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## Sulfatide-activated type II NKT cells prevent allergic airway inflammation by inhibiting type I NKT cell function in a mouse model of asthma

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**Zhang G, Nie H, Yang J, Ding X, Huang Y, Yu H, Li R, Yuan Z, Hu S.** Sulfatide-activated type II NKT cells prevent allergic airway inflammation by inhibiting type I NKT cell function in a mouse model of asthma. *Am J Physiol Lung Cell Mol Physiol* 301: L975–L984, 2011. First published August 19, 2011; doi:10.1152/ajplung.00114.2011.—Asthma is a common chronic inflammatory disease involving many different cell types. Recently, type I natural killer T (NKT) cells have been demonstrated to play a crucial role in the development of asthma. However, the roles of type II NKT cells in asthma have not been investigated before. Interestingly, type I and type II NKT cells have been shown to have opposing roles in antitumor immunity, antiparasite immunity, and autoimmunity. We hypothesized that sulfatide-activated type II NKT cells could prevent allergic airway inflammation by inhibiting type I NKT cell function in asthma. Strikingly, in our mouse model, activation of type II NKT cells by sulfatide administration and adoptive transfer of sulfatide-activated type II NKT cells result in reduced-inflammation cell infiltration in the lung and bronchoalveolar lavage fluid, decreased levels of IL-4 and IL-5 in the BALF; and decreased serum levels of ovalbumin-specific IgE and IgG1. Furthermore, it is found that the activation of sulfatide-reactive type II NKT cells leads to the functional inactivation of type I NKT cells, including the proliferation and cytokine secretion. Our data reveal that type II NKT cells activated by glycolipids, such as sulfatide, may serve as a novel approach to treat allergic diseases and other disorders characterized by inappropriate type I NKT cell activation.

natural killer T cell

ALLERGIC ASTHMA IS CHARACTERIZED by airway eosinophilia, mucus overproduction, airway hyperresponsiveness (AHR), and specific immunoglobulin E (IgE) synthesis. Myosin heavy chain class II-restricted CD4<sup>+</sup> T-helper type 2 (Th2) cells mediate inflammation in asthma by secreting key cytokines, such as IL-4 and IL-13 (16). However, the Th2 response alone does not account for the full pathogenesis of asthma (8, 21). In addition, Th2-polarized immune responses after allergen sensitization can occur independently of asthma, underscored by the fact that only 25–30% of atopic children develop asthma (13). These findings suggest that other immune cells might also play important roles in the development of asthma.

Natural killer T (NKT) cells, which are lymphocytes coexpressing both T cell receptors (TCRs) and NK cell receptors, are a potential target for immunotherapy of cancer and autoimmune diseases. These cells are widely distributed, including in the bone marrow, liver, thymus, spleen, and lung (6). NKT

cells are specific for glycolipid antigens that are presented by the myosin heavy chain class I-like protein CD1. The CD1 family consists of five distinct isoforms and is divided into two groups: group I molecules CD1a, CD1b, CD1c, and CD1e only expressed in human, and the group II molecule CD1d expressed in both humans and mice (6).

NKT cells are categorized into type I and type II NKT cells. Type I NKT cells, also known as invariant NKT, express a conserved invariant TCR, whereas type II NKT cells (noninvariant NKT) express a diverse TCR repertoire (6). Studies showed that types I and II NKT cells are likely to be activated by a different sets of ligands and may play different roles in the immune system (24, 25). Once activated, type I NKT cells rapidly produce large amounts of cytokines and chemokines, which in turn induce adaptive immune responses (6). Direct activation of pulmonary type I NKT cells was sufficient to induce AHR in mice that lack conventional CD4<sup>+</sup> T cells (22). In several mouse models of asthma, despite a normal Th2 response, mice fail to develop asthma-like symptoms in the absence of type I NKT cells (2, 19). Furthermore, recent studies in patients with asthma also revealed a significant increased in the number of pulmonary type I NKT cells (1, 20, 23). These findings indicate that type I NKT cells play a crucial role in the pathogenesis of asthma. To our knowledge, the potential roles of type II NKT cells in asthma have not been reported. Although the role of type II NKT cells in asthma is unclear, type II NKT cells and type I cells were found to have opposite and counteractive roles in antitumor immunity, antiparasite immunity, ischemia-reperfusion injury (IRI), and autoimmunity experimental models (5, 11, 12, 26, 29). Thus we hypothesize that activated type II NKT cells could inhibit allergic airway inflammation by suppressing type I NKT cell function in the asthma model.

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 (4, 7, 15). Our present study aimed to investigate whether activation of sulfatide-reactive type II NKT cells can prevent allergic airway inflammation by regulating type I NKT cells. Our data show that, in a mouse model of asthma, sulfatide administration and adoptive transfer of sulfatide-activated type II NKT cells lead to reduced inflammation cell infiltration in the lung and bronchoalveolar lavage fluid (BALF); decreased levels of IL-4, IL-5 in the BALF; and decreased serum levels of ovalbumin (OVA)-specific IgE and IgG1. Our study also reveals that sulfatide-activated type II NKT cells reduce allergic airway inflammation, possibly by suppressing the activity of type I NKT cells. Taken together, our findings indicate that activating

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type II NKT cells using sulfatide can be exploited for intervention in asthma and other inflammatory diseases.

## MATERIALS AND METHODS

**Mice.** Female BALB/c wild-type mice (6 to 8 wk old) were purchased from and maintained in the Animal Biosafety Level 3 Laboratory, the Center for Animal Experiment, Wuhan University (Wuhan, China). All mice were housed in environmentally controlled and specific pathogen-free conditions (72°F, 12-h light/12-h dark cycle). All experimental procedures were approved by the Animal Welfare Committee of Wuhan University.

**Immunization and challenge.** Mice were intraperitoneally immunized on *days 0 and 14* with 100 µg of chicken OVA (grade V; Sigma, St Louis, MO) emulsified in 2.25 mg of aluminum hydroxide (Sigma) in 200 µl of PBS. Intranasal OVA challenges (100 µg/50 µl in PBS) were administered on *days 25, 26, and 27*. Age- and sex-matched control mice were treated in the same way with PBS only. The serum and BALF were harvested 48 h after the last challenge for ELISA, and changes in cell counts in the BALF were examined. The left lungs were homogenized in Trizol for RNA extraction, and the right lungs were removed for hematoxylin-eosin (H&E) and periodic acid-Schiff (PAS) staining. Spleen mononuclear cells (MNCs) were isolated for cell proliferation analysis and cellular enzyme-linked immunosorbent spot (ELISPOT) analysis. Lung MNCs were isolated for fluorescence-activated cell sorter (FACS) analysis.

**Sulfatide treatment.** 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 PBS (200 µl/mouse) or 200 µl PBS was injected intraperitoneally 1 h before challenge on *day 25*.

**Total and differential cell count analysis in BALF.** BAL was performed 48 h after the last OVA challenge with three 0.5-ml aliquots of PBS containing 0.6 mM EDTA. The BALF collected was centrifuged at 400 g for 5 min at 4°C, and the supernatant was stored at -80°C until assayed for cytokine levels. The recovered cells were counted and washed. Differential cell counts were assessed on cytological preparations obtained by cytocentrifugation (TXD3 cytocentrifuge; Xiangyi, Changsha, China) of 100 µl of diluted BALF ( $1 \times 10^6$  cells/ml in ice-cold saline-EDTA). Slides were stained with May-Gruenwald Giemsa (Jiancheng, Nanjing, China). At least 400 cells were counted for each preparation. Numbers of eosinophils, macrophages, neutrophils, and lymphocytes were expressed as absolute numbers from total cell counts.

