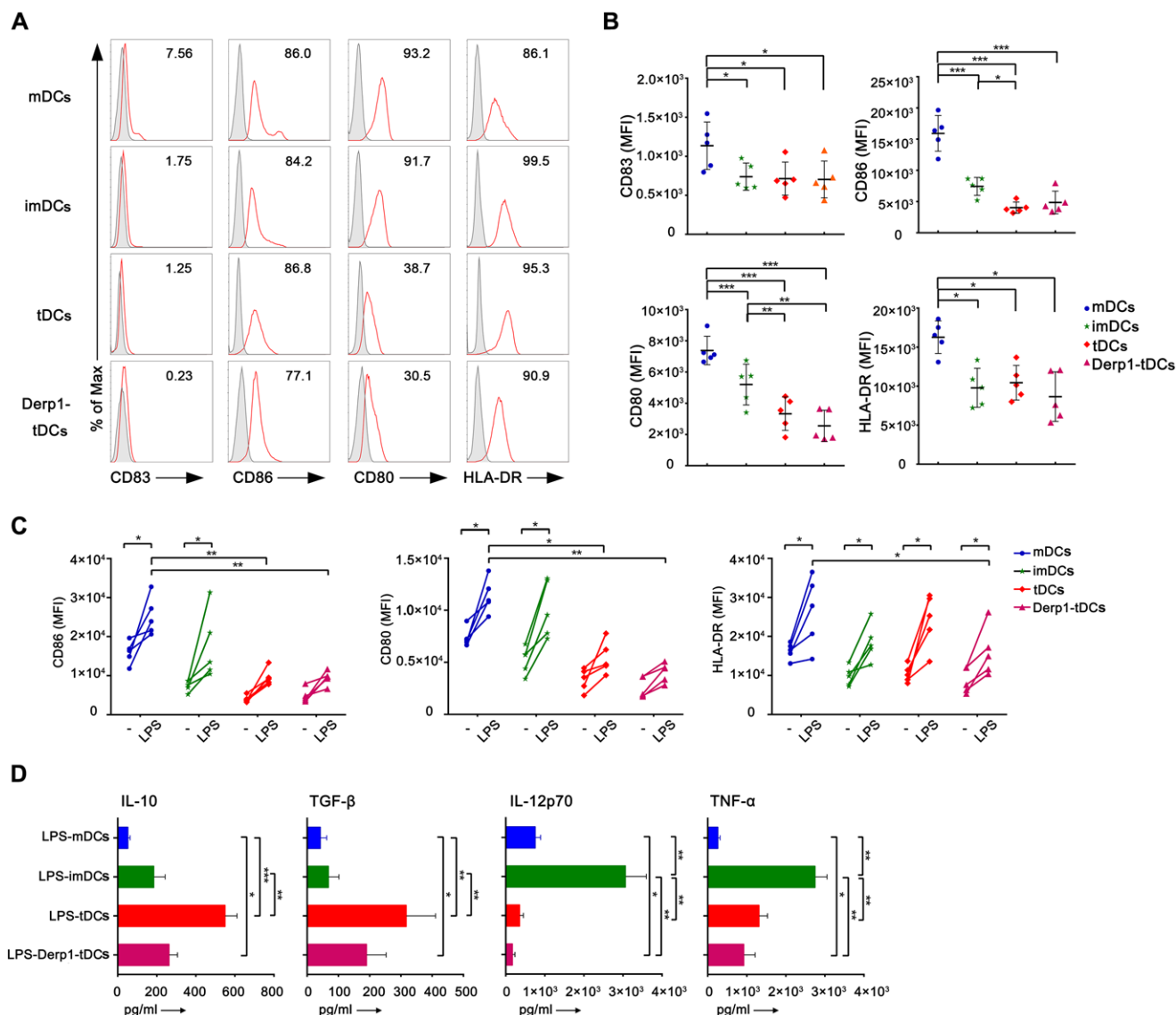


FIGURE 1. Derp1-tDCs are hypo-responsive to LPS stimulation. (A) Surface markers of mDCs, imDCs, tDCs, and Derp1-tDCs: representative histogram plots relative to isotype control (percentages shown; $n = 5$). (B) Surface markers: cumulative mean fluorescence intensities of mDCs, imDCs, tDCs, and Derp1-tDCs (MFIs; $n = 5$). (C) Surface markers of mDCs, imDCs, tDCs, and Derp1-tDCs before/after LPS stimulation ($n = 5$). (D) Cytokines in culture supernatants of mDCs, imDCs, tDCs, and Derp1-tDCs after LPS stimulation ($n = 5$). Relative MFI (rMFI) = (MFI $_{37^{\circ}\text{C}}$ - MFI $_{4^{\circ}\text{C}}$) / MFI $_{4^{\circ}\text{C}}$ ($n = 5$). Values are presented as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. Derp1-tDCs = *Dermatophagoides pteronyssinus* allergen 1 tolerogenic dendritic cells; imDCs - immature dendritic cells; LPS = lipopolysaccharide; mDCs = mature dendritic cells; MFI = mean fluorescence intensity; tDCs = tolerogenic dendritic cells.

immature DCs (imDCs), tDCs, and Derp1-tDCs (data not shown), indicating that Derp1-tDCs and tDCs had a semi-mature phenotype in vitro.

