

RESEARCH ARTICLE

Sulfatide-activated type II NKT cells suppress immunogenic maturation of lung dendritic cells in murine models of asthma

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Pan H, Zhang G, Nie H, Li S, He S, Yang J. Sulfatide-activated type II NKT cells suppress immunogenic maturation of lung dendritic cells in murine models of asthma. *Am J Physiol Lung Cell Mol Physiol* 317: L578–L590, 2019. First published August 21, 2019; doi: 10.1152/ajplung.00256.2018.—Our previous study showed that sulfatide-activated type II natural killer T (NKT) cells can prevent allergic airway inflammation in an ovalbumin (OVA)-induced murine model of asthma, but the underlying mechanism is unclear. Recently, sulfatide-activated type II NKT cells were shown to modulate the function of dendritic cells in experimental autoimmune encephalomyelitis and nonobese diabetic mice. Thus, it was hypothesized that sulfatide-activated type II NKT cells may modulate the function of lung dendritic cells (LDCs) in asthmatic mice. Our data showed that, in our mouse models, activation of type II NKT cells by sulfatide administration and adoptive transfer of sulfatide-activated type II NKT cells resulted in reduced expression of surface maturation markers and proinflammatory cytokine production of LDCs. LDCs from sulfatide-treated asthmatic mice, in contrast to LDCs from PBS-treated asthmatic mice, significantly reduced allergic airway inflammation *in vivo*. However, we found no influence of sulfatide-activated type II NKT cells on the phenotypic and functional maturation of bone marrow-derived dendritic cells *in vitro*. In addition, adoptive transfer of sulfatide-activated type II NKT cells did not influence the phenotypic and functional maturation of LDCs in CD1d^{−/−} mice, which lack both type I and II NKT cells, immunized and challenged with OVA. Our data reveal that sulfatide-activated type II NKT cells can suppress immunogenic maturation of LDCs to reduce allergic airway inflammation in mouse models of asthma, and it is possible that the immunomodulatory effect needs type I NKT cells.

asthma; dendritic cells; natural killer T cell; sulfatide

INTRODUCTION

Natural killer T (NKT) cells, a novel subpopulation of T lymphocytes, generally recognize lipid antigens presented by the major histocompatibility complex (MHC) class I-like molecule CD1d and bridge innate immune responses to adaptive immunity (3, 5). Broadly, there are two subsets of NKT cells: type I NKT cells (invariant NKT cells) express a semi-invariant T cell receptor (TCR, V α 14-J α 18 in mice and V α 24-J α 18 in humans), whereas type II NKT cells (noninvariant NKT

cells) express relatively diverse TCRs. The two subsets of NKT cells recognize different lipid antigens and play different roles in the immune system (11, 14, 16). Sulfatide, 3-sulfated galactosyl ceramide derived from myelin, can be recognized by a major subset of type II NKT cells in both mice and humans, often used to investigate the function of type II NKT cells (11, 13). Because of the limited availability of specific tools to identify type II NKT cells, the understanding of their function remains limited. However, accumulating studies have shown the immunoregulatory properties of type II NKT cells in various disorders, including tumor immunity, infectious diseases, and autoimmune and inflammatory conditions (10, 11, 22, 38). However, little is known about the role of type II NKT cells in asthma. Our previous study showed that sulfatide-activated type II NKT cells can prevent allergic airway inflammation in a murine model of asthma (41), but the underlying mechanism is fully unclear.

Dendritic cells (DCs) are the most potent antigen-presenting cells of the immune system and link innate and adaptive immunity. LDCs can stimulate CD4⁺ T cells, instruct Th2 differentiation, and initiate and maintain maladaptive immunity after allergen exposure in asthma (18, 40). Recently, Maricic et al. (23) reported that sulfatide administration can induce tolerance of DCs in the periphery in experimental autoimmune encephalomyelitis (EAE), and adoptive transfer of DCs from sulfatide-treated animals can prevent EAE in naïve mice. In spontaneously diabetic nonobese diabetic mice, sulfatide, a prototypic ligand that can activate type II NKT cells, was found to be protective by inducing tolerogenic maturation of DCs, characterized by elevated anti-inflammatory cytokine IL-10 production (37). These studies indicate that sulfatide-activated type II NKT cells may modulate the function of DCs. Thus, it was hypothesized that sulfatide-activated type II NKT cells may modulate the function of LDCs in asthma. To confirm this hypothesis, we used a combined method, including sulfatide administration, adoptive transfer of sulfatide-activated type II NKT cells, adoptive transfer of LDCs from asthmatic mice with sulfatide treatment, coculture experiment, and NKT cell-deficient mice (CD1d^{−/−} mice) to investigate the regulatory effect of sulfatide-activated type II NKT cells on the phenotypic and functional maturation of LDCs in an ovalbumin (OVA)- or house dust mite (HDM)-induced mouse model of asthma. Our data show that both sulfatide administration and adoptive transfer of sulfatide-activated type II NKT cells can reduce the expression of surface maturation markers and proinflammatory cytokines of

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LDCs in mouse models of asthma. In addition, adoptive transfer of LDCs from OVA-immunized and -challenged mice treated with sulfatide can attenuate allergic airway inflammation in asthmatic mice. However, sulfatide-activated type II NKT cells could not affect the maturation of bone marrow-derived dendritic cells (BMDCs) *in vitro*. Furthermore, adoptive transfer of sulfatide-activated type II NKT cells did not elicit the maturation of LDCs in CD1d^{-/-} mice immunized and challenged with OVA. Our data suggest that sulfatide-activated type II NKT cells can suppress immunogenic maturation of LDCs to reduce allergic airway inflammation in mouse models of asthma.

MATERIALS AND METHODS

Mice. Wild-type (WT) female BALB/c mice (6–8 wk old) were purchased from the Animal Biosafety Level 3 Laboratory, the Center for Animal Experiment, Wuhan University (Wuhan, China). CD1d^{-/-} mice (BALB/c background) were obtained from Jackson Laboratory (Bar Harbor, ME). All mice were bred and maintained in environmentally controlled and specific pathogen-free conditions (22°C, 12:12-h light-dark cycle). All experimental procedures were approved by the Animal Welfare Committee of Wuhan University.

Immunization and challenge. WT mice and CD1d^{-/-} mice were intraperitoneally sensitized on *days 0* and *14* with 20 µg of chicken OVA (grade V; Sigma, St. Louis, MO) bound to 2 mg of Imject Alum (Thermo Scientific Pierce, Rockford, IL) in 200 µL of phosphate-buffered saline (PBS). Intranasal OVA challenges (100 µg/50 µL in PBS) were administered on *days 25–27*. Mice were euthanized 24 h after the final challenge for further analysis. In some experiments, WT mice were immunized intranasally with 1 µg HDM extracts (*Dermatophagoides pteronyssinus*; Greer Laboratories) suspended in PBS on *day 1*, challenged intranasally with 10 µg HDM on *days 7–11*, and euthanized 3 days after the final challenge for further analysis (34). The control groups received sterile PBS (vehicle) at all time points.

Sulfatide administration. Sulfatide (Matreya, Pleasant Gap, PA) was dissolved in PBS supplemented with 0.5% polysorbate-20 (wt/vol), and the control vehicle was PBS supplemented with 0.5% polysorbate-20. Sulfatide (20 µg) in 200 µL PBS/mouse or 200 µL PBS were injected intraperitoneally 1 h before challenge on *day 25*.

Tetrameric CD1d-sulfatide complexes. Biotinylated mouse CD1d monomers (1 mg/mL in PBS; NIH tetramer core facility, Atlanta, GA) were mixed with sulfatide in PBS with 0.5% polysorbate-20 (pH 7.4) at a molar ratio of 1:3 (protein-lipid) and incubated at room temperature overnight. The following day, 80 µg of streptavidin-phycoerythrin (PE; eBioscience, San Diego, CA) were added to 200 µg of the CD1d-sulfatide complexes and incubated at room temperature for 4 h as previously described (20). Tetramers were stored at 4°C until use.

Type II NKT cell isolation and adoptive transfer. A single-cell suspension of splenocytes was collected from naïve WT mice and cultured at a density of 1×10^7 /well in a six-well plate in 3 ml of RPMI 1640 supplemented with 10% FBS, L-glutamine, nonessential amino acids, sodium pyruvate, streptomycin, and penicillin. Cells were activated with 20 µg/mL sulfatide for 3 h at 37°C in a 5% CO₂ atmosphere. For sorting, the splenocytes were harvest and blocked with anti-mouse FcR-γ (CD16/32; 2.4G2; BD Biosciences) and then stained with PE-labeled sulfatide-loaded CD1d tetramers and PeCy5-conjugated anti-TCR-β, sorted on a BD FACS Aria III Cell Sorter (BD Biosciences, San Diego, CA). The purity was ~80% by flow cytometry (FCM) for PE-labeled sulfatide/CD1d tetramer-positive cells double stained with PeCy5-conjugated anti-TCR-β. Type II NKT cells (3×10^6) were adoptive transferred to WT mice or CD1d^{-/-} mice via tail vein 1 h before OVA challenge on *day 25* or 1 h before HDM challenge on *day 7*.

LDC isolation, adoptive transfer, and cytokine secretion. LDCs were isolated and purified using flow cytometry. Lungs were collected

from CD1d^{-/-} and WT mice 24 h after the final OVA challenge, and single cell suspensions were prepared as previously reported (41). Previous researches have illustrated that LDCs express the DC marker CD11c but do not express the macrophage marker F4/80 (4, 17). Thus, in this study, the lung cells were first incubated with PE-conjugated anti-CD11c and allophycocyanin-Cy7-conjugated anti-F4/80; LDCs (CD11c⁺F4/80⁻ cells) were purified by flow cytometry using a BD FACS Aria III Cell Sorter (BD Biosciences). The purity was ~90%, which was determined by flow cytometry for PE-CD11c-positive cells and allophycocyanin-Cy7-F4/80-negative cells.