**Lung histology and goblet cell hyperplasia.** The right lungs were removed after BAL and fixed in 4% buffered paraformaldehyde. The specimens were then dehydrated and embedded in paraffin. Sections (4 µm thick) were cut, placed on glass slides, deparaffinized, and stained with H&E or PAS (Baso, Taiwan, China). Peribronchial and perivascular inflammation was assessed using light microscopy under  $\times 100$  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. Four sections were evaluated per lung. The circumference of the airway at the basement membrane was measured by NIH image. The results were expressed as the number of PAS-positive cells per unit of length (mm) of the basement membrane.

**ELISPOT, ELISA, and immunoglobulin analysis.** The frequency of IFN- $\gamma$  and IL-4-producing cells was determined by cellular ELISPOT assays as described previously (15, 27). For ELISA, the BALF was collected 48 h after the last OVA challenge and stored at

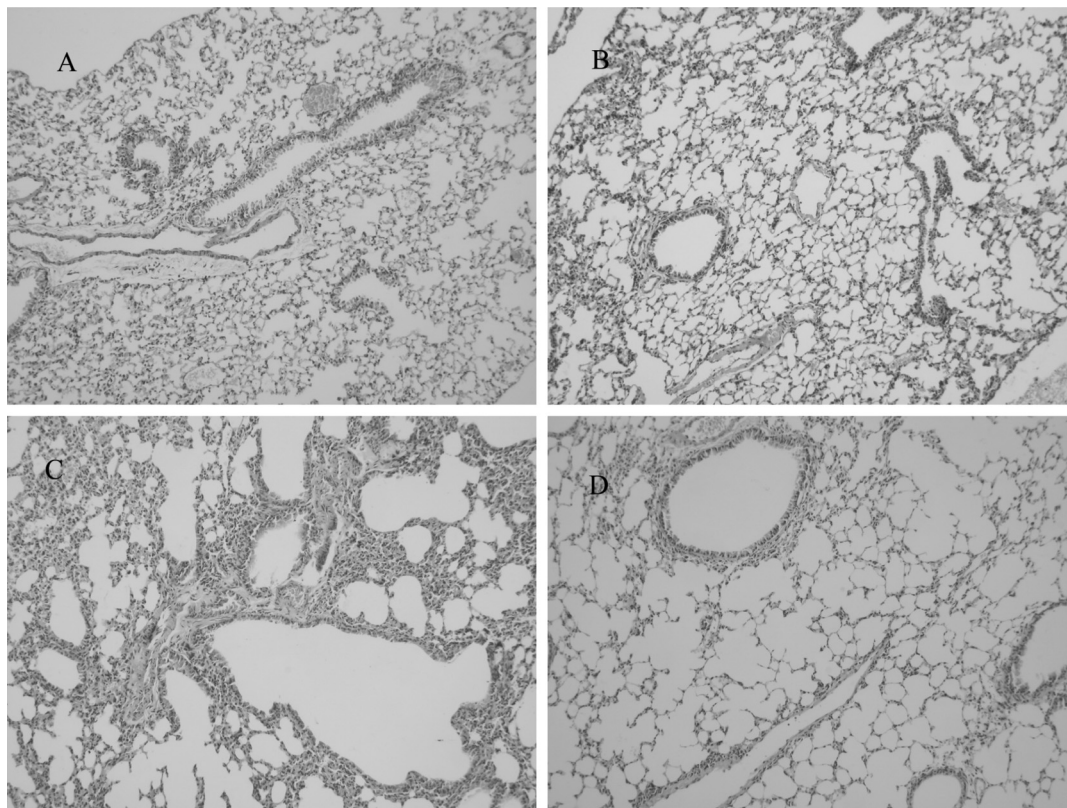


Fig. 1. Sulfatide reduces the recruitment of inflammatory cells into the lung tissue. Histological examination of the lung tissue was performed 48 h after the final ovalbumin (OVA) challenge, showing the hematoxylin and eosin (H&E) staining of the lung tissue sections from control mice treated with PBS (A) or sulfatide (B) and OVA-sensitized/OVA-challenged mice treated with PBS (C) or sulfatide (D) under  $\times 100$  magnification.

–20°C until use. The IL-4 and IL-5 levels in the BALF were quantified using a capture ELISA kit according to the manufacturer's instructions (eBioscience, San Diego, CA). The lower detection limits were 1.45 pg/ml for IL-4 and IL-5. For immunoglobulin analysis, 48 h after the last OVA challenge, the serum was harvested to determine the levels of OVA-specific IgE and IgG1 by ELISA. Briefly, 96-well plates (Corning Costar, Corning, NY) were coated with 200 µg/ml of OVA diluted in 0.1 M NaHCO<sub>3</sub> (pH 8.3). After 3-h incubation at 37°C, the plates were washed six times and blocked with 10% fetal bovine serum in PBS for 1 h at 37°C. Serum samples were then incubated overnight at 4°C. After six washes with PBS containing 0.05% Tween-20, biotinylated antibodies against mouse IgE or IgG1 (Biolegend, San Diego, CA) were added to the wells respectively and incubated for 1 h at room temperature. The plates were then washed six times and incubated with an avidin-peroxidase complex (Sigma) for 30 min at room temperature. After the final washes, tetramethylbenzidine (Sigma) was added, and coloration was allowed to develop for 15 to 20 min. The reaction was stopped by adding 2 M H<sub>2</sub>SO<sub>4</sub> and the absorbance intensity was read at 450 nm using a microplate reader (Tecan, Clontarf, Australia). Serum levels of OVA-specific IgE were expressed as optical density.

**Cell isolation.** Following euthanasia, the lungs were removed, cut into small pieces, digested in collagenase I (1 mg/ml; Invitrogen, Carlsbad, CA) at 37°C for 1 h, and filtered through a 100-µm nylon net filter. The lung MNCs were isolated by centrifugation at 800 g for 20 min at room temperature in a Lymphoprep gradient (density = 1.081 mg/ml; TBD, Tianjin, China). Splenocytes were obtained by

pressing the spleen through a 100-µm nylon net filter, and erythrocytes were lysed using a RBC lysis buffer (Sigma).

**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% Tween 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-PE (eBioscience) was added to 200 µg of the CD1d-sulfatide complexes and incubated at room temperature for 4 h as previously described (18). Tetramers were stored at 4°C until use.

**Adoptive transfer.** A single-cell suspension of splenocytes were cultured at a density of  $1 \times 10^7$ /well in a six-well plate in 3 ml of RPMI 1640 supplemented with 10% FCS, 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% CO<sub>2</sub> atmosphere. Then type II NKT cells were purified from spleen using PE-labeled sulfatide-loaded CD1d tetramers (purity was about 75% by flow cytometry for PE-labeled sulfatide/CD1d tetramer-positive cells double stained with PeCy5-conjugated anti-TCR-β), type II NKT cells ( $3 \times 10^6$ ), and were adoptively transferred into BALB/c mice via tail vein 1 h before challenge on day 25. The donors of spleen cells were naïve wild-type BALB/c mice.