After stimulation with LPS, although the expression levels of CD86, CD80, and HLA-DR were all substantially increased in mDCs and imDCs, the LPS-stimulated Derp1-tDCs and tDCs had only slightly upregulated levels of CD86 and CD80 expression (Fig. 1C). Furthermore, LPS-stimulated Derp1-tDCs and tDCs produced significantly higher levels of the immunomodulatory cytokine TGF- β and IL-10, whereas they secreted lower levels of pro-inflammatory IL-12p70 and TNF- α , compared with LPS-imDCs and LPS-mDCs (Fig. 1D).

Derp1-tDCs possess enhanced tolerogenic properties and inhibit CD4 $^{+}$ T-cell responses in vitro

The aforementioned findings led us to explore whether Derp1-tDCs express cell-surface inhibitory molecules.^{18,19} We found that expression levels of ILT3, ILT4, and ICOS-L were significantly increased on Derp1-tDCs and tDCs compared with those on mDCs, whereas expression of PD-L1 was similar among all tested groups (Fig. 2A and B). We then investigated the immunostimulatory capacity of Derp1-tDCs and tDCs on allogeneic CD4 $^{+}$ T-cell responses. The results show that mDCs had significant alloantigen-driven T-cell proliferation. In contrast, Derp1-

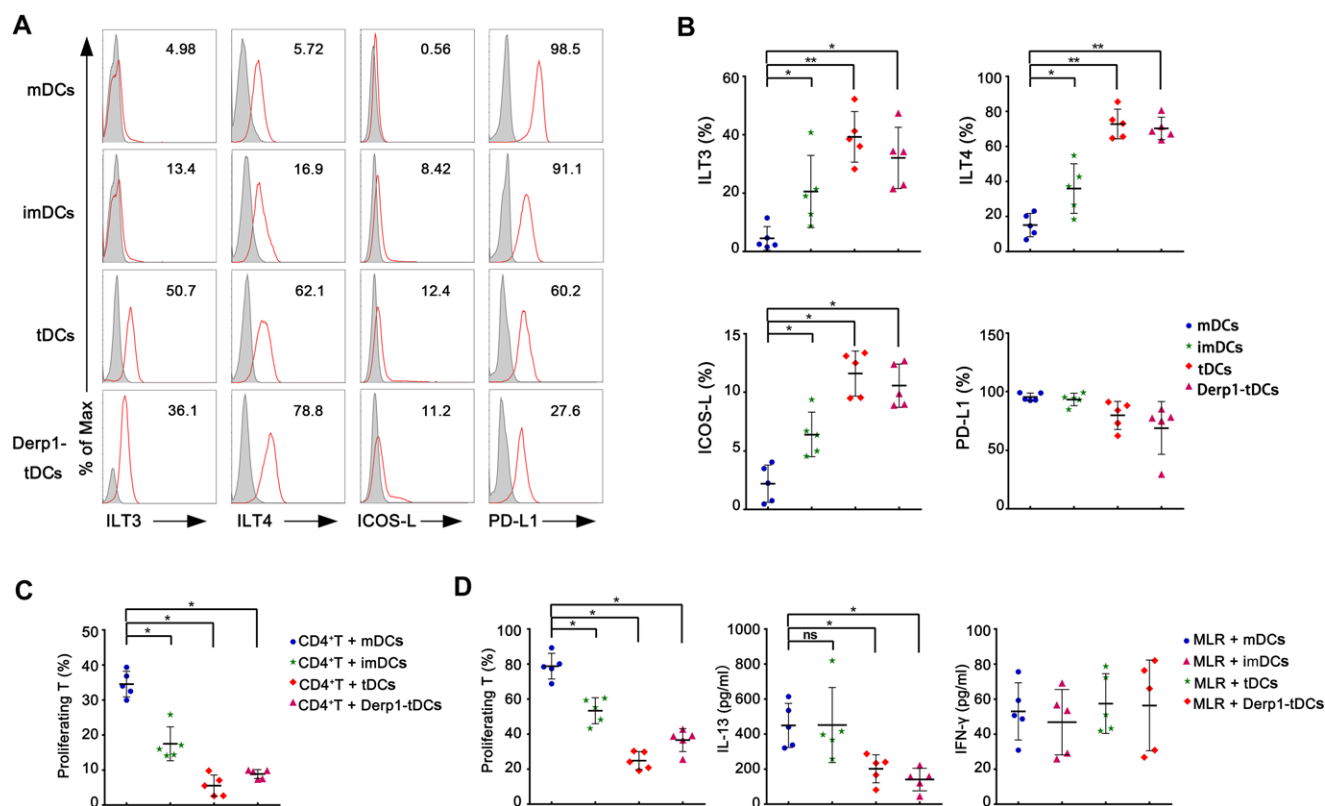


FIGURE 2. Derp1-tDCs possess tolerogenic surface markers and inhibit CD4⁺ T-cell responses. (A) Tolerogenic surface markers of mDCs, imDCs, tDCs, and Derp1-tDCs: representative histogram plots relative to isotype control (percentages shown; $n = 5$). (B) Tolerogenic surface markers: cumulative data of mDCs, imDCs, tDCs, and Derp1-tDCs ($n = 5$). (C) CFSE-labeled proliferation of CD4⁺ T cells stimulated by autologous mDCs, imDCs, tDCs, or Derp1-tDCs ($n = 5$). (D) CFSE-labeled proliferation and cytokines production of CD4⁺ T cells stimulated by allogeneic APCs (MLRs) and mDCs, imDCs, tDCs or Derp1-tDCs ($n = 5$). Values are presented as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. APCs = antigen-presenting cells; CFSE = carboxyfluorescein diacetate succinimidyl ester; Derp1-tDCs = *Dermatophagoides pteronyssinus* allergen 1 tolerogenic dendritic cells; mDCs = mature dendritic cells; imDCs = immature dendritic cells; sMLRs = mixed-leukocyte reactions; SEM = standard error of the mean; tDCs = tolerogenic dendritic cells.

