

RESEARCH ARTICLE | Biomarkers in Lung Diseases: From Pathogenesis to Prediction to New Therapies

Invariant natural killer T cells promote immunogenic maturation of lung dendritic cells in mouse models of asthma

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He Q, Liu L, Yang Q, Wang A, Chen S, Li R, Huang Y, Zhang G, Ding X, Yu H, Hu S, Nie H. Invariant natural killer T cells promote immunogenic maturation of lung dendritic cells in mouse models of asthma. *Am J Physiol Lung Cell Mol Physiol* 313: L973–L990, 2017. First published September 14, 2017; doi: 10.1152/ajplung.00340.2016.—Our previous study showed that invariant natural killer T (iNKT) cells might act as an adjuvant to promote Th2 inflammatory responses in an OVA-induced mouse model of allergic asthma, but the mechanism remains unknown. To clarify the underlying mechanism through which iNKT cells promote Th2 inflammatory responses, we investigated the modulatory influence of iNKT cells on phenotypic and functional maturation of lung dendritic cells (LDCs) using iNKT cell-knockout mice, specific iNKT cell activation, coculture experiments, and adoptive transfer of iNKT cells in mouse models of asthma. Our data showed that iNKT cell deficiency could downregulate surface maturation markers and proinflammatory cytokine secretion of LDCs from a mouse model of asthma. However, elevated activation of iNKT cells by α -galactosylceramide and adoptive transfer of iNKT cells could upregulate surface maturation markers and proinflammatory cytokine secretion of LDCs from mouse models of asthma. Meanwhile, iNKT cells significantly influenced the function of LDCs, markedly enhancing Th2 responses in vivo and in vitro. In addition, iNKT cell can induce LDCs expression of CD206 and RELM- α , reflecting alternative activation of LDCs in a mouse model of asthma. α -Galactosylceramide treatment significantly enhanced expression of CD40L of lung iNKT cells from a mouse model of asthma, and the coculture experiment of LDCs with iNKT cells showed that the blockade of CD40L strongly suppressed surface maturation markers and proinflammatory cytokine production by LDCs. Our data suggest that iNKT cells can promote immunogenic maturation of LDCs to enhance Th2 responses in mouse models of asthma.

asthma; invariant natural killer cells; dendritic cells; Th2 responses; immunogenic maturation

INTRODUCTION

Invariant natural killer T (iNKT) cells, a unique subpopulation of T lymphocytes, possess important immunoregulatory functions (5, 7) and have been implicated in a wide array of

disorders, including asthma, cancer, autoimmunity, and infectious diseases (15, 39, 51). iNKT cells express semi-invariant T cell receptors (TCRs) encoded by *V α 14-J α 18* gene segments in mice or *V α 24-J α 18* in humans, which recognize glycolipid antigens presented by the nonpolymorphic major histocompatibility complex (MHC) class I-like molecule CD1d (6, 36). It has been determined that iNKT cells recognize α -galactosylceramide (α -GalCer), a prototypic glycolipid originally identified in marine sponges (5). iNKT cells are well known to link innate and adaptive immune responses due to their capacity to stimulate a broad range of innate and adaptive immune cells, such as dendritic cells (DCs), natural killer cells, T cells, and B cells. This occurs through the prompt secretion of copious quantities of Th1 and Th2 cytokines such as interferon (IFN)- γ and IL-4 (5, 48). Recently, the activation of NKT cells by α -GalCer can enhance the conventional CD4⁺ and CD8⁺ T cell response to soluble antigens through the regulation of DC function (20). Fujii et al. (13) reported that activation of NKT cells by α -GalCer rapidly induces full maturation of DCs and that this stimulatory effect accounts for the production of combined CD4⁺ and CD8⁺ T cell immune responses to coadministered proteins. In addition, the combined use of NKT cell activation with α -GalCer and different Toll-like receptor ligands promoted the capacity of DCs to activate naïve T cells in vitro (19). Although these reports indicated that iNKT cell activation can provide potent stimulatory signals to enhance DC function, much less is known about the role of iNKT cells in modulating LDC function during the development of asthma.