**Flow cytometry.** Lung MNCs were resuspended in the FACS buffer ( $1-2 \times 10^6$  cells/ml). Cells were first blocked with anti-CD16/CD32 (clone2.4G2; BD Biosciences, San Diego, CA) and then labeled with isotype controls or the following antibodies: FITC-conjugated anti-IL-4, FITC-conjugated anti-IFN-γ, FITC-conjugated anti-CD69,

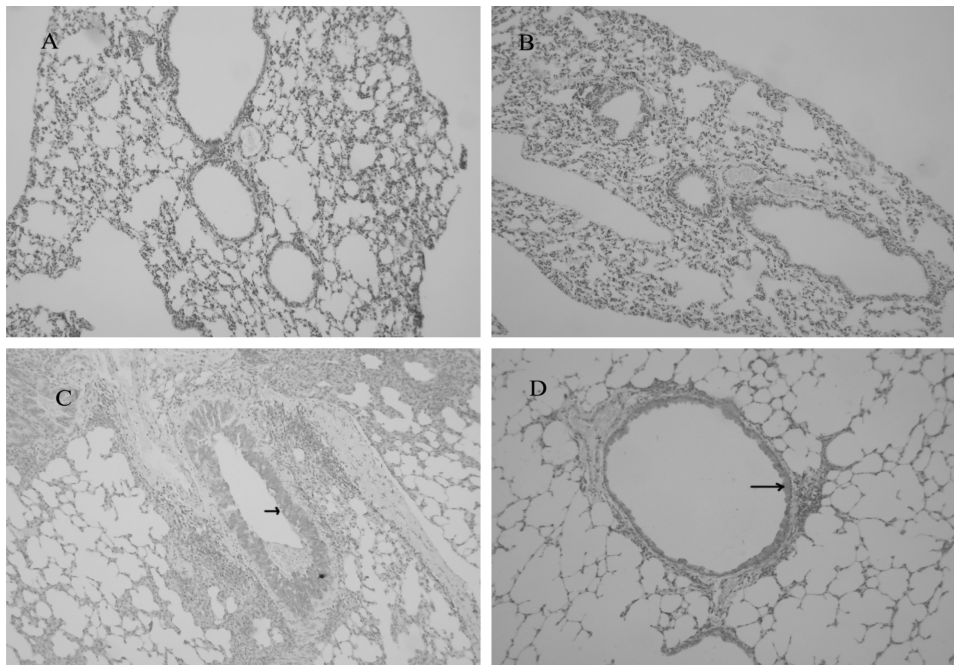
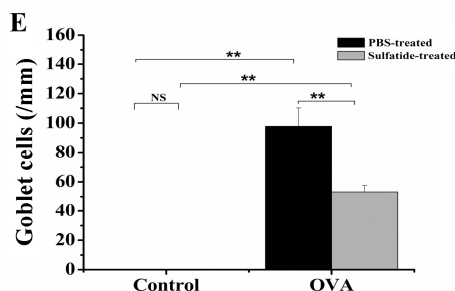


Fig. 2. Sulfatide reduces goblet cell hyperplasia in the airway epithelium. Histological examination of the lung tissue was performed 48 h after the final OVA challenge, showing the periodic acid-Schiff (PAS) staining of the lung tissue sections from control mice treated with PBS (A) or sulfatide (B) and OVA-sensitized/OVA-challenged mice treated with PBS (C) or sulfatide (D) under  $\times 100$  magnification. E: pulmonary goblet cell hyperplasia is expressed as the number of PAS-positive cells per unit of length (mm) of the basement membrane. Data are expressed as means  $\pm$  SD of 5 mice per group. \*\* $P < 0.01$ ; NS, not significant.



PeCy5-conjugated anti-TCR- $\beta$  (eBioscience), PE-labeled PBS-57( $\alpha$ -GalCer analog)/CD1d tetramer (NIH tetramer core facility), and PE-labeled sulfatide/CD1d tetramer. Dead cells were excluded by forward scatter and side scatter. Type I NKT cells were measured as PBS-57/CD1d tetramer and TCR- $\beta$  double-positive cells, whereas type II NKT cells were measured as sulfatide/CD1d tetramer and TCR- $\beta$  double-positive cells. For intracellular cytokine staining, lung MNCs were cultured for 4 to 6 h with 500 ng/ml ionomycin and 50 ng/ml PMA in the presence of monensin (1  $\mu$ l/ml). Cells were harvested and washed, and intracellular staining was performed according to the manufacturer's protocol (eBioscience). Cells were analyzed by flow cytometry (Epics Altra; Beckman, Seattle, WA).

**Quantitative real-time PCR.** Total RNA was extracted with Trizol (Invitrogen), and the cDNAs were synthesized using a Revertaid first-strand cDNA synthesis kit (ShineGene, Shanghai, China). Quantitative real-time PCR was conducted in triplicate using SYBR Green Supermix (ShineGene) in a 50- $\mu$ l reaction volume containing 600 nM of each primer and 5 ng of cDNA. The thermal cycling was initiated with a 4-min denaturation at 94°C, followed by 35 cycles of 94°C for 20 s, 60°C for 30 s, and 72°C for 30 s. The  $\beta$ -actin gene was used as an endogenous control. The following primer pairs were used: eosinophil peroxidase (EPO): forward 5'-ACTGTTTCCTGCTAGAGCTTTTGC-3', reverse 5'-AGAGTGCTGCTGCTTCCTCAGG-3'; and  $\beta$ -actin: forward 5'-GAGACCTTCAACACCCAGC-3', reverse 5'-ATGTCACGCACGATTT CCC-3'. The EPO mRNA expression level was assessed relative to  $\beta$ -actin expression level.

**Measurement of proliferation responses in vitro.** The spleen cell proliferation assays were performed using a Cell Counting Kit-8 (CCK-8; Donjindo, Kumamoto, Japan) according to the manufacturer's instructions. The spleen cells were seeded at a concentration of  $3 \times 10^5$  cells/100  $\mu$ l per well in 96-well culture plates and challenged with  $\alpha$ -GalCer (100 ng/ml; Enzo Life Sciences, Ann Arbor, MI), a specific ligand for type I NKT cells, in the absence or presence of IL-2 (5 ng/ml; Peprotech, Rocky Hill, NJ). After 72 h, the cultured wells were treated with 10  $\mu$ l/well of CCK-8 solution for 2 h, and the optical density of wells was measured at 450 nm using a microplate reader

(Tecan). All measurements were performed in sextuplicate. Results were expressed as mean  $A450 \pm$  SD.

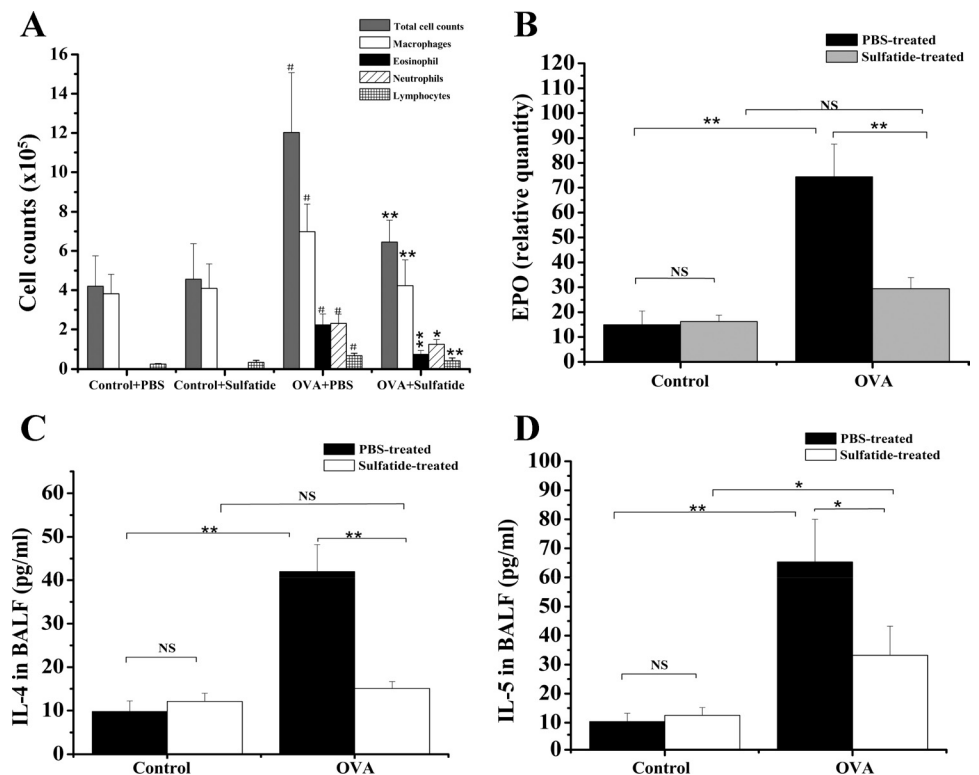
**Statistical analysis.** All data were presented as means  $\pm$  SD. Statistical significance between groups was determined using Student's paired *t*-test (two groups) or one-way ANOVA (four groups) followed by the least-significant difference test in the condition of variance homogeneity and Dunnett's T3 test in variance heterogeneity. Data were analyzed using the SPSS13.0 software. A *P* value  $<0.05$  was considered statistically significant.