For adoptive transfer, LDCs (2×10^5 cells/mouse) were transferred to mice via intranasal injection 1 h before challenge on *day 25*.

LDCs from different groups of mice were cultured in complete RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 25 µg/mL gentamicin, 50 U/mL penicillin, 2 mM L-glutamine, 50 µM mercaptoethanol, and 0.1 mM nonessential amino acids in 96-well plates (2×10^5 cells/well) for 72 h in the presence of OVA (100 µg/mL). Supernatants were collected to measure IL-10, IL-12p70, IL-6, and TNF-α by enzyme-linked immunosorbent assay (ELISA; eBioscience).

Coculture of type II NKT cells and BMDCs. Sulfatide-activated type II NKT cells were purified from spleen by flow cytometry as above. BMDCs were differentiated from bone marrow cells according to the procedure described (24, 28), with slight modification. Briefly, bone marrow cells were flushed from the femurs and tibias of naïve WT BALB/c mice, washed, and cultured in complete RPMI 1640 supplement containing 10% heat-inactivated FBS, 50 µM 2-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin in the presence of 10 ng/mL recombinant mouse GM-CSF and 10 ng/mL recombinant mouse IL-4. Nonadherent granulocytes were removed after 48 h of culture, and culture media were replaced by fresh complete medium containing 10 ng/mL recombinant mouse GM-CSF and 10 ng/mL recombinant mouse IL-4 every other day. All cultures were incubated at 37°C in 5% humidified CO₂. After 7 days, the nonadherent cells were collected, centrifuged, and resuspended in fresh medium. The purity of the DCs was demonstrated to be >90% by CD11c staining. The immature BMDCs (CD11c⁺ cells) were stimulated with lipopolysaccharide (LPS, 1 µg/mL), cocultured with sulfatide-activated type II NKT cells (DC-to-type II NKT cell ratio, 1:2), or left untreated (immature BMDCs). For cytokine analysis, aliquots of culture supernatants were collected after 20 h of BMDC culture and assayed for the presence of IL-12p70, IL-10, IL-6, and TNF-α by ELISA (eBioscience).

Flow cytometry analysis. To detect the surface maturation markers of lung DCs and BMDCs, isolated lung mononuclear cells and BMDCs cocultured with sulfatide-activated type II NKT cells in the presence or absence of LPS were resuspended in FCM buffer ($1-2 \times 10^6$ cells/mL), preincubated with Fc_γ receptor-blocking mAb (CD16/32; 2.4G2; BD Biosciences) for 15 min at 4°C to reduce nonspecific binding, and then labeled with isotype controls or the following antibodies for 30 min at 4°C: PE-conjugated anti-CD11c, allophycocyanin-Cy7-conjugated anti-F4/80, FITC-conjugated anti-CD80, FITC-conjugated anti-CD40, and FITC-conjugated anti-MHC-II, PeCy5-conjugated anti-CD86 (eBioscience). Cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences).

Measurement of airway hyperresponsiveness. Airway hyperresponsiveness (AHR) was detected by invasive measurement of airway resistance, using the FinePointe RC system (Wilmington, NC), as previously described (19). Mice were anesthetized 24 h after the final OVA challenge, and a 20-gauge polyethylene catheter was inserted in the trachea, followed by mechanical ventilation. Airway resistance (R_L) and dynamic compliance (C_{dyn}; reflecting the elasticity of the lung) to increasing doses of aerosolized methacholine (MCh), ranging from 3.125 to 50 mg/mL in PBS, for 3 min were measured to assess AHR. Nonspecific airway responsiveness was measured by exposing mice to aerosolized PBS to set a baseline value.

Bronchoalveolar lavage and lung histological analysis. Bronchoalveolar lavage was performed using a catheter with three 0.5-ml aliquots of PBS containing 0.6 mM EDTA, as previously described (41). The bronchoalveolar lavage fluid (BALF) was collected and centrifuged, and the supernatant was stored at -80°C until assayed for cytokine levels. The recovered cells were counted and stained with May–Grunwald Giemsa (Jiancheng, Nanjing, China) for differential cell counts. Numbers of eosinophils, macrophages, neutrophils, and lymphocytes were expressed as absolute numbers from total cell counts.

The lung histological analysis was performed as previously mentioned (41). Briefly, the right lungs were fixed in 4% buffered paraformaldehyde and embedded in paraffin. Sections (4 μm thick) were obtained and stained with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS; Baso, Taiwan, China). Peribronchial and perivascular inflammation was assessed using light microscopy under $\times 200$ magnification. Goblet cell hyperplasia was determined by counting the number of PAS-positive cells in the epithelium of the central airway on a digital image under $\times 200$ magnification. The circumference of the airway at the basement membrane was measured by National Institutes of Health Image. The results were shown as the number of PAS-positive cells per unit length (mm) of the basement membrane.

ELISA and immunoglobulin analysis. Measurement of IL-4, IL-5, IL-13, IFN- γ , and IL-10 in the BALF and IL-12p70, IL-10, IL-6, and TNF- α in supernatants was performed using ELISA kits, according to the manufacturer's protocols (eBioscience). For immunoglobulin (Ig) analysis, the serum was collected 24 h after the last OVA challenge or 3 days after the final HDM challenge to detect the concentrations of IgE and IgG1 by ELISA, as previously described (41). Serum levels of Igs were expressed based on optical density measurements.

Statistical analyses. All data are expressed as means $\pm$ SE. Statistical analyses were performed with Student's unpaired t test (two groups) or one-way ANOVA (four groups) followed by the least-significant difference test in the condition of variance homogeneity and Dunnett's T3 test in variance heterogeneity. Data were analyzed using the SPSS 22.0 software. Statistical graphs were plotted by GraphPad Prism 5.0 software. $P < 0.05$ was considered statistically significant.

RESULTS

Sulfatide treatment reduces surface maturation marker expression and proinflammatory cytokine secretion of LDCs from WT mice immunized and challenged with OVA. The phenotypic maturation of LDCs is largely based on surface maturation ligands such as costimulatory molecules CD80, CD86, and CD40 along with the MHC II molecules (7, 15). To investigate the influence of sulfatide administration on the phenotypic maturation of LDCs, the number and surface maturation ligand expression of LDCs from OVA-immunized and -challenged mice treated with sulfatide or PBS were detected by flow cytometry. As shown in Fig. 1, A and C, the number of LDCs from sulfatide- and PBS-treated asthmatic mice was similar ($P > 0.05$), whereas the number of LDCs from OVA-immunized and -challenged mice was dramatically higher than those from unimmunized and unchallenged mice ($P < 0.01$; Fig. 1, A and C). Furthermore, LDCs from OVA-immunized and -challenged mice treated with sulfatide showed decreased expression of MHC II, CD40, CD80, and CD86 compared with those in OVA-immunized and -challenged mice treated with PBS ($P < 0.05$ or 0.01 ; Fig. 1, B and D). LDCs from OVA-immunized and -challenged mice exhibited significantly higher expression of MHC II, CD40, CD80, and CD86 than those in unimmunized and unchallenged mice ($P < 0.01$; Fig.

1, B and D). The number and surface maturation marker expression of LDCs from unimmunized and unchallenged mice treated with sulfatide and PBS were similar ($P > 0.05$).

The functionally mature DCs exhibit the ability to produce high levels of inflammatory or immunostimulatory cytokines (e.g., IL-12, IL-6, and TNF- α) but secrete no or negligible amounts of tolerogenic or anti-inflammatory cytokine IL-10 (21). To determine the effect of sulfatide administration on the functional maturation of LDCs, we administered sulfatide or PBS to mice 1 h before the first OVA challenge on *day 25*. After the final OVA challenge (24 h), LDCs were isolated and cultured for 72 h, and then the supernatants were harvested to detect the cytokine production of IL-10, IL-12p70, TNF- α , and IL-6. As shown in Fig. 1, A and E, the levels of IL-12p70, TNF- α , and IL-6 in the culture supernatant of LDCs from OVA-immunized and -challenged mice treated with sulfatide were lower than those in OVA-immunized and -challenged mice treated with PBS ($P < 0.05$ or 0.01), whereas the concentration of IL-10 in the culture supernatant of LDCs from OVA-immunized and -challenged mice treated with sulfatide was higher than that in OVA-immunized and -challenged mice treated with PBS ($P < 0.05$). However, the concentrations of IL-10, IL-12p70, TNF- α , and IL-6 in the culture supernatant of LDCs from unimmunized and unchallenged mice were negligible. The above data suggest that sulfatide administration can reduce surface maturation marker expression and proinflammatory cytokine production of LDCs from OVA-immunized and -challenged mice. Sulfatide, 3-sulfated galactosyl ceramide derived from myelin, binds to mouse CD1d and human CD1a, CD1b, and CD1c and is recognized by type II NKT cells (2, 11). In our previous study, sulfatide was confirmed to activate type II NKT cells in OVA-immunized and -challenged mice (41). These indicate that the modulation of sulfatide treatment on LDCs in OVA-immunized and -challenged mice is mediated by sulfatide-activated type II NKT cells.