The role of iNKT cells in the development of asthma has been studied intensively. Akbari et al. (2) reported that the number of iNKT cells is significantly increased in the airways of patients with asthma. Furthermore, two studies suggested that iNKT cells play an important role in the initiation of airway hyperresponsiveness (AHR) and allergic airway inflammation (3, 32). Our previous data revealed that iNKT cells could serve as an adjuvant to promote Th2 inflammatory responses in an OVA-induced mouse model of allergic asthma (39). iNKT cells can facilitate T cell responses when activated by a powerful stimulus such as the synthetic glycolipid α -GalCer, suggesting that iNKT cells could provide the stimulatory signals required for DC maturation and activation (13, 20).

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Thus, it was hypothesized that iNKT cells might promote the function of LDCs in asthmatics.

To confirm this hypothesis, we used a combined approach involving iNKT cell-knockout mice, specific iNKT cell activation, coculture experiments, and adoptive transfer of iNKT cells to investigate the modulatory effect of iNKT cells on the phenotypic and functional maturation of LDCs in an OVA- or house dust mite (HDM)-induced mouse model of asthma. Our data demonstrate that iNKT cells can promote the immunogenic maturation of lung DCs to enhance Th2 responses 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 of the Center for Animal Experiments, Wuhan University (Wuhan, China). CD1d^{-/-} mice and OVA-specific TCR transgenic DO11.10 mice (BALB/c background) were obtained from The 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 animal care and handling protocols were approved by the Institutional Ethics Committee of Wuhan University.

OVA or HDM sensitization and airway challenge. WT mice and CD1d^{-/-} mice were immunized intraperitoneally on days 0 and 14 with 20 µg of chicken ovalbumin (OVA) (grade V; Sigma, St. Louis, MO) bound to 2 mg of Inject Alum (Thermo Scientific Pierce, Rockford, Rockford, IL) in 200 µl of phosphate-buffered saline (PBS). This was followed by intranasal OVA challenges (100 µg in 50 µl of PBS) on days 25, 26, and 27. Mice were euthanized 24 h after the final challenge for further analysis. In some experiments, WT mice and CD1d^{-/-} mice were sensitized intranasally with 1 µg of HDM extracts (*Dermatophagoides pteronyssinus*; Greer Laboratories) on day 1 and subsequently challenged intranasally with 10 µg of HDM on days 7–11 to induce allergic airway inflammation, and 3 days after the final challenge they were euthanized for dissection (42). Littermate mice were similarly handled and challenged with PBS only as controls.

α-GalCer administration. A stock solution of α-GalCer (Enzo Life Sciences, Ann Arbor, MI) was diluted to 0.01 mg/ml in 0.5% polysorbate-20 and stored at -20°C. Mice received a single intraperitoneal injection of 2 µg of α-GalCer 1 h before challenge on day 25. Control mice were intraperitoneally administered with an identical volume of vehicle solution alone (0.5% polysorbate-20 in PBS).

LDC isolation, adoptive transfer, and cytokine secretion. LDCs were isolated and purified using magnetic bead purification combined with fluorescence-activated cell sorting (FACS). CD1d^{-/-} mice and WT mice were euthanized 24 h after the final OVA challenge. Lungs were collected and single-cell suspensions were prepared, as previously reported (52). Whole lung cells were first incubated with CD11c microbeads (Miltenyi Biotec, Auburn, CA) and then passed through MASC columns (Miltenyi Biotec) for the positive selection of CD11c⁺ cells. Previous studies have demonstrated that LDCs express the DC marker CD11c but do not express the macrophage marker F4/80 (4, 29). Following enrichment, CD11c⁺F4/80⁻ cells were gated as LDCs and sorted using a BD FACS Aria III Cell Sorter (BD Biosciences, San Diego, CA). The purity of the isolated LDCs was >99%, which was determined by flow cytometry for APCcy7-CD11c-positive cells and PEcy5-F4/80-negative cells.

For adoptive transfer, LDCs isolated from different groups of mice were first washed in protein-free PBS and then transferred into mice via intranasal injections (2 × 10⁵ LDCs/mouse) 1 h before challenge on day 25.