## RESULTS

**Sulfatide treatment reduces the recruitment of inflammatory cells into the lung.** To determine whether sulfatide could modulate antigen-induced airway inflammation, we administered sulfatide or control vehicle into mice 1 h before challenge on day 25. No apparent inflammatory cell infiltration, including lung eosinophil infiltration, was observed in PBS-treated (Fig. 1A) or sulfatide-treated (Fig. 1B) control mice. As expected, PBS-treated OVA-challenged mice showed marked inflammatory cell infiltration in the airways and vessels (Fig. 1C). Strikingly, a single injection of sulfatide remarkably reduced airway inflammation in the lungs of OVA-challenged mice (Fig. 1D).

**Sulfatide treatment reduces goblet cell hyperplasia.** To evaluate airway mucus hypersecretion and goblet cell hyperplasia, we stained the lung sections with PAS. No PAS-positive cells were observed in the lungs of PBS-treated (Fig. 2A) or sulfatide-treated (Fig. 2B) control mice. OVA exposure led to numerous PAS-positive goblet cells in the epithelium of OVA-sensitized and challenged mice. However, sulfatide treatment significantly inhibited goblet cell hyperplasia in the airway compared with control PBS treatment (Fig. 2, C and D). Mucus overproduction, observed as a violet color, was also much

Fig. 3. Sulfatide reduces cellular infiltration and IL-4, IL-5 secretion in bronchoalveolar lavage fluid (BALF). A: numbers of total cells, macrophages, eosinophils, neutrophils, and lymphocytes in the BALF from control mice injected with PBS or sulfatide, and OVA-challenged mice injected with PBS or sulfatide. Data are expressed as means  $\pm$  SD,  $n = 5$  per group. # $P < 0.01$  (compared with the control + PBS group). \* $P < 0.05$ , \*\* $P < 0.01$  (compared with the OVA + PBS group). B: eosinophil peroxidase (EPO) mRNA levels in the lungs from control or OVA-challenged mice treated with PBS or sulfatide, normalized to  $\beta$ -actin. Data are shown as means  $\pm$  SD,  $n = 5$  per group. \*\* $P < 0.01$ ; NS, not significant. C and D: levels of IL-4 and IL-5 in the BALF from control or OVA-challenged mice treated with PBS or sulfatide were assessed by ELISA. Data are expressed as means  $\pm$  SD,  $n = 5$  per group. \* $P < 0.05$ ; \*\* $P < 0.01$ ; NS, not significant.



more severe in PBS-treated than in sulfatide-treated OVA-challenged mice. The number of PAS-positive cells per unit of length (mm) of the basement membrane ( $53.2 \pm 4.55$ ; Fig. 2E) in sulfatide-treated OVA-challenged mice is significantly lower than that in PBS-treated OVA-challenged mice ( $97.8 \pm 12.5$ ; Fig. 2E). Taken together, our findings that sulfatide reduces pulmonary inflammatory cell infiltration (Fig. 1) and goblet cell hyperplasia (Fig. 2) in OVA-challenged mice suggest that sulfatide may inhibit airway inflammation in asthma.

*Sulfatide treatment reduces the levels of cells and cytokines in the BALF.* We next examined the effects of sulfatide on the cell number and components of the BALF. Cellular profiles in the BALF upon OVA challenge were assessed by May-Gruenwald-Giemsa stained cytopins. As expected, OVA challenges induced eosinophilic airway inflammation, characterized by a significant increase in the number of eosinophils, lymphocytes, neutrophils, and total cells in the BALF 48 h after the last OVA challenge (Fig. 3A, all  $P < 0.01$ ). Strikingly, this increase in OVA-challenged mice was significantly abolished by sulfatide administration (Fig. 3A, total cells, macrophages, eosinophils, and neutrophils:  $P < 0.01$ ; lymphocytes:  $P < 0.05$ ). The recruitment of eosinophils into the lung was further confirmed by determining the mRNA levels of EPO, an eosinophil activation marker. In OVA-challenged mice, EPO mRNA levels were significantly elevated 48 h after the last OVA challenge, compared with the levels in PBS-treated or sulfatide-treated control mice (Fig. 3B,  $P < 0.01$ ). Sulfatide administration in OVA-challenged mice effectively reduced the mRNA levels of EPO by about 60% (Fig. 3B,  $P < 0.01$ ). In addition, we determined whether sulfatide affected lung inflammatory cytokine production. The BALF supernatant was collected 48 h after the last OVA challenge, and the levels of IL-4, IL-5 in the BALF were analyzed by ELISA. In PBS-treated OVA-challenged mice, higher levels of Th2 cytokines (IL-4 and IL-5) were detected, compared with the levels in PBS- or sulfatide-treated control mice (Fig. 3C, all  $P < 0.01$ ). In OVA-challenged mice, administration of sulfatide significantly reduced the levels of IL-4 and IL-5 in the BALF 48 h after the last OVA challenge (Fig. 3C, IL-4:  $P < 0.01$ ; IL-5:  $P < 0.05$ ). In control mice, there is no difference in the levels of IL-4, IL-5 in the BALF in sulfatide-treated mice and PBS-treated mice (Fig. 3C).

*Sulfatide treatment reduces OVA-specific IgE and IgG1.* Allergic airway inflammation is typically accompanied by increased serum levels of IgE and IgG1, whose synthesis heavily depends on Th2 cells. Thus we examined whether sulfatide injection influenced OVA-specific IgE and IgG1 release. OVA-specific Igs, although not detectable in control mice, were readily detected in OVA-challenged mice by ELISA (Fig. 4). Sulfatide treatment significantly reduced the levels of OVA-specific IgE and IgG1 in OVA-challenged mice (Fig. 4, all  $P < 0.01$ ).