Adoptive transfer of sulfatide-activated type II NKT cells reduces surface maturation marker expression and proinflammatory cytokine secretion of LDCs from WT mice immunized and challenged with OVA. To confirm that the effect of sulfatide administration on LDCs is dependent on sulfatide-activated type II NKT cells, we adoptively transferred sulfatide-activated type II NKT cells to OVA-immunized and -challenged mice; the purity of type II NKT cells (specifically, sulfatide/mCD1d tetramer⁺ TCR- β ⁺ cells) was greater than 80% (Fig. 2A). Next, the surface maturation marker expression and spontaneous cytokine production of LDCs were determined. As shown in Fig. 2, B and C, the expression of MHC II, CD40, CD80, and CD86 was lower in OVA-immunized and -challenged mice treated with adoptive transfer of sulfatide-activated type II NKT cells than those in OVA-immunized and -challenged mice treated with PBS ($P < 0.05$ or 0.01). Furthermore, adoptive transfer of sulfatide-activated type II NKT cells significantly reduced the production of IL-12p70, TNF- α , and IL-6 in the culture supernatant of LDCs ($P < 0.05$ or 0.01 ; Fig. 2D). In contrast, the concentration of IL-10 in the culture supernatant of LDCs from OVA-immunized and -challenged mice treated with adoptive transfer of sulfatide-activated type II NKT cells was higher than that in OVA-immunized and -challenged mice treated with PBS ($P < 0.01$; Fig. 2D). Taken together, adoptive transfer of sulfatide-activated type II NKT cells can reduce the expression of surface maturation makers

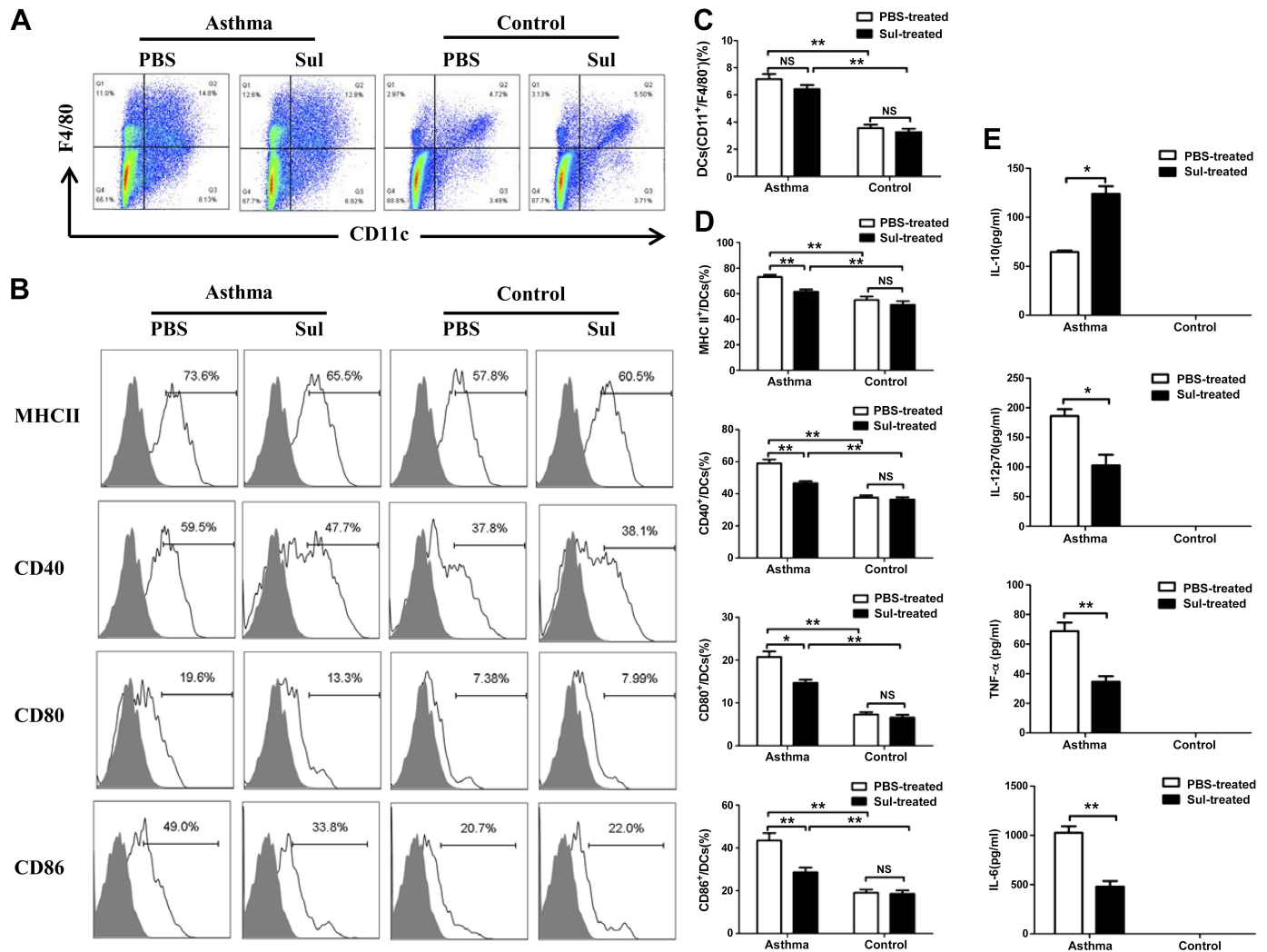


Fig. 1. Sulfatide treatment reduces surface maturation marker expression and proinflammatory cytokine secretion of lung dendritic cells (LDCs) from wild-type (WT) mice immunized and challenged with ovalbumin (OVA). OVA-immunized and -challenged WT mice were treated with sulfatide or PBS 1 h before the first challenge on day 25. Lungs were collected from mice 24 h after the final OVA challenge, and mononuclear cells (MNCs) were prepared. LDCs (CD11c⁺F4/80⁺ cells) were purified by flow cytometry using a BD FACSARIA III Cell Sorter. **A:** representative dot plots of LDCs in lung MNCs from OVA-immunized and -challenged mice treated with sulfatide or PBS. **B:** expression of CD80, CD86, CD40, and major histocompatibility complex (MHC) II was detected in CD11c⁺F4/80⁺ cells (line) and isotype controls (shaded histograms) from OVA-immunized and -challenged mice treated with sulfatide or PBS. Nos. above parentheses indicate the percentages of positive cells. **C:** percentage of LDCs in lung MNCs from OVA-immunized and -challenged mice treated with sulfatide or PBS. **D:** percentage of CD80, CD86, CD40, and MHC II in LDCs from OVA-immunized and -challenged mice treated with sulfatide or PBS. **E:** purified LDCs from OVA-immunized and -challenged mice treated with sulfatide or PBS were cultured in vitro for 72 h; the levels of IL-10, IL-12p70, TNF- α , and IL-6 in the culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA). Data are presented as means $\pm$ SE from 3 independent experiments ($n = 5$ /group), and 1 representative experiment is indicated. * $P < 0.05$ and ** $P < 0.01$; NS, not significant.

and proinflammatory cytokine secretion of LDCs from OVA-immunized and -challenged mice.

Adoptive transfer of sulfatide-activated type II NKT cells depresses surface maturation marker expression and proinflammatory cytokine secretion of LDCs and attenuates allergic airway inflammation in WT mice immunized and challenged with HDM. To further validate these findings in a model with more relevance for human asthma, we used HDM to induce an experimental asthma model. Sulfatide-activated type II NKT cells were adoptively transferred to WT mice via tail vein injections 1 h before HDM challenge on day 7. After the last challenge (3 days), mice were euthanized, and the surface maturation marker expression and spontaneous cytokine secretion of LDCs, allergic airway inflammation, and AHR were

detected. We found that adoptive transfer of sulfatide-activated type II NKT cells reduced the expression of MHC II, CD40, CD80, and CD86 on LDCs from HDM-immunized and -challenged mice ($P < 0.01$; Fig. 3, A and B). In addition, the concentrations of IL-12p70, TNF- α , and IL-6 in the culture supernatant of LDCs from HDM-immunized and -challenged mice treated with adoptive transfer of sulfatide-activated type II NKT cells were markedly lower than those of LDCs from nonrecipient mice ($P < 0.05$ or 0.01 ; Fig. 3C). In contrast, the level of IL-10 in the culture supernatant of LDCs from HDM-immunized and -challenged mice treated with adoptive transfer of sulfatide-activated type II NKT cells was significantly higher than that in the supernatant of LDCs from nonrecipient mice ($P < 0.01$; Fig. 3C). In addition, our data showed that