LDCs sorted 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 (all from Invitrogen) in 96-well plates at 2 × 10⁵ cells/well for 72 h in the presence or absence of OVA (100 µg/ml). Supernatants were collected to measure IL-12p40, IL-12p70, IL-10, IL-6, and TNF-α by ELISA (eBioscience, San Diego, CA).

iNKT cell isolation and adoptive transfer. To isolate iNKT cells, WT mice were euthanized 24 h after the final OVA challenge, and their spleens were removed. Splenocytes were collected by pressing the spleen through a 100-µm nylon net filter, and erythrocytes were lysed using red blood cell lysis buffer (Sigma). iNKT cells were harvested using magnetic bead purification according to the manufacturer's protocol (Miltenyi Biotec). The purity of the iNKT cells collected after sorting was ~95%, which was determined by flow cytometry for PE-PBS57/mCD1d tetramer-positive cells double-stained with FITC-TCR-β. iNKT cells were adoptively transferred into mice via tail vein injections (1 × 10⁶ cells/mouse) 1 h before challenge on day 25.

Coculture of LDCs and iNKT cells. Isolated LDCs (5 × 10⁵) were cultured with iNKT cells (1 × 10⁶) in the presence or absence of OVA (100 µg/ml) in 96-well plates with or without anti-CD40L mAb (50 µg/ml) for 48 h. For cytokine analysis, aliquots of culture supernatants were collected to measure the concentrations of IL-12p70, IL-6, and TNFα by ELISA (eBioscience, San Diego, CA).

Bone marrow-derived DC generation and bone marrow-derived DC-iNKT cell coculture. To generate bone marrow-derived DC (BMDCs), bone marrow cells from naïve WT BALB/c mice were cultured in complete RPMI 1640 supplemented with FBS in the presence of 10 ng/ml rmGM-CSF and 10 ng/ml rmIL-4 for 7 days. Nonadherent cells were removed, and adherent cell populations containing >90% CD11c⁺ cells (immature BMDCs) were used. BMDCs were cocultured with iNKT cells isolated from WT mice immunized and challenged with OVA [bone marrow-derived DC (BMDC)/iNKT cell ratio, 1:2] in the presence or absence of OVA (100 µg/ml). Immature BMDCs treated with OVA (100 µg/ml) or PBS (untreated BMDCs) were used as controls. For cytokine analysis, aliquots of culture supernatants were harvested after 20 h of BMDC culture and assayed for IL-12p40, IL-12p70, IL-10, IL-6, and TNFα by ELISA (eBioscience).

Coculture of DCs and T cells. To determine the capacity of LDCs to prime OVA-specific T cell responses, LDCs were cocultured with CD4⁺CD25⁻ T cells isolated from OVA-immunized mice. To enhance the number of OVA-specific CD4⁺ T cells in vivo, DO11.10 CD4⁺ T cells were obtained from the spleens of naïve OVA-specific TCR transgenic DO11.10 mice using magnetic bead purification, following the manufacturer's protocol (Miltenyi Biotec). DO11.10 CD4⁺ T cells were adoptively transferred into WT mice via tail vein injections (10 × 10⁶ cells/mouse) 24 h before the first immunization. For immunization, WT mice were injected intraperitoneally on days 0 and 14 with 20 µg of chicken ovalbumin (OVA) (grade V; Sigma) bound to 2 mg of Inject Alum (Thermo Scientific Pierce) in 200 µl of phosphate-buffered saline (PBS). Two weeks after the second immunization, their spleens were harvested and processed into single-cell suspensions. CD4⁺CD25⁻ T cells were obtained using magnetic bead purification according to the manufacturer's protocol (Miltenyi Biotec). LDCs, sorted from CD1d^{-/-} and WT mice immunized and challenged with OVA, were irradiated (40 Gy) and then cocultured with purified CD4⁺CD25⁻ T cells (LDC/T cell ratio, 1:10) in complete RPMI 1640 medium in the presence or absence of OVA (100 µg/ml) in 96-well plates for 48 h. CD4⁺CD25⁻ T cells were cultured with an identical volume of PBS as control. For cytokine analysis, aliquots of culture supernatants were collected to analyze IL-4, IL-5, IL-13, IL-17A, and IFN-γ levels by ELISA (eBioscience).