tDCs and tDCs exhibited a severely impaired stimulatory capacity (Fig. 2C), which was not due to induction of T-cell apoptosis (data not shown).

We next explored whether Derp1-tDCs could exert sufficient tolerogenic functions by assessing the ability of Derp1-tDCs to inhibit MLRs²⁰ consisting of naive CD4⁺ T cells stimulated with allogeneic APCs.²¹ Remarkably, both Derp1-tDCs and tDCs could suppress the robust T-cell-proliferative responses and the IL-13 release induced by allogeneic APCs, whereas the mDCs had no striking inhibitory effects (Fig. 2D). These data highlight a dominant tolerogenic effect of tDCs that mitigates the stimulatory capacities of fully competent APCs.

Derp1-tDCs generate aTregs and exert immunosuppressive function via a TGF- β /IL-10-dependent pathway but not intercellular contact

We next sought to delineate whether Derp1-tDC-educated CD4⁺ T cells acquired the suppressive properties or characteristics of Tregs. The results show a significantly increased proportion of aTregs among the total population

of Derp1-tDC-educated CD4⁺ T cells as well as among the Derp1-tDC-educated CD4⁺CD25⁻ T cells (Fig. 3A and B). Subsequently, we observed that Derp1-tDCs significantly suppressed anti-CD3/CD28 mAb-driven CD4⁺ T-cell proliferation in an APC-free system, which was abolished by neutralizing antibodies against TGF- β or IL-10R. In contrast, blocking cell-cell contact via a Transwell filter did not produce a marked proliferation recovery (Fig. 3C). In parallel, Derp1-tDCs were added to fresh MLRs in the presence or absence of neutralizing antibodies against TGF- β , IL-10R, ILT3, or ICOS-L. The presence of anti-TGF- β or anti-IL-10R blocking antibodies significantly impaired the capacity of Derp1-tDCs to restrain allogeneic T-cell responses in MLRs, whereas neither anti-ILT3 nor anti-ICOSL had that effect (Fig. 3D).

Subsequently, we isolated the Derp1-tDC-educated CD4⁺ T cells that were generated in the presence of neutralizing antibodies against TGF- β or IL-10R and added them to fresh MLRs, which largely abolished their suppressive effects on alloantigen-driven T-cell responses (Fig. 3E). Similarly, the addition of CM obtained from the supernatants of Derp1-tDCs/CD4⁺ T-cell cultures to fresh