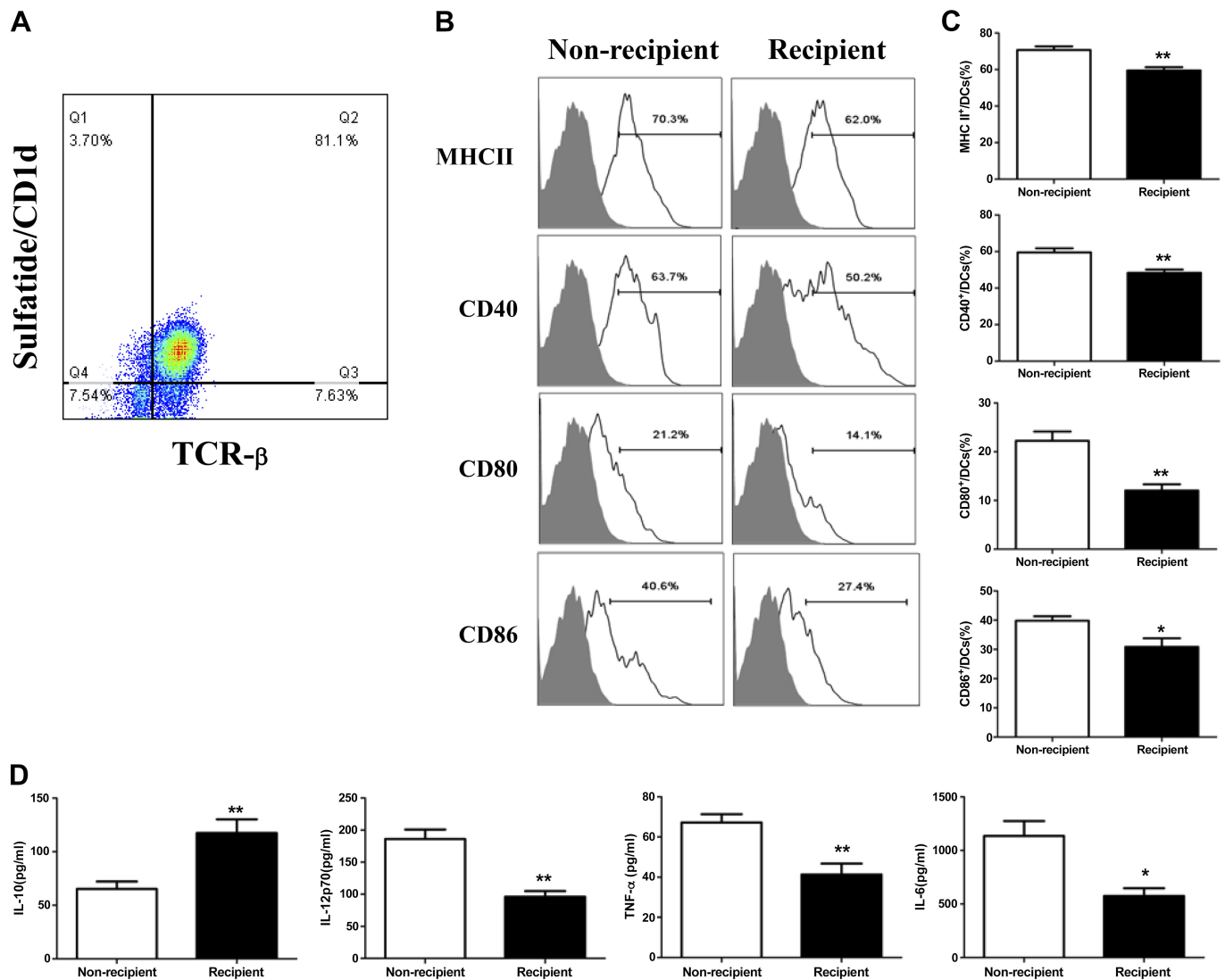


Fig. 2. Adoptive transfer of sulfatide-activated type II NKT cells reduces surface maturation marker expression and proinflammatory cytokine secretion of lung dendritic cells (LDCs) from wild-type (WT) mice immunized and challenged with ovalbumin (OVA). OVA-immunized and -challenged WT mice were treated with adoptive transfer of sulfatide-activated type II NKT cells or PBS 1 h before the first challenge on day 25. Lung mononuclear cells (MNCs) were isolated 24 h after the last challenge, and LDCs were purified by flow cytometry. **A**: purity of the transferred sulfatide-activated type II NKT cell population. Flow cytometry showed that 81.1% of the transferred cells stained with sulfatide/CD1d tetramers and anti-T cell receptor (TCR)-β antibody. **B**: expression of CD80, CD86, CD40, and major histocompatibility complex (MHC) II on lung CD11c⁺F4/80⁺ cells (line) and isotype controls (shaded histograms) from OVA-immunized and -challenged WT mice treated with adoptive transfer of sulfatide-activated type II NKT cells or PBS. Nos. above parentheses indicate the percentages of positive cells. **C**: percentage of CD80, CD86, CD40, and MHC II in LDCs from OVA-immunized and -challenged WT mice treated with adoptive transfer of sulfatide-activated type II NKT cells or PBS. **D**: purified LDCs from OVA-immunized and -challenged WT mice treated with adoptive transfer of sulfatide-activated type II NKT cells or PBS were cultured in vitro for 72 h; the concentrations of IL-10, IL-12p70, TNF-α, and IL-6 in the culture supernatants were detected by enzyme-linked immunosorbent assay (ELISA). Data are presented as means ± SE from 3 independent experiments ($n = 4\text{--}5/\text{group}$), and 1 representative experiment is indicated. * $P < 0.05$ and ** $P < 0.01$.

adoptive transfer of sulfatide-activated type II NKT cells significantly attenuated allergic airway inflammation, including reduced inflammatory cell infiltration in the lung tissues and BALF (total cells, eosinophils, lymphocytes, and neutrophils: $P < 0.01$; macrophages: $P < 0.05$; Fig. 4, A and B), decreased PAS-positive cells in the epithelium of the airway ($P < 0.01$; Fig. 4C), decreased HDM-specific IgG1 and IgE ($P < 0.01$; Fig. 4D), and reduced Th2 cytokine (IL-4, IL-5, and IL-13) levels in BALF ($P < 0.05$ or 0.01; Fig. 4E). In contrast, the level of IFN-γ did not significantly change between HDM-immunized and -challenged mice treated with adoptive transfer of sulfatide-activated type II NKT cells and nonre-

cipient mice ($P > 0.05$; Fig. 4E). Finally, AHR was determined by measurement of R_L and C_{dyn} . R_L and C_{dyn} to increasing concentrations of MCh, ranging from 3.125 to 50 mg/mL in PBS, were detected. Decreased R_L to MCh was observed in HDM-immunized and -challenged mice treated with adoptive transfer of sulfatide-activated type II NKT cells compared with nonrecipient mice ($P < 0.05$ or 0.01; Fig. 4F). In contrast, no difference was found in terms of C_{dyn} between the two groups ($P > 0.05$; Fig. 4F). Thus, our data indicate that adoptive transfer of sulfatide-activated type II NKT cells can reduce the levels of surface maturation ligands and proinflammatory cytokine secretion of