To analyze intracellular cytokines, Brefeldin A (20 µg/ml; Sigma) was added to the cultures for the last 4 h for intracellular cytokine accumulation. The cytokine secretion pattern of CD4⁺CD25⁻ T cells was assessed using intracellular cytokine staining, following the manufacturer's instructions (eBioscience).

To investigate whether immature BMDCs adopted a tolerogenic maturation status upon interaction with iNKT cells alone, the naive T cell-priming capacity of DCs was measured using another DC-CD4⁺ T cell coculture system (DCs/CD4⁺ T cell ratio, 1:10), as previously mentioned (24). Immature BMDCs cocultured with iNKT cells in the absence or presence of OVA or immature BMDCs treated with PBS (untreated BMDCs) were isolated and purified using CD11c microbeads (Miltenyi Biotec), and DO11.10 CD4⁺ T cells were obtained from the spleens of naive DO11.10 OVA-specific TCR-transgenic mice using magnetic bead purification, following the manufacturer's protocol (Miltenyi Biotec), and then DO11.10 CD4⁺ T cells were cocultured with BMDCs treated with PBS (untreated BMDCs) or BMDCs cocultured with iNKT cells in the absence or presence of OVA (100 µg/ml). After 48 h, culture supernatants were collected to measure the concentrations of IL-4 and IFNγ by ELISA (eBioscience).

Flow cytometry. To determine the number of LDCs, CD11c⁺ cell suspensions from lung tissue were resuspended in FACS buffer. All staining reactions were performed at 4°C. The cells were first blocked with an anti-CD16/CD32 antibody (BD Biosciences) to reduce nonspecific binding and then labeled with isotype controls or PE-cy5-conjugated anti-F4/80 and APC-cy7-conjugated anti-CD11c (eBioscience). To further analyze the expression of various surface markers, freshly isolated LDCs from the indicated experimental groups were stained with anti-MHC-II-PE, anti-CD40-PE, anti-CD80-PE, anti-CD86-PE, and anti-CD206-PE or respective isotype controls (eBioscience).

To analyze the effect of α-GalCer administration on the number of lung iNKT cells, CD40L expression on lung iNKT cells, and the production of IFN-γ and IL-4 in lung iNKT cells, lung MNCs were obtained and resuspended in the FACS buffer at a density of 2×10^6 cells/ml. Cells were first blocked with anti-CD16/CD32 antibody (BD Biosciences) to reduce nonspecific binding and subsequently labeled with isotype controls or antibodies FITC-IL-4, FITC-IFNγ, PEcy5-TCRβ (eBioscience), and PE-PBS57 (α-GalCer analog)/mCD1d tetramer (National Institutes of Health tetramer core facility), and PE-CD40L. iNKT cells were determined as TCRβ and PBS57/mCD1d tetramer double-positive cells. For intracellular cytokine staining, lung MNCs were cultured for 4 to 6 h with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) and 500 ng/ml ionomycin together with monensin (1 µl/ml). Cells were collected and washed, and intracellular staining was performed, following the manufacturer's instructions (eBioscience).

In vitro restimulation of mediastinal lymph node cells and cytokine analysis. To detect cytokine secretion, single-cell suspensions were prepared from mediastinal lymph node (MLNs) and then cultured at a density of 1×10^6 cells/ml in the presence or absence of OVA (100 µg/ml). After 72 h of incubation, the supernatants were collected to measure concentrations of IL-4, IL-5, IL-13, IL-17A, and IFNγ by ELISA (eBioscience).