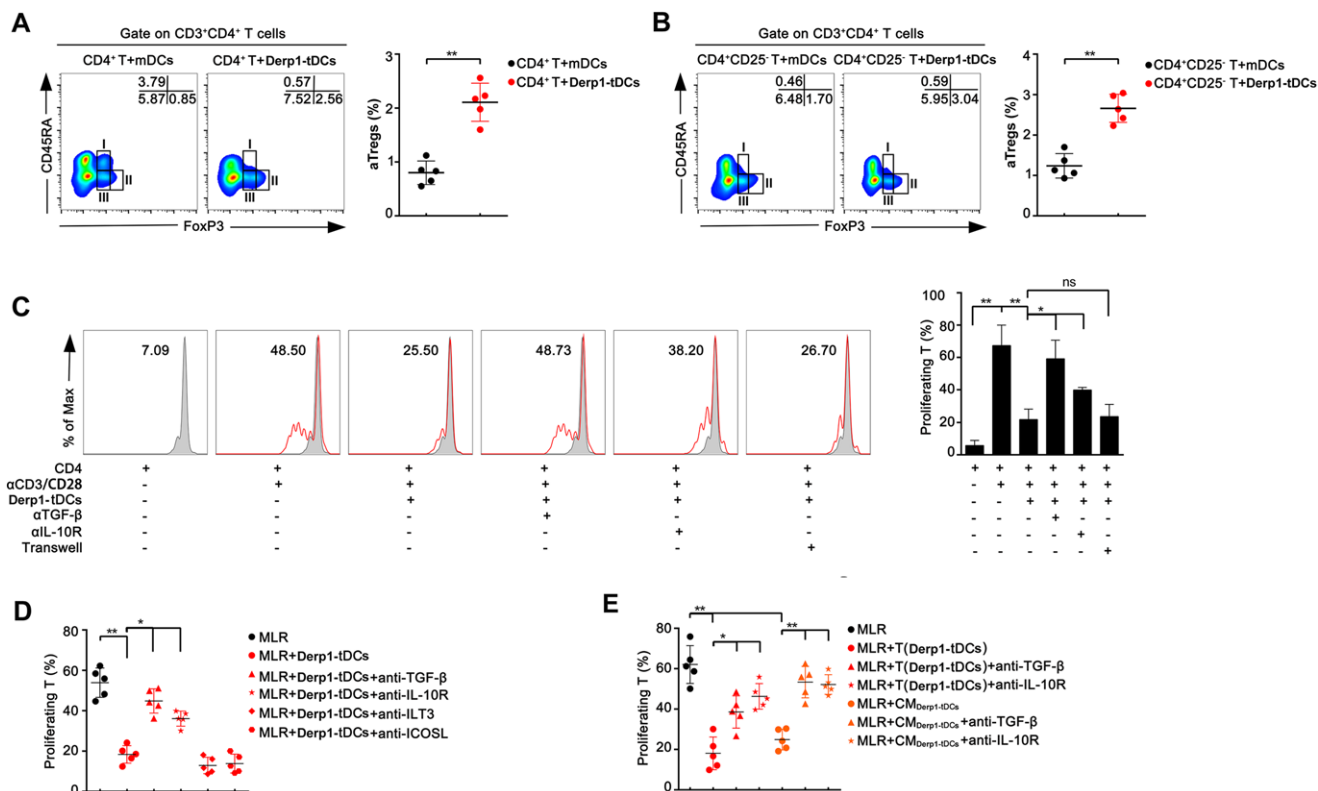


FIGURE 3. Derp1-tDCs induce aTreg and exert immunosuppressive function via a TGF- β /IL-10-dependent pathway but not intercellular contact. (A) Representative Foxp3⁺CD4⁺ T-cell subsets (I = rTregs, II = aTregs, III = nTregs) of CD4⁺ T cells exposed to mDCs and Derp1-tDCs (left panel) and summary data of aTreg frequency (right panel, $n = 5$). (B) Representative Foxp3⁺CD4⁺ T-cell subsets (I = rTregs, II = aTregs, III = nTregs) of CD4⁺CD25⁻ T cells exposed to mDCs and Derp1-tDCs (left panel) and summary data of aTreg frequency (right panel, $n = 5$). (C) Representative anti-CD3/CD28-driven proliferating CD4⁺ T cells with/without Derp1-tDCs and neutralizing antibodies against TGF- β or IL-10R and representative proliferating CD4⁺ T cells in a transwell culture system with Derp1-tDCs (percentages shown, $n = 5$) (left panel) and summary data of proliferating CD4⁺ T-cell frequency (right panel, $n = 5$). (D) Statistical analysis for proliferating T-cell percentage in MLR with/without Derp1-tDCs and neutralizing antibodies against TGF- β , IL-10R, ILT3, or ICOSL ($n = 5$). (E) Statistical analysis for proliferating T-cell percentage in MLR with/without Derp1-tDC-educated T cells [T(Derp1-tDCs)], conditioned medium of Derp1-tDCs/CD4⁺ T-cell cocultures (CMDerp1-tDCs), and neutralizing antibodies against TGF- β or IL-10R ($n = 5$). Values are presented as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. aTregs = activated regulatory T cells; CM = conditioned medium; Derp1-tDCs = *Dermatophagoides pteronyssinus* allergen 1 tolerogenic dendritic cells; IL-10R = interleukin-10 receptor; MLR = mixed-leukocyte reaction; TGF- β = transforming growth factor-beta; SEM = standard error of the mean.

MLRs induced a significant decrease in allogeneic T-cell proliferation, which was abolished by anti-TGF- β or anti-IL-10R (Fig. 3E). Moreover, CTLA-4, HLA-G, ICOS, and PD-1 were equivalently expressed among groups of CD4⁺ T cells educated by different types of DCs (see Fig. S1 in the supplementary material online).

Adoptive transfer of BM-tDCs suppresses inflammation in allergic airway mouse model

To assess whether upregulating tDCs in vivo could relieve allergic airway inflammation, we utilized a mouse model of OVA-induced allergic airway inflammation (Fig. 4A). We then adoptively transferred BM-tDCs of normal mice to OVA-induced allergic model mice to determine whether BM-tDCs played a pivotal role in the immunomodulation of allergic airway inflammation. The adoptive transfer of BM-tDCs suppressed allergic nasal symptoms (Fig. 4B) and reduced inflammatory cell infiltrates and goblet cell hyperplasia in the lungs as well as eosinophilic infiltrates in the nose in OVA-induced allergic mice (Fig. 4C and D).

We next examined the effect of BM-tDCs on OVA-induced inflammatory cell profiles in the BALF and NALF. We observed a significant inhibition of eosinophilic and neutrophilic airway inflammation in the OVA-induced allergic model mice after adoptively transferring BM-tDCs into these mice (Fig. 5A). Furthermore, we confirmed a significant elevation in OVA-specific serum IgE levels in our allergic model mice, and these levels were significantly decreased after the transfer of BM-tDCs (Fig. 5B).