*Sulfatide treatment activates type II NKT cells in vivo.* To confirm that the amount of sulfatide used in this study (20  $\mu\text{g}/\text{mouse}$ ) could activate type II NKT cells in vivo, the percentage of lung type II NKT cells, measured as sulfatide/CD1d tetramer and TCR- $\beta$  double-positive cells, was examined 48 h after the last challenge in control or OVA-challenged mice. In the absence of sulfatide, no change was observed in the percentage of lung type II NKT cells (Fig. 5, A and B) or cytokine secretion by (Fig. 5, C and D) lung type II NKT cells

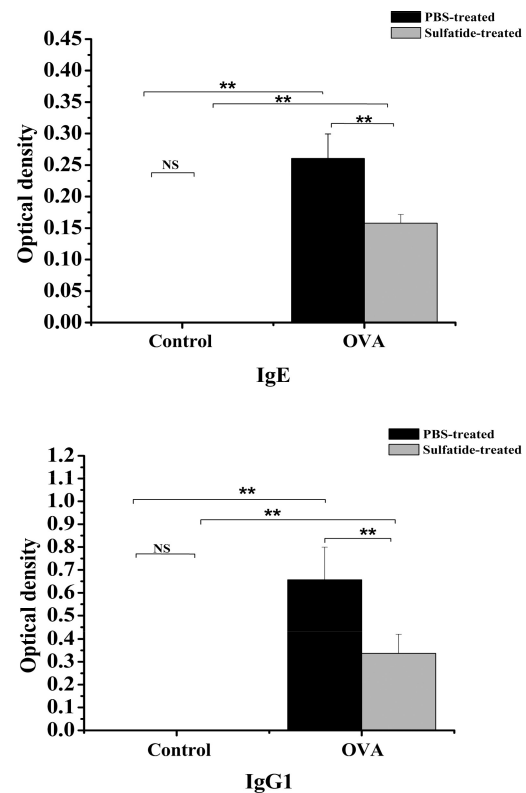


Fig. 4. Sulfatide reduces the serum levels of OVA-specific IgE and IgG1. The serum was collected 48 h after the last OVA challenge. The levels of OVA-specific IgE and IgG1 were then analyzed by ELISA. Data are shown as means  $\pm$  SD ( $n = 5-8$ ). \*\* $P < 0.01$ ; NS, not significant.

between control and OVA-challenged mice, suggesting that type II NKT cells were not activated upon OVA challenge without sulfatide injection.

After sulfatide injection, the percentage of lung type II NKT cells was slightly increased compared with the value after PBS treatment, but the difference was not significant (Fig. 5, A and B). In addition, there was a marked increase in the level of IFN- $\gamma$ -producing lung type II NKT cells (sulfatide/CD1d tetramer-, TCR- $\beta$ -, and IFN- $\gamma$ -positive cells) in sulfatide-treated mice compared with in PBS-treated mice (Fig. 5, C and D,  $P < 0.01$ ). A similar increase was also observed in the level of IL-4-producing lung type II NKT cells (sulfatide/CD1d tetramer-, TCR- $\beta$ -, and IL-4-positive cells) (Fig. 5, C and D,  $P < 0.01$ ). These data suggest that sulfatide activates type II NKT cells in control and OVA-challenged mice.

To further examine the activation of sulfatide-reactive T cells, ELISPOT analysis was carried out on splenocytes from OVA-challenged mice injected with sulfatide or PBS. As shown in Fig. 5E, administration of sulfatide to mice expanded a T cell population in splenocytes that produces IFN- $\gamma$  and IL-4 in response to an in vitro recall with sulfatide. The frequency of sulfatide-reactive T cells is three- to fivefold higher in sulfatide-treated mice than in PBS-treated mice (Fig. 5E,  $P < 0.01$ ). These data clearly indicate that sulfatide-reactive type II NKT cells are activated and secrete cytokines following recognition of the self-glycolipid sulfatide.

*Adoptive transfer of sulfatide-activated type II NKT cells prevents allergic airway inflammation.* To confirm that the effect of sulfatide administration on allergic airway inflamma-