Quantitative RT-PCR. Total RNA was extracted from purified LDCs with Tri reagent (Applied Biosystems, Carlsbad, CA), following the manufacturer's instructions. The isolated RNA was subsequently purified using the RNeasy Microprep kit (Qiagen, Hilden, Germany). cDNA was synthesized with SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's protocols. Real-time PCR was performed with the Lightcycler Fast Start DNA Master^{PLUS} SYBR Green I reaction mix (Roche) on a Lightcycler 480II (Roche). The mRNA expression levels of *RELM-α* and *Arg1* were determined with primers obtained from Life Technologies (Gaithersburg, MD). The primer sequences used are as follows: *RELM-α* (sense, 5'-CTTCCATCACCTTGCCAATC-3'; antisense, 5'-GCATATCTGCCAAGACATCG-3'), *Arg1* (sense, 5'-TGG-GAAGACAGCAGAGGAGGTG-3'; antisense, 5'-GCTTATGGT-TACCCTCCCGTTG-3'), and GAPDH (sense, 5'-AGGAGC-GAGACCCCACTAACA-3'; antisense, 5'-AGGGGGGCTAAGCA-GTTGGT-3'). All data were analyzed using SDS software (Applied

Biosystems). Gene expression was quantified as the average of triplicate samples after standard curve interpolation and normalization to GAPDH gene expression.

Measurement of AHR. AHR was evaluated by invasive measurement of airway resistance, as previously mentioned (31). Mice were anesthetized 24 h after the final OVA challenge, and a 20-gauge polyethylene catheter was inserted into the trachea, which was followed by mechanical ventilation. Airway resistance (RL) and dynamic compliance (C_{dyn}; reflecting the elasticity of the lung) to increasing doses of aerosolized methacholine (Mch), ranging from 3.12 to 50 mg/ml in PBS, for 3 min were examined using the FinePointe (Wilmington, NC) RC system to assess AHR. Nonspecific airway responsiveness was examined by exposing mice to aerosolized PBS to determine the baseline value.

Bronchoalveolar lavage and lung histologic analysis. After AHR was measured, lungs were lavaged using a catheter with a total volume of 1.5 ml of PBS containing 1 mM sodium EDTA, which was followed by lung resection, as previously described (52). The supernatants were collected and stored at -80°C to measure cytokine concentrations. Cells recovered from bronchoalveolar lavage fluid (BALF) were harvested and stained with May-Grunwald Giemsa (Jiancheng, Nanjing, China) for differential cell counts. The numbers of eosinophils, macrophages, neutrophils, and lymphocytes were expressed as the absolute number based on the total cell counts.

The right lungs were excised after bronchoalveolar lavage and immediately fixed in 4% buffered paraformaldehyde. The specimens were then dehydrated and embedded in paraffin. Lung tissue sections were stained with routine hematoxylin and eosin staining to assess peribronchial and perivascular inflammation by light microscopy and periodic acid-Schiff staining (Baso, Taiwan, China) to analyze goblet cell hyperplasia. Goblet cell hyperplasia was determined by counting the number of PAS-positive cells in the epithelium of the central airway using a digital image. The circumference of the airway at the basement membrane was measured by ImageJ software (National Institutes of Health, Bethesda, MD). The results were expressed 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, IL-17A, and IFNγ in the BALF was performed using an ELISA kit according to the manufacturer's protocols (eBioscience). For immunoglobulin (Ig) measurement, the serum was collected 24 h after the final OVA or HDM challenge to detect the concentrations of IgE and IgG1 by ELISA (Biolegend, San Diego, CA), as previously described (8, 52). Serum levels of Igs were expressed based on optical density measurements.