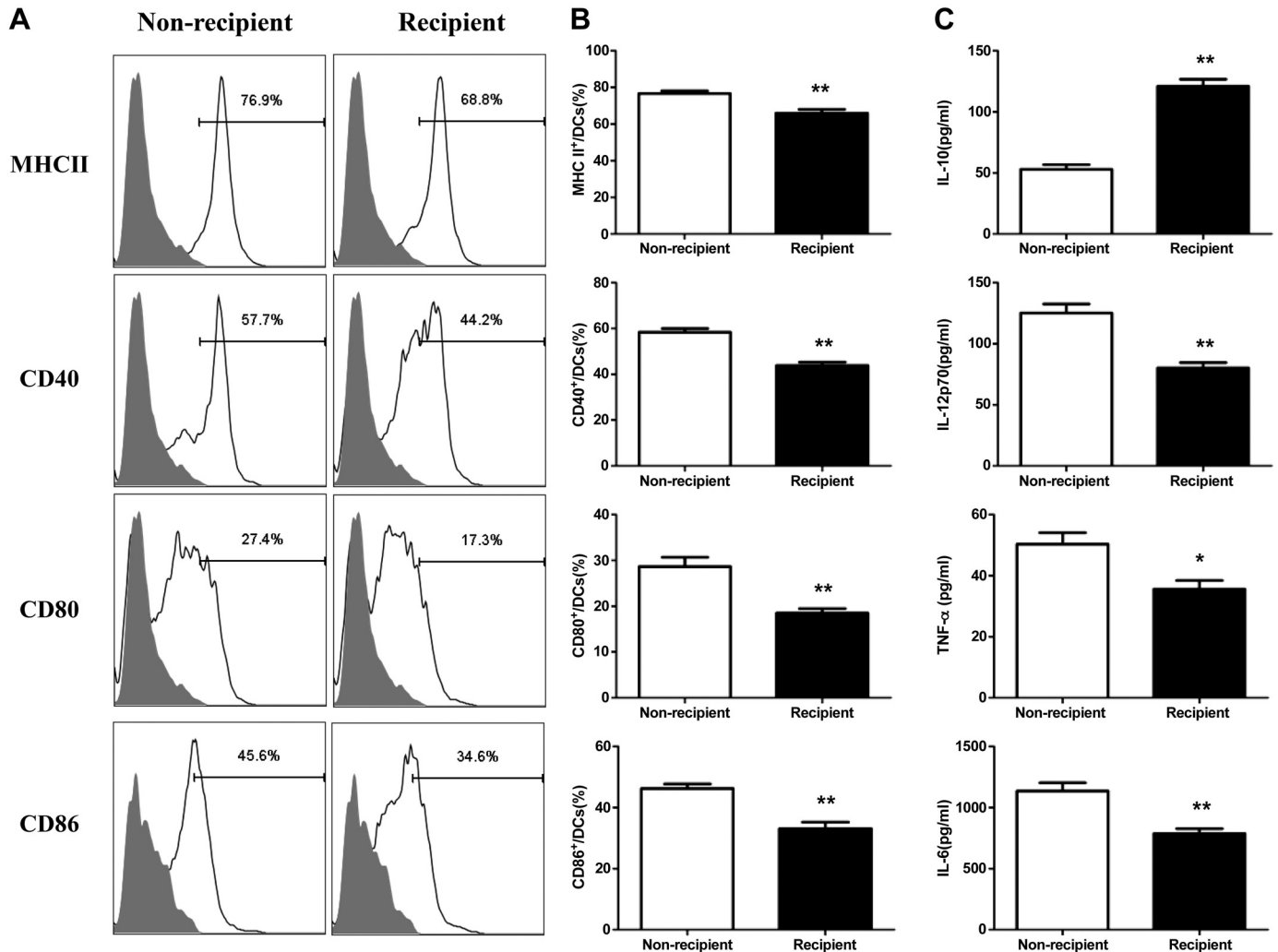


Fig. 3. Adoptive transfer of sulfatide-activated type II NKT cells reduces surface maturation marker expression and proinflammatory cytokine secretion of lung dendritic cells (LDCs) from wild-type (WT) mice immunized and challenged with house dust mite (HDM). Sulfatide-activated type II NKT cells (3×10^6 /mouse) or PBS were adoptive transferred in WT mice immunized and challenged with HDM 1 h before the first challenge on day 7. A: expression of CD80, CD86, CD40, and major histocompatibility complex (MHC) II on lung CD11c⁺F4/80[−] cells (line) and isotype controls (shaded histograms) from HDM-immunized and -challenged mice treated with adoptive transfer of sulfatide-activated type II NKT cells or PBS. Nos. above parentheses indicate the percentages of positive cells. B: percentage of CD80, CD86, CD40, and MHC II in LDCs from HDM-immunized and -challenged mice treated with adoptive transfer of sulfatide-activated type II NKT cells or PBS. C: purified LDCs from HDM-immunized and -challenged mice treated with adoptive transfer of sulfatide-activated type II NKT cells or PBS were cultured in vitro for 72 h; the production of IL-10, IL-12p70, TNF-α, and IL-6 in the culture supernatants was assayed by enzyme-linked immunosorbent assay (ELISA). Data are presented as means $\pm$ SE from 3 independent experiments ($n = 5$ /group), and 1 representative experiment is indicated. * $P < 0.05$ and ** $P < 0.01$.

LDCs and prevent allergic airway inflammation in HDM-immunized and -challenged mice.

Adoptive transfer of LDCs from OVA-immunized and -challenged mice treated with sulfatide alleviates allergic airway inflammation in WT mice immunized and challenged with OVA. To further investigate the effect of sulfatide-activated type II NKT cells on LDCs initiating allergic airway inflammation in vivo, LDCs ($>90\%$ CD11c⁺F4/80[−]; Fig. 5A) from asthmatic mice treated with sulfatide or PBS were adoptively transferred to WT mice immunized and challenged with OVA, and then the allergic airway inflammation and AHR were determined. As shown in Fig. 5, adoptive transfer of LDCs from asthmatic mice treated with sulfatide, compared with adoptive transfer of LDCs from asthmatic mice treated with PBS, significantly alleviated allergic airway inflammation in

WT mice immunized and challenged with OVA, characterized by reduced inflammatory cell infiltration in the lung tissue and BALF (Fig. 5, B and C) (total cells, eosinophils, macrophages, neutrophils: $P < 0.01$, lymphocytes: $P < 0.05$), decreased production of OVA-specific IgE and IgG1 ($P < 0.05$ or 0.01 ; Fig. 5D), decreased PAS⁺ cells in the airway epithelium ($P < 0.01$; Fig. 5E), lowered concentrations of IL-4, IL-5, and IL-13 in BALF ($P < 0.05$ or 0.01 ; Fig. 5F) but no significant change in the level of IFN-γ in BALF ($P > 0.05$; Fig. 5F), and elevated production of IL-10 in BALF ($P < 0.05$; Fig. 5F). However, in contrast to asthmatic mice treated with PBS, asthmatic mice treated with adoptive transfer of LDCs from asthmatic mice treated with sulfatide displayed augmented allergic airway inflammation, including more inflammatory cell infiltration in the lung and BALF ($P < 0.05$ or 0.01 ; Fig.

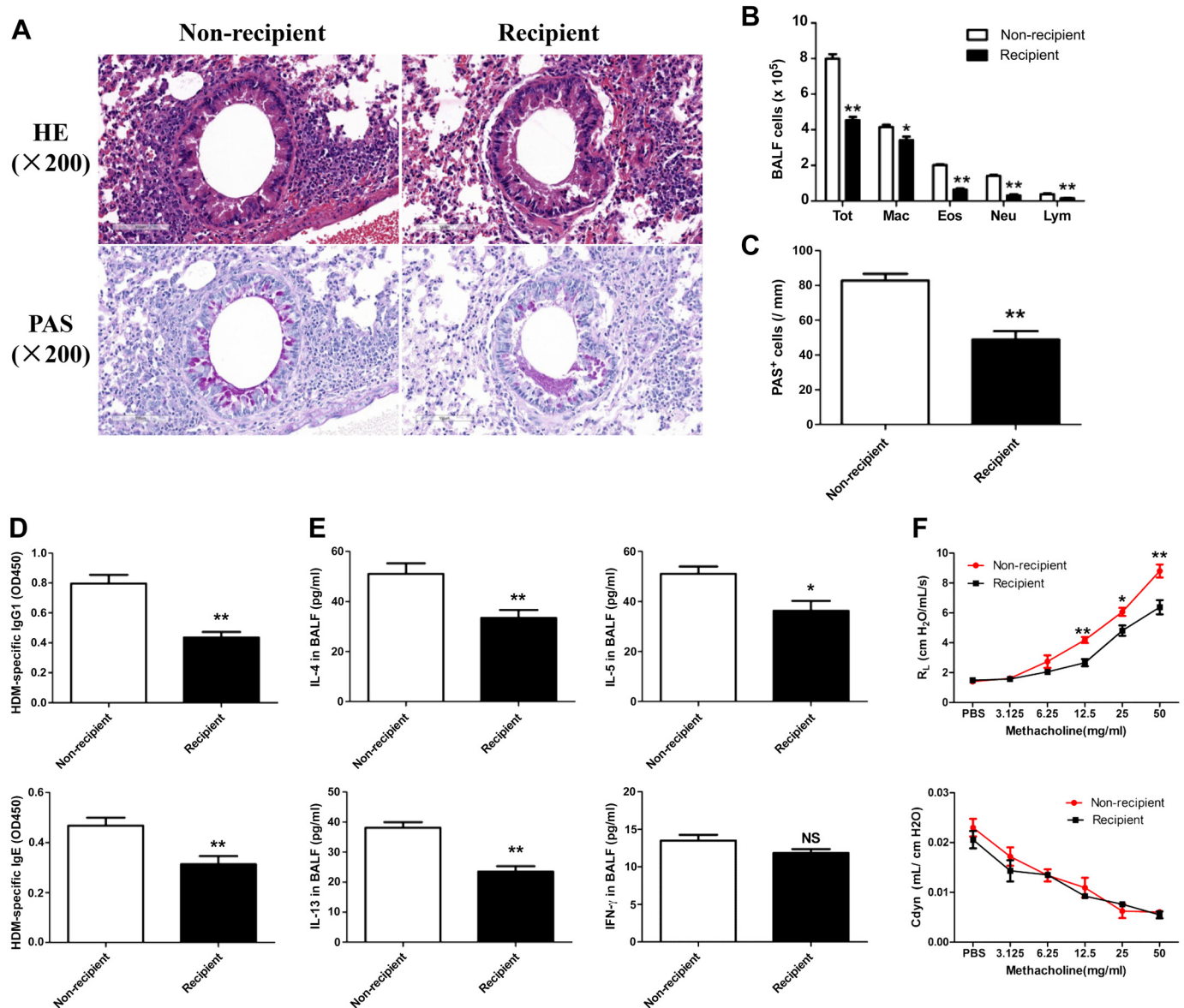


Fig. 4. Adoptive transfer of sulfatide-activated type II NKT cells alleviates allergic airway inflammation in wild-type (WT) mice immunized and challenged with house dust mite (HDM). Sulfatide-activated type II NKT cells or PBS were adoptive transferred in WT mice via tail vein injections 1 h before HDM challenge on day 7. Mice were euthanized 3 days after the last challenge, and then allergic airway inflammation and airway hyperresponsiveness (AHR) were determined. **A:** histopathological analysis of lung tissue from HDM-immunized and -challenged mice treated with adoptive transfer of sulfatide-activated type II NKT cells or PBS, showing hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) staining (magnification ×200). **B:** total and differential cell counting in bronchoalveolar lavage fluid (BALF) of HDM-immunized and -challenged mice treated with adoptive transfer of sulfatide-activated type II NKT cells or PBS. Tot, total cell counts; Mar, macrophages; Eos, eosinophils; Neu, neutrophils; Lym, lymphocytes. **C:** no. of PAS-positive cells/unit of length (mm) of the basement membrane in each group. **D:** concentrations of HDM-specific IgE and IgG1 in serum of asthmatic mice treated with adoptive transfer of sulfatide-activated type II NKT cells or PBS. **E:** concentrations of IL-4, IL-5, IL-13, and IFN-γ in BALF from HDM-induced asthmatic mice treated with adoptive transfer of sulfatide-activated type II NKT cells or PBS were assayed by enzyme-linked immunosorbent assay (ELISA). **F:** airway response to increasing concentrations of methacholine was examined. Data are expressed as means ± SE from 3 independent experiments ($n = 5$ /group), and 1 representative experiment is indicated. * $P < 0.05$ and ** $P < 0.01$; NS, not significant.