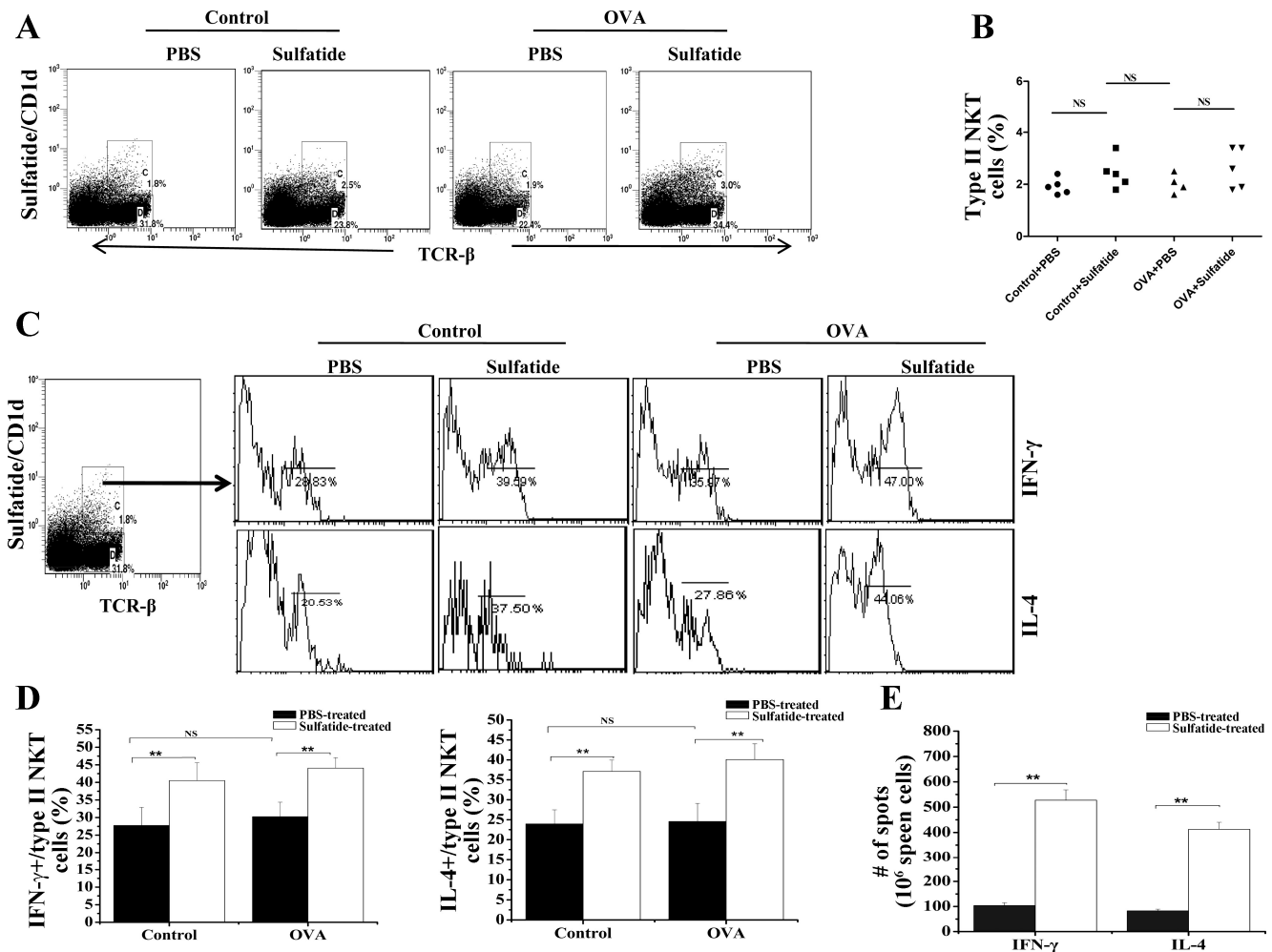


Fig. 5. Activation of type II natural killer T (NKT) cells following sulfatide injection in vivo. **A:** lung mononuclear cells (MNCs) were isolated 48 h after the last challenge from control and OVA-challenged mice injected with PBS or sulfatide, and stained using sulfatide/CD1d tetramers and anti- T cell receptor (TCR)- $\beta$  antibody. The gate used for type II NKT cells (gate C) and the corresponding percentages are shown in each dot plot. **B:** percentage of type II NKT cells in lung MNCs from control and OVA-challenged mice (OVA-challenged mice treated with PBS:  $n = 4$ ; other groups:  $n = 5$ ). Data are expressed as means  $\pm$  SD. NS, not significant. **C:** IFN- $\gamma$ - and IL-4-producing lung type II NKT cells 48 h after the last challenge. Sulfatide/CD1d tetramer and TCR- $\beta$  double-positive cells are examined for IFN- $\gamma$  (top) and IL-4 (bottom) secretion. Numbers below brackets indicate the percentage of positive cells. **D:** percentage of IFN- $\gamma$ - and IL-4-producing lung type II NKT cells. Data are expressed as mean  $\pm$  SD (control mice:  $n = 5$  per group; OVA-challenged mice:  $n = 6$  per group;  $**P < 0.01$ ; NS, not significant). **E:** increased frequency of cytokine-secreting, sulfatide-reactive lymphocytes after immunization with sulfatide. Mice ( $n = 5$  per group) were immunized with 20  $\mu$ g of sulfatide or PBS alone. 48 h after the last challenge, splenocytes were isolated, recalled in vitro with sulfatide (40  $\mu$ g/ml), and subjected to enzyme-linked immunosorbent spot (ELISPOT) analysis. The average number of spots/10<sup>6</sup> spleen cells for both IFN- $\gamma$  and IL-4 in each group is shown. Data are expressed as means  $\pm$  SD.  $**P < 0.01$ .

tion is dependent on sulfatide-activated type II NKT cells, we adoptive transferred sulfatide-activated type II NKT cells to OVA-sensitized BALB/c mice and challenged with OVA. Adoptive transfer of sulfatide-activated type II NKT cells that bound sulfatide-loaded CD1d tetramer and TCR- $\beta$  (>75% tetramer and TCR- $\beta$  double-positive; Fig. 6A) significantly attenuated allergic airway inflammation, including reduced inflammatory cell infiltration in the lung tissues and BALF (Fig. 6, B and C, total cells, eosinophils, lymphocytes:  $P < 0.01$ ; macrophages and neutrophils:  $P < 0.05$ ), decreased goblet cell hyperplasia in the airway (Fig. 6A), reduced EPO mRNA expression in the lung tissues (Fig. 6B,  $P < 0.05$ ), as well as decreased IL-4 and IL-5 levels in BALF (Fig. 6D, all  $P < 0.01$ ), and OVA-specific IgE and IgG1 levels in serum (Fig. 6E, IgE:  $P < 0.01$ ; IgG1:  $P < 0.05$ ). These results

indicate that adoptive transfer of sulfatide-activated type II NKT cells prevents allergic airway inflammation.

**Sulfatide-activated type II NKT cells suppresses type I NKT cell function.** We first confirmed that type I NKT cells were activated and recruited into the lungs upon OVA challenge. As expected, type I NKT cells, measured as PBS-57/CD1d tetramer and TCR- $\beta$  double-positive cells, accumulated in the lungs upon OVA challenge (Fig. 6, A and B). The early activation marker CD69 was also markedly upregulated in lung type I NKT cells in OVA-challenged mice compared with in control mice (Figs. 6D,  $P < 0.01$ ). Furthermore, OVA-challenged mice had more lung type I NKT cells producing IFN- $\gamma$  and IL-4 than control mice (Fig. 6, C and D,  $P < 0.01$ ).

We next investigated whether/how type I NKT cells were mediated by type II NKT cells upon sulfatide treatment.

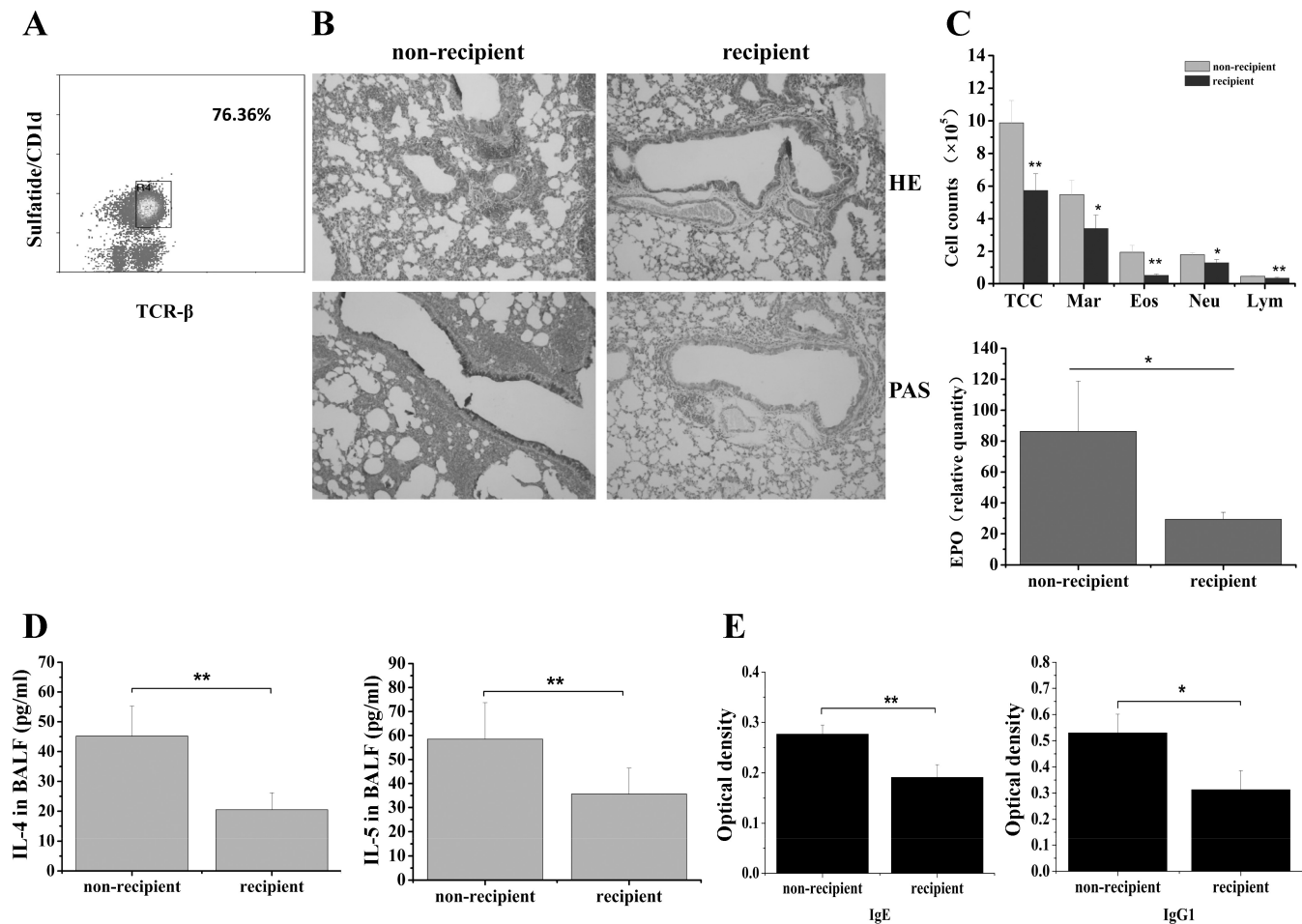


Fig. 6. Adoptive transfer of sulfatide-activated type II NKT cells prevents allergic airway inflammation. **A**: purity of the transferred sulfatide-activated type II NKT cell population. Flow cytometry showed that 75% of the transferred cells stained with sulfatide/CD1d tetramers and anti-TCR- $\beta$  antibody. **B**: histological examination of the lung tissue was performed 48 h after the final OVA challenge, showing the H&E and PAS staining of the lung tissue sections from mice with or without sulfatide-activated type II NKT cells adoptive transfer (magnification,  $\times 100$ ). **C**: adoptive transfer of sulfatide-activated type II NKT cells prevents inflammatory cell infiltration in BALF and EPO mRNA expression. Data are expressed as means  $\pm$  SD,  $n = 5$  per group. \* $P < 0.05$ , \*\* $P < 0.01$ . TCC, total cell counts; Mar, macrophages; Eos, eosinophils; Neu, neutrophils; Lym, lymphocytes. **D**: adoptive transfer of sulfatide-activated type II NKT cells prevents IL-4 and IL-5 secretion in BALF. Data are expressed as means  $\pm$  SD,  $n = 5$  per group. \*\* $P < 0.01$ . **E**: adoptive transfer of sulfatide-activated type II NKT cells reduces the serum levels of OVA-specific IgE and IgG1. Data are shown as mean  $\pm$  SD,  $n = 5$  per group. \* $P < 0.05$ , \*\* $P < 0.01$ .