In addition, we determined the levels of Th-cell-derived cytokines that were present in the BALF and NALF. Compared with normal mice, untreated OVA-induced model mice showed higher levels of IL-4, IL-5, and IL-13 in both BALF and NALF, which are characteristic of Th2 allergic inflammation. Notably, the transfer of BM-tDCs significantly reduced the IL-4, IL-5, and IL-13 levels in both the BALF and NALF. Similar findings were obtained for IFN- γ in the BALF (Fig. 5C and D). In contrast, the levels of IL-10 and TGF- β , which are typical immunoregulatory cytokines, were increased in both BALF and NALF after the transfer of BM-tDCs (Fig. 5C and D), indicating that

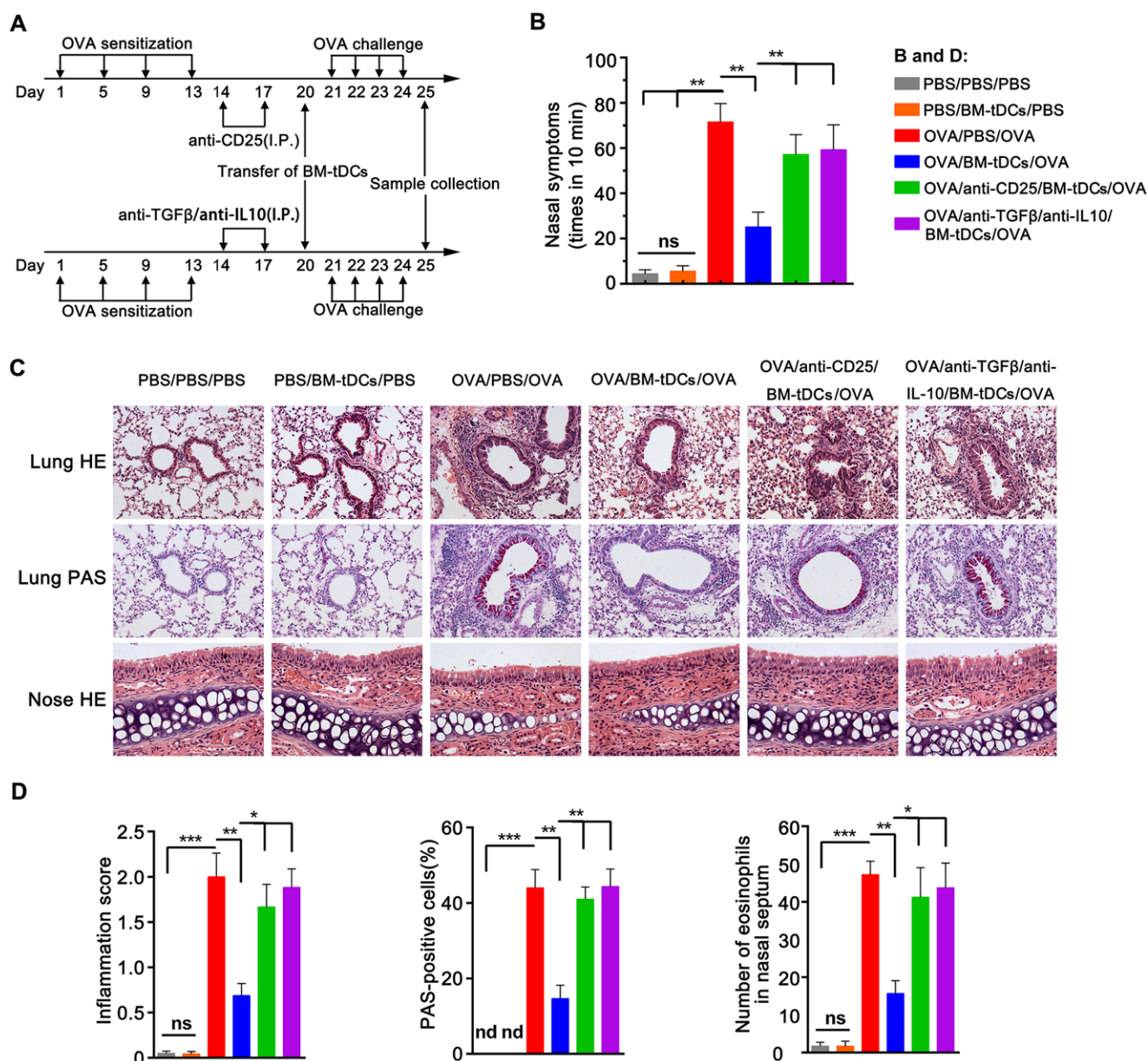


FIGURE 4. Adoptive transfer of BM-tDCs attenuated nasal symptoms and OVA-induced airway inflammation of lungs and noses. (A) Experimental protocol for allergic mice models. (B) Nasal symptom score of mice in different groups ($n = 6$). (C) Representative photomicrographs of H&E- and PAS-stained lung sections and H&E-stained nose sections of different groups. (D) Statistical analysis for inflammation score and mucus hypersecretion as quantified by PAS scores of lungs, and eosinophilic infiltration of nasal submucosa ($n = 6$). Values are presented as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. BM-tDCs = bone marrow-derived tolerogenic dendritic cells; H&E = hematoxylin-eosin; OVA = ovalbumin; PAS = periodic acid-Schiff; SEM = standard error of the mean.

BM-tDCs may mediate a potent direct anti-inflammatory activity via secretion of TGF- β and IL-10.