5, **B** and **C**), more PAS⁺ cells in the airway epithelium ($P < 0.05$; Fig. 5E), higher levels of OVA-specific IgE and IgG1 ($P < 0.05$; Fig. 5D), and increased production of IL-4, IL-5, and IL-13 in BALF ($P < 0.05$ or 0.01 ; Fig. 5F). In contrast, the concentrations of IFN-γ and IL-10 did not change significantly between the two groups ($P > 0.05$; Fig. 5F). Finally, AHR was determined by direct measurement of R_L and C_{dyn} . R_L and C_{dyn} to increasing concentrations of MCh, ranging from 3.125 to 50

mg/mL in PBS, was detected. Decreased R_L to MCh was observed in OVA-immunized and -challenged mice receiving adoptive transfer of LDCs from asthmatic mice treated with sulfatide compared with OVA-immunized and -challenged mice receiving adoptive transfer of LDCs from asthmatic mice treated with PBS ($P < 0.01$; Fig. 5G). However, no difference was found in terms of C_{dyn} between the two groups ($P > 0.05$; Fig. 5G). In conclusion, LDCs from asthmatic mice treated with

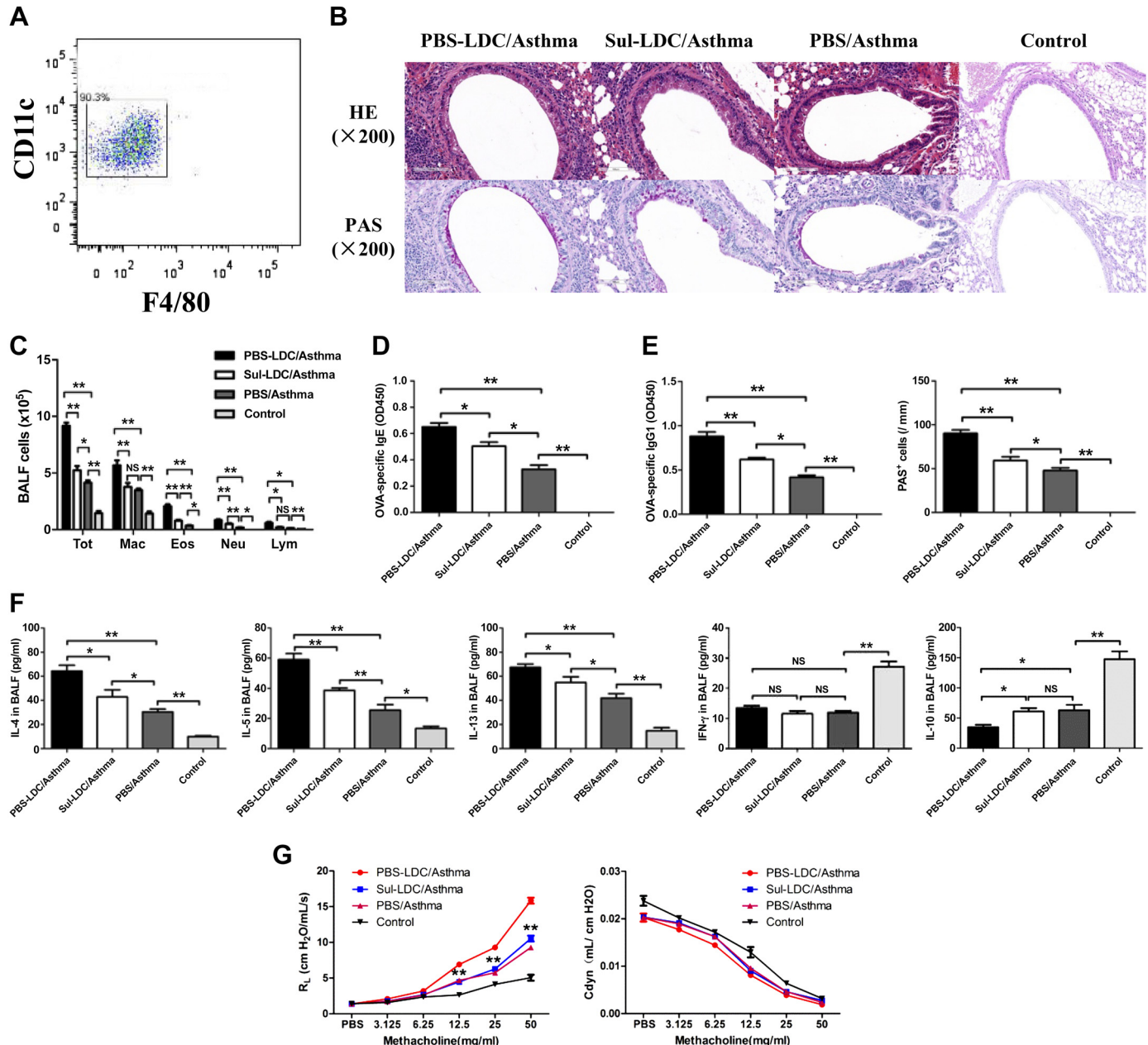


Fig. 5. Adoptive transfer of lung dendritic cells (LDCs) from ovalbumin (OVA)-immunized and -challenged mice treated with sulfatide alleviates allergic airway inflammation in OVA-induced asthmatic mice. LDCs (2×10^5) obtained from OVA-immunized and -challenged mice treated with sulfatide or PBS were adoptive transferred to asthmatic mice via intranasal injection, 1 h before the first OVA challenge on day 25; mice were euthanized 24 h after the final OVA challenge; and then allergic airway inflammation and airway hyperresponsiveness (AHR) were determined. **A**: flow cytometry verified the purity of the adoptively transferred cell population, which was stained positive with CD11c and negative with F4/80 (i.e., the proportion of LDCs was $>90\%$). **B**: histopathological analysis of lung tissue from mice treated with adoptive transfer of LDCs or PBS by hematoxylin and eosin (H&E) staining and periodic acid-Schiff (PAS) staining (magnification $\times 200$). **C**: total and differential cell counting in bronchoalveolar lavage fluid (BALF) of mice treated with adoptive transfer of LDCs or PBS. Tot, total cell counts; Mac, macrophages; Eos, eosinophils; Neu, neutrophils; Lym, lymphocytes. **D**: production of OVA-specific IgE and IgG1 in serum of mice treated with adoptive transfer of LDCs or PBS. **E**: no. of PAS-positive cells/unit of length (mm) of the basement membrane in mice treated with adoptive transfer of LDCs or PBS. **F**: concentrations of IL-4, IL-5, IL-13, IFN- γ , and IL-10 in BALF from mice treated with adoptive transfer of LDCs or PBS. **G**: airway response to increasing concentrations of methacholine was examined. Data are presented as means $\pm$ SE from 3 independent experiments ($n = 5/\text{group}$), and 1 representative experiment is indicated. * $P < 0.05$ and ** $P < 0.01$; NS, not significant.

sulfatide mediated reduced allergic airway inflammation in vivo, in contrast to LDCs from asthmatic mice treated with PBS.

Sulfatide-activated type II NKT cells alone have no influence on the surface maturation marker expression and proinflammatory cytokine secretion of BMDCs in vitro. To further investigate whether sulfatide-activated type II NKT cells can

modulate surface maturation marker expression and spontaneous cytokine secretion of BMDCs in vitro, coculture experiment was performed with purified sulfatide-activated type II NKT cells and immature BMDCs in the presence and absence of LPS ($1 \mu\text{g/mL}$), and immature BMDCs were cultured with or without LPS ($1 \mu\text{g/mL}$) for 20 h. As shown in Fig. 6, A and

B, BMDCs cultured with LPS show significantly higher expression levels of MHC II, CD40, CD80, and CD86 than those on immature BMDCs ($P < 0.01$). The concentration of proinflammatory cytokines such as IL-12p70, TNF- α , and IL-6 in the supernatant of BMDCs cultured with LPS were also increased (Fig. 5C). Although it was found that BMDCs cultured with LPS also produced tolerogenic cytokine IL-10, the level of IL-10 was very limited. In contrast, the levels of IL-10, IL-12p70, TNF- α , and IL-6 in the supernatant of immature BMDCs cocultured with type II NKT cells, immature BMDCs cultured alone, and type II NKT cells cultured in the presence or absence of LPS were negligible (Fig. 6C), which indicated that the cytokines (IL-10, IL-12p70, TNF- α , and IL-6) were mainly secreted by mature BMDCs but not type II NKT cells. The above results suggest that immature BMDCs undergo immunogenic maturation in the presence of LPS. Unexpectedly, the expression levels of MHC II, CD40, CD80, and CD86

on BMDCs cocultured with type II NKT cells and LPS were similar to those on BMDCs cultured with LPS ($P > 0.05$; Fig. 6, A and B). In addition, the levels of proinflammatory cytokine (IL-12p70, TNF- α , and IL-6) and tolerogenic cytokine (IL-10) in the supernatant of BMDCs cocultured with type II NKT cells and LPS were similar to those in the supernatant of BMDCs cultured with LPS ($P > 0.05$; Fig. 6C). In conclusion, sulfatide-activated type II NKT cells alone cannot directly modulate the surface maturation marker expression and proinflammatory cytokine secretion of BMDCs in vitro. Thus, how sulfatide-activated type II NKT cells influence the maturation of LDCs in asthmatic mice needs further investigation.