Although sulfatide administration did not affect the percentage of type I NKT cells in lung MNCs (Fig. 7, *A* and *B*) or the expression of CD69 in lung type I NKT cells (Fig. 7*D*), IFN- $\gamma$  and IL-4-producing type I NKT cells were significantly reduced after sulfatide treatment (Fig. 7, *C* and *D*,  $P < 0.01$ ). To further examine the activation of  $\alpha$ -GalCer-reactive T cells (type I NKT cells), ELISPOT analysis was carried out on splenocytes from OVA-challenged mice injected with sulfatide or PBS. As shown in Fig. 7*E*, the frequency of IFN- $\gamma$  and IL-4-secreting cells was significantly decreased following sulfatide injection ( $P < 0.01$ ). We next examined whether the recruited type I NKT cells in the lung were suppressed by assessing their proliferation in response to an *in vitro* challenge with  $\alpha$ -GalCer in the presence or absence of IL-2. Our results showed that the proliferation of type I NKT cells in response to  $\alpha$ -GalCer was significantly inhibited by sulfatide injection, and this inhibition could be reversed by adding IL-2 into cultures from sulfatide-treated mice (Fig. 7*F*). Taken together, these data clearly indicate that the function of type I NKT cells is inhibited by sulfatide injection.

## DISCUSSION

In the present study, we demonstrate that sulfatide, a specific ligand for type II NKT cells, prevents allergic airway inflammation in a mouse model of asthma. Sensitized BALB/c mice treated with sulfatide showed significant reductions in pulmonary inflammatory cell infiltration, goblet cell hyperplasia, Th2 cytokine levels in the BALF, and OVA-specific IgE and IgG1 levels in the serum. In addition, it was found that adoptive transfer of sulfatide-activated type II NKT cells also prevented allergic airway inflammation. Furthermore, we demonstrate that sulfatide-activated type II NKT cells suppressed the proliferation and cytokine secretion of type I NKT cells. Our data indicate that sulfatide-activated type II NKT cells may be used in a novel therapeutic target for allergic diseases, such as asthma, by inhibiting the activity of type I NKT cells.

Several studies have characterized the roles of type II NKT cells in different immune responses, including infectious and autoimmune diseases (10–12, 15, 26). Recently, sulfatide-activated type II NKT cells were shown to prevent IRI in