Taken together, our findings show that the transfer of BM-tDCs suppressed allergic airway inflammation in the OVA-induced model mice and suggest that tDCs could be a therapeutic target for AR treatment.

TGF- β /IL-10 signaling controls differentiation of Tregs by BM-tDC in the mouse model

To learn more about the tolerogenic and immunosuppressive properties of BM-tDCs, we further investigated whether

BM-tDCs play a direct role in reducing the amount of Tregs via TGF- β /IL-10 signaling in vivo.

The depletion of Tregs by anti-CD25-neutralizing antibodies or blocking TGF- β /IL-10 signaling with anti-TGF- β /anti-IL-10R concomitant neutralizing antibodies abrogated the capacity of BM-tDCs to inhibit allergic inflammation in OVA-induced allergic mice, including allergic nasal symptoms, eosinophilic infiltrates in lung and nose, inflammatory cell and Th2 cytokines in BALF and NALF, and OVA-specific serum IgE levels (Figs. 4 and 5). Collectively, these data suggest a potential direct role for Tregs

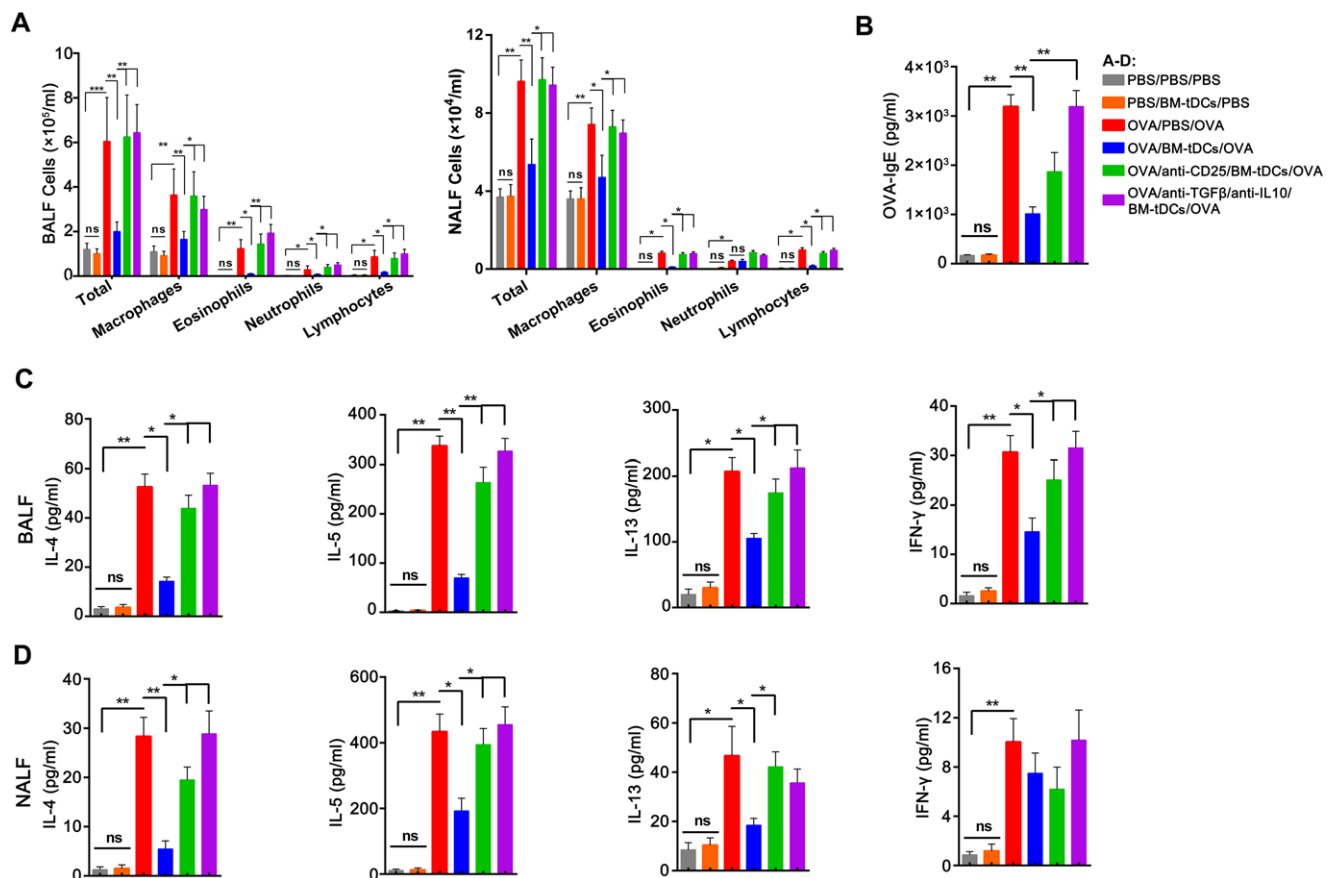


FIGURE 5. Adoptive transfer of BM-tDCs decreased OVA-specific IgE levels and inhibited inflammatory cell infiltration and cytokine production in the BALF and NALF. (A) Inflammatory cell counts in BALF and NALF ($n = 6$). (B) Serum OVA-specific IgE ($n = 6$). (C) Inflammatory cytokines in BALF ($n = 6$). (D) Inflammatory cytokines in NALF ($n = 6$). Values are presented as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. BALF = bronchoalveolar lavage fluid; BM-tDCs = bone marrow-derived tolerogenic dendritic cells; IgE = immunoglobulin E; NALF = nasal lavage fluid; OVA = ovalbumin; SEM = standard error of the mean.