Adoptive transfer of sulfatide-activated type II NKT cells did not affect the surface maturation marker expression and proinflammatory cytokine secretion of LDCs from CD1d^{-/-} mice immunized and challenged with OVA. Our previous studies showed that type I NKT cells can promote immunogenic

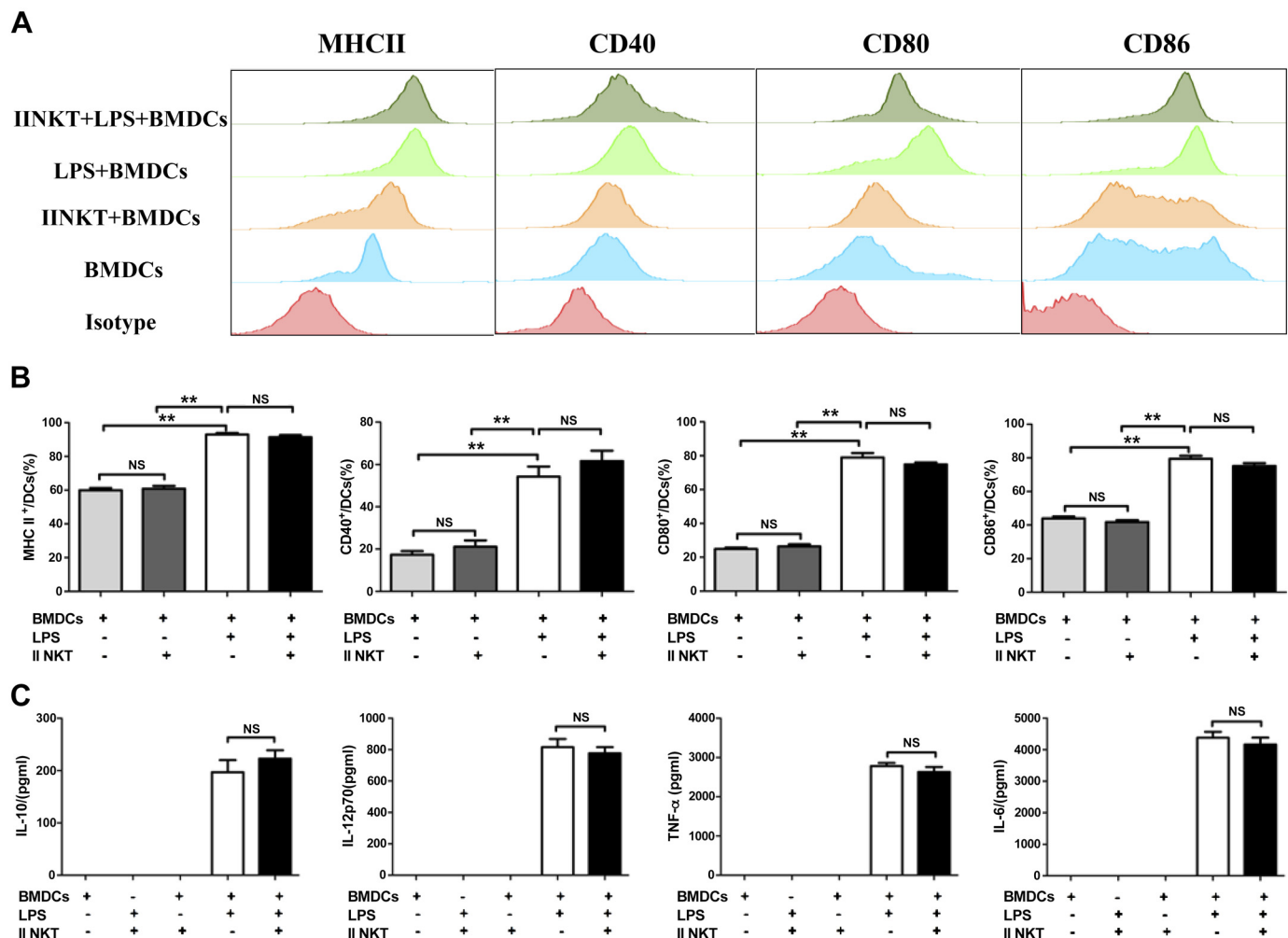


Fig. 6. Sulfatide-activated type II NKT cells alone have no influence on the surface maturation marker expression and proinflammatory cytokine secretion of bone marrow-derived dendritic cells (BMDCs) in vitro. Immature BMDCs were cocultured with sulfatide-activated type II NKT cells (BMDC-to-type II NKT cell ratio, 1:2) in the presence and absence of LPS (1 μ g/mL); immature BMDCs cultured in the presence of LPS (1 μ g/mL) or PBS for 20 h were used as controls. A: expression of CD80, CD86, CD40, and major histocompatibility complex (MHC) II on dendritic cells (DCs; CD11c⁺ cells), and isotype control is shown from different DC specimens, measured by flow cytometry as indicated. B: expression of CD80, CD86, CD40, and MHC II on DCs from different DCs cocultured with type II NKT cells with or without LPS or cultured with LPS or PBS for 20 h. C: culture supernatants were harvested from different DCs cocultured with type II NKT cells with or without LPS or cultured with LPS or PBS for 20 h, and the concentrations of IL-10, IL-12p70, TNF- α , and IL-6 were determined by enzyme-linked immunosorbent assay (ELISA). Data are expressed as means $\pm$ SE of 3 independent experiments ($n = 6$ /group), and 1 representative experiment is indicated. ** $P < 0.01$; NS, not significant.

maturation of LDCs in mouse models of asthma (15), and sulfatide-activated type II NKT cells could inhibit the activity of type I NKT cells in an OVA-induced murine model of asthma (41). Therefore, we further investigate whether the effect of sulfatide-activated type II NKT cells on LDCs in asthmatic mice needs type I NKT cells. Briefly, $CD1d^{-/-}$ mice (deficient in both type I and II NKT cells) were immunized and challenged with OVA, and sulfatide-activated type II NKT cells were adoptive transferred to $CD1d^{-/-}$ mice via tail vein injections 1 h before OVA challenge on day 25. After the last challenge (24 h), LDCs were isolated, and the surface maturation markers and spontaneous cytokine secretion in the culture supernatant of LDCs were detected. Surprisingly, adoptive transfer of sul-

fatide-activated type II NKT cells did not influence the expression of MHC II, CD40, CD80, or CD86 on LDCs from $CD1d^{-/-}$ mice immunized and challenged with OVA ($P > 0.05$; Fig. 7, A and B). Furthermore, the concentrations of proinflammatory cytokines, such as IL-12p70, TNF- α , and IL-6, in the culture supernatant of LDCs were similar in $CD1d^{-/-}$ asthmatic mice treated with adoptive transfer of sulfatide-activated type II NKT cells and PBS ($P > 0.05$; Fig. 7C); the levels of IL-10 in the culture supernatant of LDCs from two groups were similar as well ($P > 0.05$; Fig. 7C). Collectively, sulfatide-activated type II NKT cells cannot modulate the phenotypic and functional maturation of LDCs in $CD1d^{-/-}$ mice immunized and challenged with OVA. Thus, the immunoregulatory role of sulfatide-acti-