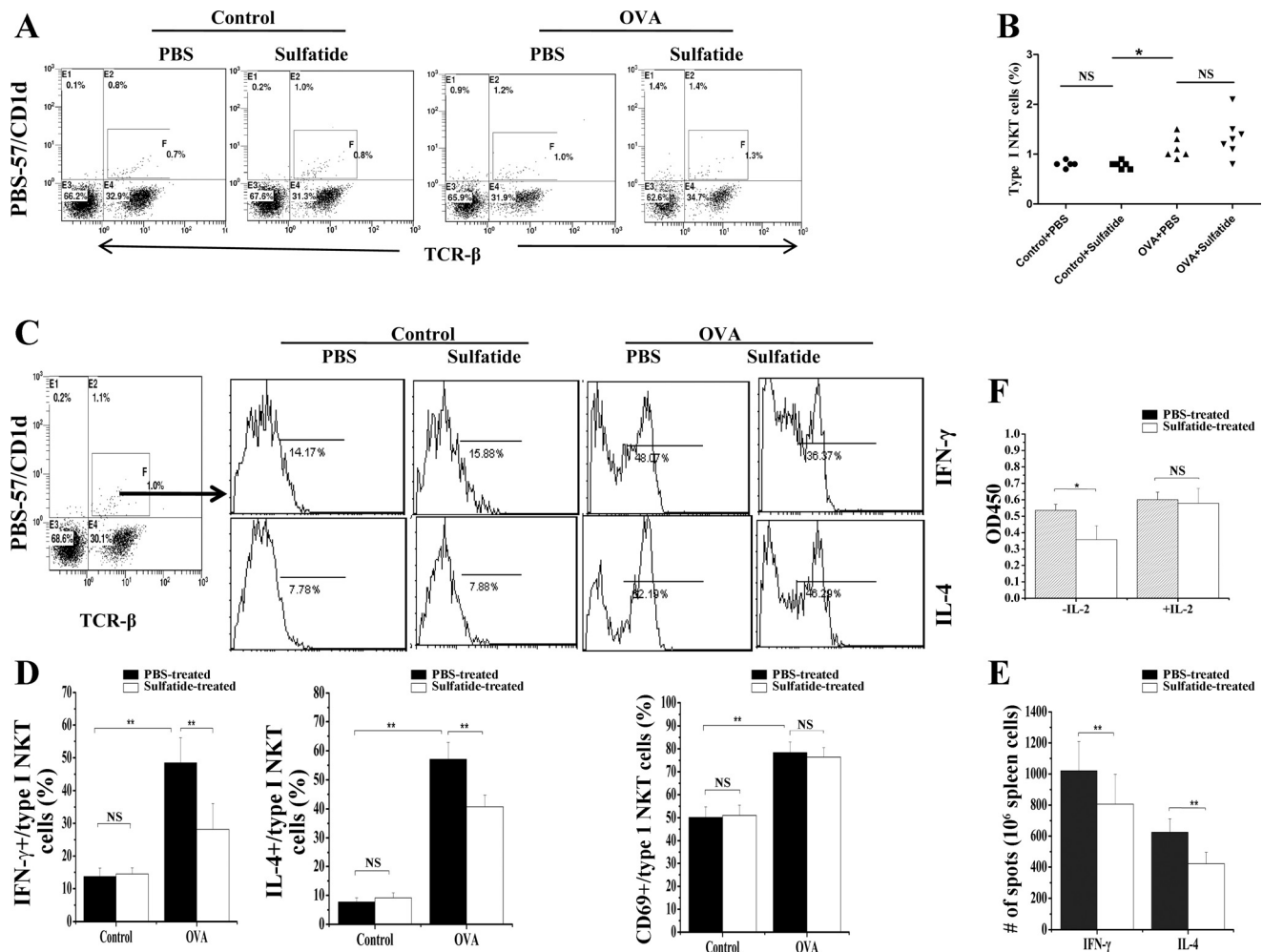


Fig. 7. Suppression of type I NKT cells by sulfatide in OVA-challenged mice. **A**: lung MNCs were isolated 48 h after the last challenge from control and OVA-challenged mice injected with PBS or sulfatide and stained using PBS-57/CD1d tetramers and anti-TCR-β antibody. The gate used for type I NKT cells (gate F) and the corresponding percentages are shown in each dot plot. **B**: percentage of type I NKT cells in lung MNCs from control and OVA-challenged mice (PBS-treated control mice:  $n = 5$ ; sulfatide-treated OVA-challenged mice:  $n = 7$ ; other groups:  $n = 6$ ). Data are expressed as means  $\pm$  SD. \* $P < 0.05$ ; NS, not significant. **C**: lung type I NKT cells producing IFN-γ and IL-4 48 h after the last challenge. PBS-57/CD1d and TCR-β double-positive cells are examined for IFN-γ (top) and IL-4 (bottom) secretion. Numbers below brackets indicate the percentage of positive cells. **D**: percentage of IFN-γ-, IL-4-, and CD69-expressing lung type I NKT cells. Data are expressed as means  $\pm$  SD ( $n = 5$  per group; \*\* $P < 0.01$ ; NS, not significant). **E**: decreased frequency of cytokine-secreting type I NKT cells after immunization with sulfatide. Mice ( $n = 5$  per group) were immunized with 20 μg of sulfatide or PBS alone. 48 h after the last challenge, splenocytes were isolated, recalled in vitro with α-GalCer (100 ng/ml), and subjected to ELISPOT analysis. The average number of spots/10<sup>6</sup> spleen cells for both IFN-γ and IL-4 in each group is shown. Data are expressed as means  $\pm$  SD. \*\* $P < 0.01$ . **F**: proliferation of splenocytes in response to in vitro challenge with α-GalCer (100 ng/ml) in the absence or presence of IL-2 (5 ng/ml). Splenocytes were isolated from OVA-challenged mice 48 h after the last challenge. Data shown are means  $\pm$  SD from 3 independent experiments ( $n = 5$  per group). \* $P < 0.05$ ; NS, not significant.

kidney and hepatic IRI models (5, 29). However, in the context of asthma, this is the first study to investigate the role of sulfatide-reactive type II NKT cells. A significant hallmark of asthma is accumulation of eosinophils, neutrophils, lymphocytes, and macrophages in the lung (9). In our mouse model, we detected profoundly reduced levels of inflammatory cells into the lung tissue in sulfatide-treated mice compared with asthma mice without sulfatide treatment (Figs. 1 and 2). Th2-biased immune response characterized by production of Th2 cytokines IL-4, IL-5, and IL-13, plays a critical role in the pathophysiology of asthma (17). As expected, the productions of the Th2 cytokines were dramatically reduced in the BALF in sulfatide-treated mice (Fig. 3). Furthermore, the serum levels of OVA-specific IgE and IgG1 were reduced in asthma mice administrated with sulfatide (Fig. 4). In addition, sulfatide

treatment did not have any effect on the histology (Figs. 1 and 2), cytokine secretion (Fig. 3), and OVA-specific Ig production (Fig. 4) in control mice, suggesting that sulfatide administration did not cause apparent adverse effects in our mouse model. These results collectively suggest that sulfatide treatment is safe and can prevent the allergic airway inflammation in our mouse model.

To reveal the underlying mechanisms, we measured the activation of type II NKT cells in the presence or absence of sulfatide. We first confirmed that type II NKT cells were not activated by OVA challenge alone, manifested by similar percentages of lung type II NKT cells and similar levels of cytokine secretion between control and OVA-challenged mice in the absence of sulfatide (Fig. 5). We then examined the activation of type II NKT cells upon sulfatide treatment. The

percentage of lung type II NKT cells in sulfatide-treated mice was a little higher than that in PBS-treated mice, but the difference was not significant (Fig. 5, A and B). However, sulfatide treatment significantly increased the level of IFN- $\gamma$  and IL-4-producing type II NKT cells in lung MNCs (Fig. 5, C and D) and the frequency of cytokine-secreting, sulfatide-reactive lymphocytes (Fig. 5E), suggesting that type II NKT cells were activated upon sulfatide treatment. These results are consistent with a recent study in which splenic cells from wild-type but not CD1d-deficient mice proliferated and produced IFN- $\gamma$  and IL-4 in response to sulfatide (15) and another study in which the percentage of IFN- $\gamma$ -producing type II NKT cells was increased after sulfatide injection (12). In addition, it was found that adoptive transfer of sulfatide-activated type II NKT cells to OVA-sensitized BALB/c mice, significantly attenuated allergic airway inflammation, including reduced inflammatory cell infiltration in the lung tissues and BALF (Fig. 6, B and C), decreased goblet cell hyperplasia in the airway (Fig. 6A), reduced EPO mRNA expression in the lung tissues (Fig. 6B), as well as decreased IL-4 and IL-5 levels in BALF (Fig. 6D) and OVA-specific IgE and IgG1 levels in serum (Fig. 6E). These data collectively indicate that sulfatide-activated type II NKT cells prevent allergic airway inflammation.

Furthermore, we investigated how sulfatide-activated type II NKT cells prevented allergic airway inflammation. Consistent with previous studies, which has suggested the importance of type I NKT cells in asthma (1, 2, 14, 19, 20, 22, 23, 28), our study showed that lung type I NKT cells were activated in OVA-challenged mice, evidenced by the increased percentage of pulmonary type I NKT cells (Fig. 7, A and B), upregulation of CD69 (Fig. 7D), and increased cytokine secretion (Fig. 7D). In a recent study, activation of sulfatide-reactive type II NKT cells induces anergy of type I NKT cells, thereby preventing experimental inflammatory liver disease (12). In addition, sulfatide-mediated activation of type II NKT cells prevented IRI in hepatic IRI models by suppressing type I NKT cells function (5). In tumor immunity, sulfatide-activated type II NKT cells appeared to suppress the activation of type I NKT cells in vitro and suppressed the immunosurveillance of type I NKT cells in vivo (3). Accompanied with these researches, in our mouse model, it was found that activation of type II NKT cells by sulfatide resulted in inhibition of the activation of type I NKT cells including the following: 1) lack of cytokine secretion in lung type I NKT cells (Fig. 7, C and D); 2) decreased frequency of cytokine-secreting splenic lymphocytes in response to  $\alpha$ -GalCer in vitro (Fig. 7E); and 3) failure to proliferate in response to  $\alpha$ -GalCer in vitro in the absence of exogenously added IL-2 (Fig. 7F). These data suggest that sulfatide-activated type II NKT cells affected the type I NKT cell population, thereby preventing allergic airway inflammation.

In this study, we showed that sulfatide-activated type II NKT cells reduced allergic airway inflammation. However, many questions remain to be answered. Whether sulfatide-activated type II NKT cells also reduce AHR, another important feature of asthma, remains to be tested. Another interesting question is, in addition to type I NKT cells, whether sulfatide-activated type II NKT cells also affect other important cells in asthma, such as CD4<sup>+</sup> CD25<sup>+</sup> Tregs, dendritic cells, Th1, and Th2 cells. To fully capture the dynamic of the immune cascade in the lung following sulfatide stimulation, different doses of

sulfatide and different time points should be examined in future studies.

In conclusion, our present study shows in a mouse model of asthma that sulfatide-activated type II NKT cells prevent OVA-induced allergic airway inflammation, possibly by inhibiting the function of type I NKT cells. The activation of type II NKT cells may provide a promising new approach to treating patients with diseases characterized by unwanted type I NKT cell activation, such as allergic disorders and asthma.

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#### DISCLOSURES

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

#### AUTHOR CONTRIBUTIONS

Author contributions: G.Z. and Z.Y. performed experiments; G.Z. and Y.H. analyzed data; G.Z. interpreted results of experiments; G.Z. drafted manuscript; H.N., J.Y., and S.H. conception and design of research; H.N., H.Y., and R.L. edited and revised manuscript; X.D. and Y.H. prepared figures.

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