and TGF- β /IL-10 signaling in the anti-inflammatory activity of BM-tDCs. Moreover, blocking TGF- β /IL-10 signaling by neutralizing antibodies can significantly inhibit the generation of Tregs rather than tDCs in the lung (see Fig. S2 in Supplementary Material online), suggesting a critical role for TGF- β /IL-10 signaling in the tDC-mediated expansion or generation of Tregs. Overall, our data suggest that BM-tDCs could reduce allergic airway inflammation by inducing Tregs through TGF- β /IL-10 signaling.

Discussion

Previous studies have demonstrated a pivotal role for Tregs in suppressing polarized Th2-cell responses and inducing immune tolerance to allergens.^{23,24} However, little is known about how to reverse the Treg defect in patients with AR. Increasing evidence from basic and clinical research has revealed the limitations, disadvantages, and risks of inducing and/or expanding Tregs by SIT⁸ or the adoptive transfer of exogenous Treg,⁹ so it is necessary to explore new therapeutic targets for AR immunotherapy.

Advances in immunology over the past decade have revealed a fundamental role for DCs in sensing pathogens and

directing host immune responses toward either immunity or tolerance.¹³ One hypothesis is that allergen-specific DC specialization promotes and sustains the polarized Th-cell responses that characterize AR²⁵ and chronic rhinosinusitis with nasal polyps (CRSwNP).²⁶ Although much is known about the control of Th1- and Th2-cell responses by inflammatory DCs,^{23,27,28} the presence and the functional specialization of tDCs in AR remain obscure. Our study suggests that tDCs restrained Th2-cell effector functions and favored the generation of Tregs through the TGF- β /IL-10 signaling pathway. Restoring defective immunomodulatory functions of tDCs may emerge as a powerful tool to redress the aberrant respiratory immunity and to control excessive respiratory allergic inflammation.

The *in vitro* study showed that Derp1-tDCs exhibited a semimature phenotype and secreted lower levels of pro-inflammatory cytokines and higher levels of inhibitory cytokines in response to the maturation stimuli of LPS. Consistently, studies showed that tDCs exhibit an incomplete mature and inhibitory phenotype, called the “steady state.”²⁹ We hypothesized that the observed decrease in costimulatory molecule expression may be associated with the enhanced immunosuppressive functions of Derp1-tDCs.

In line with our hypothesis, the results show that Derp1-tDCs were able to induce allogeneic CD4⁺ or CD4⁺CD25⁻ T cells to differentiate into immunosuppressive aTregs in vitro and suppress the robust T-cell responses of MLRs or anti-CD3/CD28 mAb-driven T-cell proliferation and downregulate the production of Th2-cell-derived cytokines via TGF- β - and IL-10-dependent mechanisms. In further analyses of the immunosuppressive function of Derp1-tDC-educated CD4⁺ T cells, we found that they exhibited a strong inhibitory function, although CD4⁺ T cells stimulated with Derp1-tDCs did not express enhanced levels of CTLA-4, HLA-G, ICOS, and PD-1. Collectively, our findings suggest that Derp1-tDCs may exert their immunosuppressive function via inducing Tregs.

To identify whether tDCs could restrain allergic inflammation in vivo by inducing suppressive Tregs in AR and to better understand the tolerogenic properties and potential therapeutic function of tDCs, we then utilized an