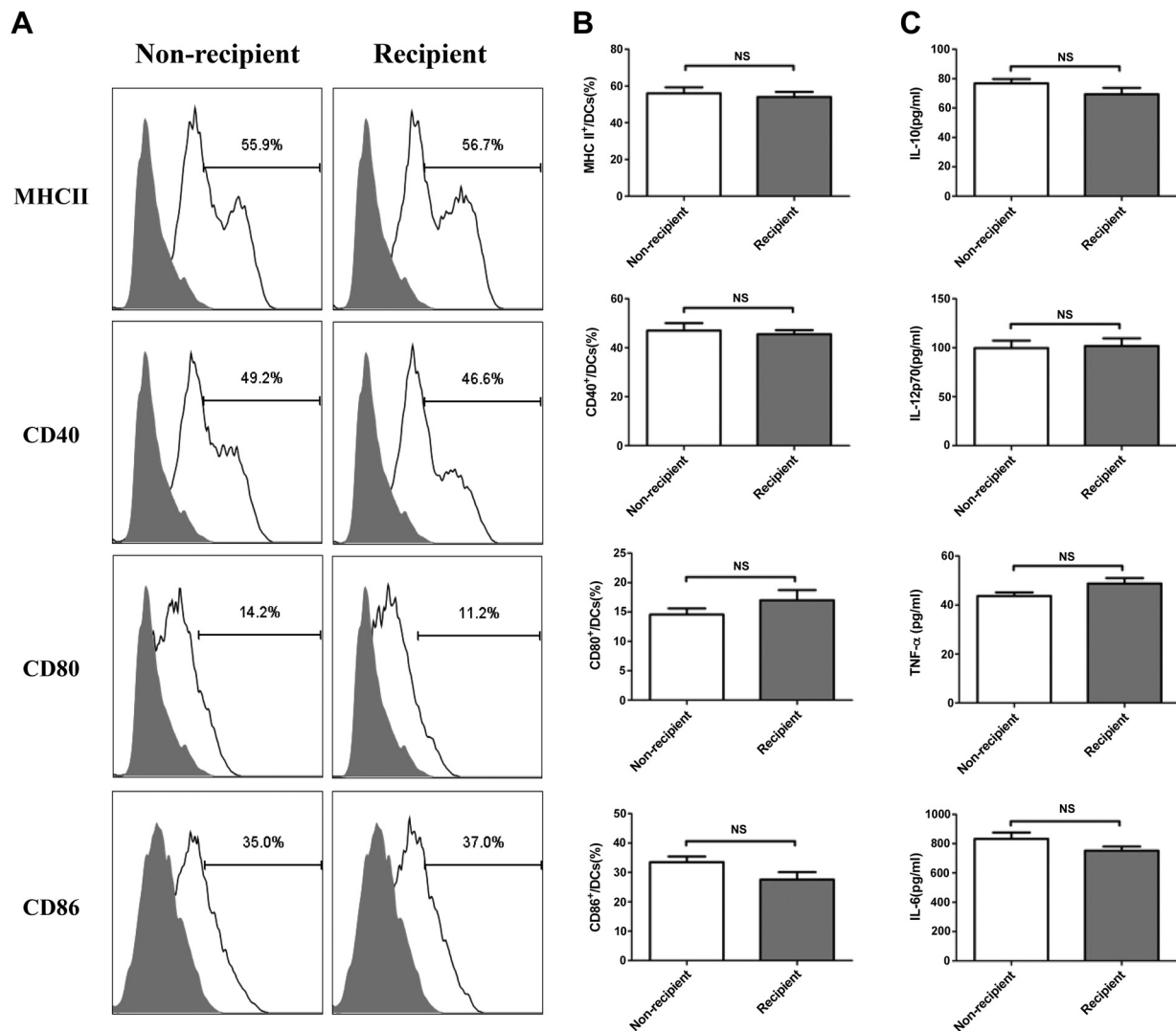


Fig. 7. Adoptive transfer of sulfatide-activated type II NKT cells did not affect the surface maturation marker expression and proinflammatory cytokine secretion of lung dendritic cells (LDCs) from $CD1d^{-/-}$ mice immunized and challenged with ovalbumin (OVA). Sulfatide-activated type II NKT cells and PBS were adoptive transferred to $CD1d^{-/-}$ asthmatic mice 1 h before the first challenge on day 25, and mice were euthanized 24 h after the last challenge for further analysis. A: lung mononuclear cells (MNCs) were isolated and stained for maturation-associated surface markers, and the expression of CD80, CD86, CD40, and major histocompatibility complex (MHC) II on $CD11c^{+}F4/80^{-}$ cells (line) and isotype controls (shaded histograms) was analyzed by flow cytometry analysis. Nos. above parentheses indicate the percentage of positive cells. B: percentage of CD80-, CD86-, CD40-, and MHC II-positive cells in LDCs from $CD1d^{-/-}$ asthmatic mice treated with adoptive transfer of sulfatide-activated type II NKT cells or PBS. C: LDCs from $CD1d^{-/-}$ asthmatic mice treated with adoptive transfer of sulfatide-activated type II NKT cells or PBS were purified by flow cytometry and cultured in vitro for 72 h; the concentrations of IL-10, IL-12p70, TNF- α , and IL-6 in the culture supernatants were detected by enzyme-linked immunosorbent assay (ELISA). Data are expressed as means $\pm$ SE of 3 independent experiments ($n = 5$ /group), and 1 representative experiment is indicated. NS, not significant.

vated type II NKT cells on LDCs in murine models of asthma may need the presence of type I NKT cells.

DISCUSSION

In the present study, we showed that sulfatide administration and adoptive transfer of sulfatide-activated type II NKT cells could downregulate the expression of surface maturation markers and proinflammatory cytokines of LDCs in OVA- and HDM-induced mouse models of asthma. Furthermore, sulfatide-activated type II NKT cells could reduce allergic airway inflammation by inhibiting the immunogenic maturation of LDCs in vivo. However, sulfatide-activated type II NKT cells could not influence the maturation of BMDCs in vitro. In addition, adoptive transfer of sulfatide-activated type II NKT cells did not elicit the maturation of LDCs in *CD1d*^{-/-} mice immunized and challenged with OVA. Thus, our data suggest that sulfatide-activated type II NKT cells can suppress immunogenic maturation of LDCs to reduce allergic airway inflammation in murine models of asthma.

DCs are currently categorized into tolerogenic immature and immunogenic mature differentiation stages. The distinction between tolerogenic immature and immunogenic mature DCs is according to the changes occurring on two crucial levels: phenotypic and functional (12, 21, 25, 27, 36). In our present study, LDCs from WT mice unimmunized and unchallenged with OVA displayed low levels of surface maturation markers and negligible secretion of proinflammatory cytokines and tolerogenic cytokine IL-10, suggesting that they were tolerogenic immature DCs (7, 12, 21). In contrast, LDCs from WT mice immunized and challenged with OVA showed significant high level of surface maturation markers, high secretion of proinflammatory cytokines, and low production of tolerogenic cytokine IL-10, thereby indicating that they were immunogenic mature DCs (9, 25, 27). We further investigated whether sulfatide-activated type II NKT cells could modulate the phenotypic and functional maturation of LDCs in asthma (41). In the present study, our data showed that sulfatide administration and adoptive transfer of sulfatide-activated type II NKT cells could reduce the expression of surface maturation markers and proinflammatory cytokines of LDCs in WT mice immunized and challenged with OVA. In addition, the similar effects on LDCs and allergic airway inflammation were replicated in a HDM-induced mouse model of asthma treated with adoptive transfer of sulfatide-activated type II NKT cells. Also, our data showed that LDCs from asthmatic mice treated with sulfatide mediated reduced allergic airway inflammation compared with LDCs from asthmatic mice treated with PBS in vivo. Collectively, sulfatide-activated type II NKT cells can suppress immunogenic maturation of LDCs to reduce allergic airway inflammation in vivo.

To investigate the immunomodulatory role of sulfatide-activated type II NKT cells on DCs in vitro, we used coculture experiment of sulfatide-activated type II NKT cells and BMDCs in the presence or absence of LPS, which can induce phenotypic and functional maturation of DCs and thus often be used to investigate the function of DCs (29, 32, 33). In our present study, BMDCs cultured with PBS for 20 h showed lower expressions of MHC II, CD40, CD80, and CD86 and negligible concentrations of IL-12p70, TNF- α , IL-6, and IL-10, indicating that they were tolerogenic immature DCs (29,

39). In contrast, BMDCs cultured with LPS for 20 h displayed immunogenic maturation, characterized by high levels of MHC II, CD40, CD80, and CD86 and high production of IL-12p70, TNF- α , and IL-6 (29). However, our results also showed that sulfatide-activated type II NKT cells could not influence the expression of surface maturation markers and proinflammatory cytokine secretion of BMDCs in the presence or absence of LPS in vitro, which indicated that it is possible that sulfatide-activated type II NKT cells cannot directly modulate the maturation of LDCs in asthma. In our previous study, we found that sulfatide-activated type II NKT cells could inhibit the activity of type I NKT cells in a murine model of asthma (41). In this study, we found that adoptive transfer of sulfatide-activated type II NKT cells could not influence the phenotypic and functional maturation of LDCs in OVA-immunized and -challenged *CD1d*^{-/-} mice, which are short of the MHC class I restricting element needed by type I NKT cells and thereby lack type I NKT cells (1). Similar to our results, Maricic et al. (23) reported that sulfatide-activated type II NKT cells could induce anergy of type I NKT cells and tolerance of DCs, and then prevent EAE, and adoptive transfer of DCs from sulfatide-treated mice can prevent EAE in naïve mice. However, the suppressive role of sulfatide-activated type II NKT cells in EAE is lost in mice lacking type I NKT cells (23), which indicates that the type I NKT cells are required for the immunomodulatory effect of type II NKT cells on DCs in EAE. Thus, our data indicate that it is possible that sulfatide-activated type II NKT cells inhibit the immunogenic maturation of LDCs by suppressing the function of type I NKT cells in asthmatic mice.

Lung dendritic cells (LDCs) can stimulate CD4⁺ T cells, instruct Th2 differentiation, and initiate and maintain maladaptive immunity after allergen exposure in asthma (40). Thus, suppressing the phenotypic and functional maturation of LDCs may result in alleviated Th2 immunity, and then allergic airway inflammation in asthma mice. In our current study, sulfatide-activated type II NKT cells were shown to suppress the phenotypic and functional maturation of LDCs in murine models of asthma (Figs. 1, 2, and 3); we also found sulfatide-activated type II NKT cells can prevent allergic airway inflammation in murine models of asthma (Ref. 41 and Fig. 4). In addition, LDCs from asthmatic mice treated with sulfatide reduced Th2 immune response (Fig. 6). Thus, the above results suggest that sulfatide-activated type II NKT cells mediated suppression of phenotypic and functional maturation on LDCs and may lead to reduced Th2 immune response and the following alleviated allergic airway inflammation in mouse models of asthma. On the other hand, DCs can directly participate in the generation of functional regulatory T (Treg) cells by using different mechanisms (31). In our present study, we also found that sulfatide administration upregulated the production of IL-10 in BALF and the level of mRNA expression of IL-10 in lung tissue in OVA-immunized and -challenged mice (data not shown). IL-10 is an anti-inflammatory cytokine that elicits immunosuppression, and the protective role of IL-10 is very well established in the context of allergic asthma. Besides DCs, Treg cells can produce IL-10. IL-10-producing Treg cells not only suppress Th2 immune responses but also inhibit allergic responses to different allergenic sources such as birch pollen or related food allergens, bee venom, or HDM (30). Different models of high-dose exposure to allergens

illustrated that the generation of functional IL-10-secreting Treg cells correlates with the generation of tolerance to allergens (26, 35). Thus, IL-10-secreting Treg cells may be involved in the mechanism of sulfatide-activated type II NKT cell-mediated prevention of allergic airway inflammation, which needs further investigation.

In this study, we illustrate that sulfatide-activated type II NKT cells can inhibit immunogenic maturation of LDCs in a mouse models of asthma, and the modulation effect may need the presence of type I NKT cells. However, there are some limitations of this study. First, in this study, CD1d^{-/-} mice, which lack both type I and type II NKT cells, were used to investigate the role of type I NKT cells in sulfatide-activated type II NKT cell-mediated modulation on LDCs in asthmatic mice. Precisely, if J α 18^{-/-} mice, which are only deficient in type I NKT cells (6, 8), were used to substitute CD1d^{-/-} mice in this study, the results may be more convincing. Second, the underlying mechanism of how type II NKT cells induce anergy of type I NKT cells and how type I NKT cells influence the maturation of LDCs need to be further clarified.

In conclusion, our present study indicates that sulfatide-activated type II NKT cells can suppress immunogenic maturation of LDCs in mouse models of asthma, possibly by inhibiting the activity of type I NKT cells.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

G.Z. and H.N. conceived and designed research; G.Z., H.P., S.L., and S.H. performed experiments; G.Z. analyzed data; G.Z. interpreted results of experiments; G.Z. and H.P. prepared figures; G.Z. drafted manuscript; G.Z. edited and revised manuscript; G.Z. and J.Y. approved final version of manuscript.

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