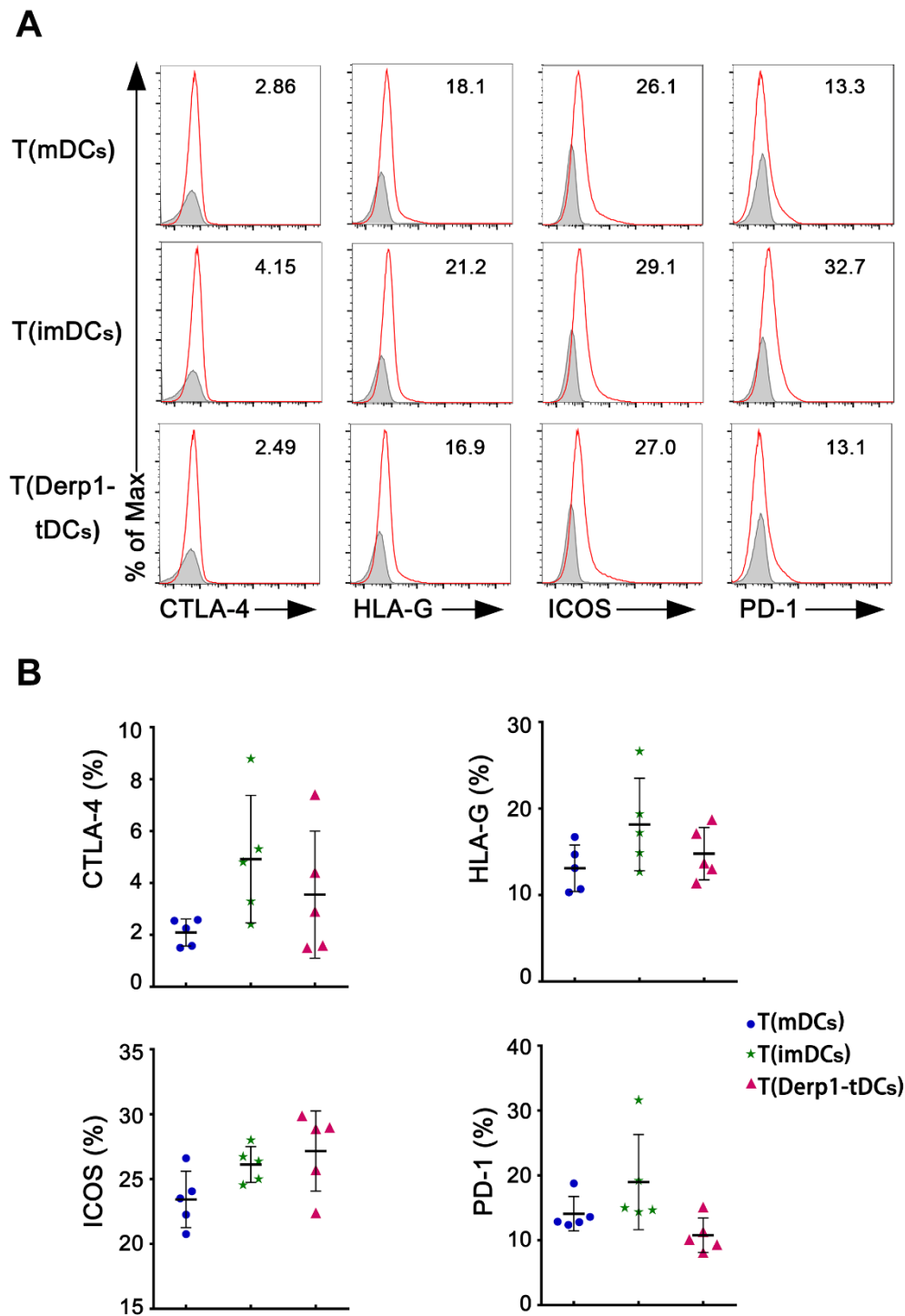
OVA-induced allergic airway disorder mouse model^{22,30} and treated the animals with an adoptive transfer of exogenous BM-tDCs. We found that the adoptive transfer of BM-tDCs significantly restrained the allergic airway inflammation and induced Foxp3⁺CD4⁺ Tregs in the lungs through TGF- β - and IL-10-dependent mechanisms. Overall, the results of our adoptive transfer experiment suggest that autologous tDC-based therapy may emerge as a powerful tool to control allergic inflammation in patients with AR.

In conclusion, our studies have confirmed tDCs as critical orchestrators of an immunosuppressive cell network in AR. The tDCs could restrain allergic respiratory inflammation by inducing immunosuppressive aTregs in a TGF- β - and IL-10-dependent manner. We propose that strategies targeting the differentiation and/or expansion of tDCs, as well as re-establishing the balance between immunity and tolerance in AR, may be valuable in future immunotherapy applications.

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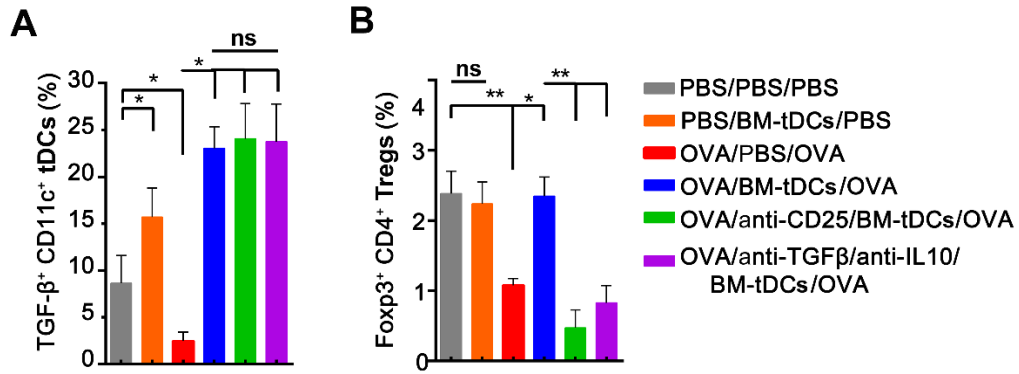
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Supplementary figures and figure legends



Supplementary Figure S1. Tolerogenic surface molecules of DCs educated T cells.

Representative histogram plots relative to **isotype** control (percentages shown; A) and cumulative data of mDCs, imDCs, and Derp1-tDCs (n=5; B).



Supplementary Figure S2. Adoptive transfer of BM-tDCs induced Foxp3 $^+$ CD4 $^+$ Tregs through TGF- β - and IL-10-dependent mechanisms. Flow cytometric analysis of TGF- β^+ CD11c $^+$ tDCs (A) and Foxp3 $^+$ CD4 $^+$ Tregs (B) in the lungs (n=6). Values are presented as means $\pm$ SEMs. *P<0.05, **P<0.01, and ***P<0.001.