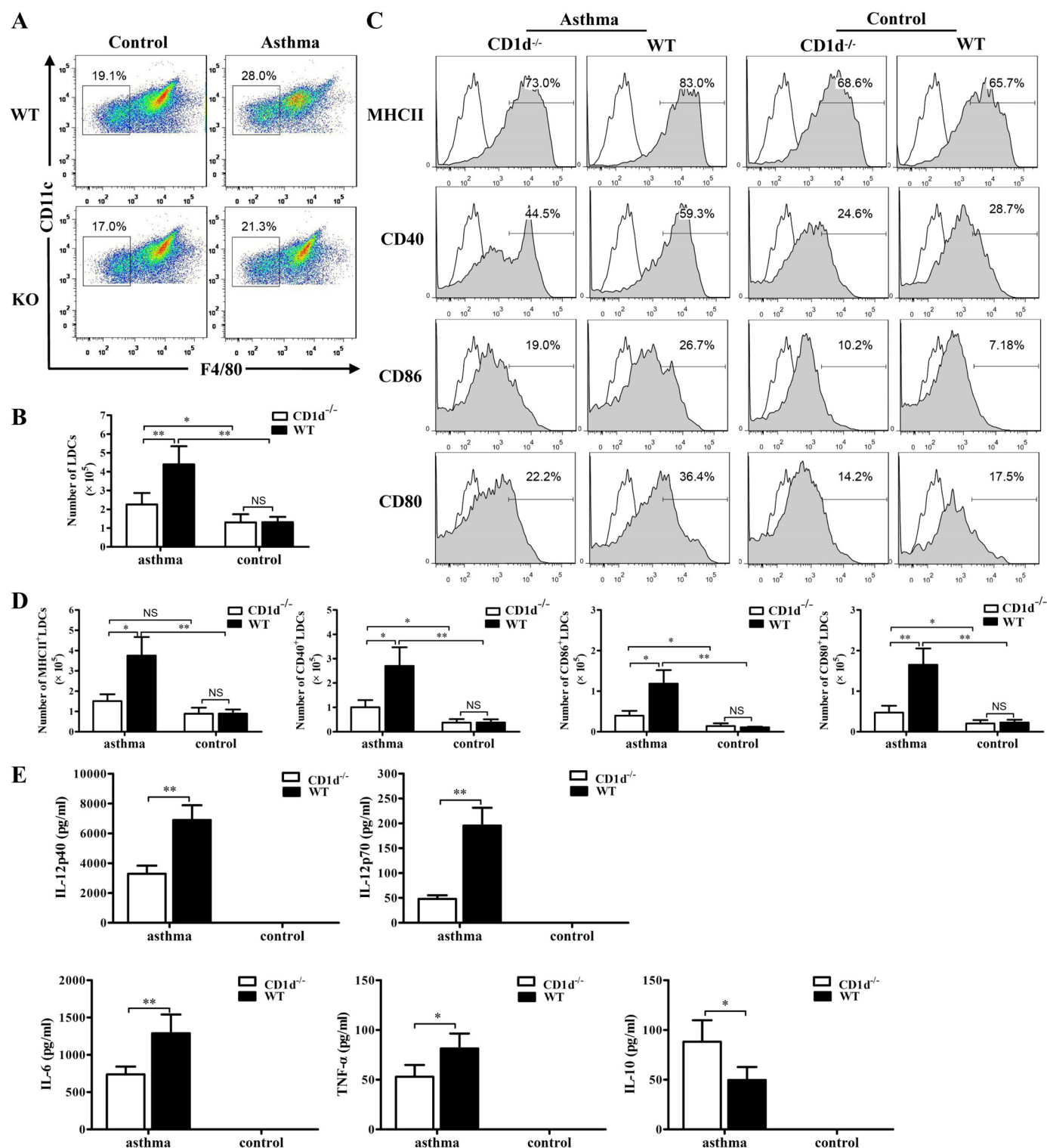
Statistical Analyses. All data are expressed as means ± SD. Statistical analyses were performed with Student's unpaired *t*-test or one-way ANOVA using GraphPad Prism 5 (GraphPad Software, San Diego, CA) software. *P* < 0.05 was considered statistically significant.

RESULTS

Reduction in surface maturation markers and proinflammatory cytokine secretion of LDCs from CD1d^{-/-} mice immunized and challenged with OVA. The phenotypic maturation of DCs is dependent largely on surface markers, such as costimulatory molecules CD80, CD86, and CD40, as well as MHC II molecules (11, 33). To determine the modulatory influence of iNKT cells on the phenotypic maturation of LDCs, the number of LDCs from CD1d^{-/-} and WT mice were immunized and challenged with OVA, and corresponding surface maturation marker expression was analyzed. As shown in Fig. 1, A and B, the number of LDCs from CD1d^{-/-} mice was lower than that from WT mice when immunized and challenged with OVA (*P* < 0.05), whereas the numbers of LDCs from unimmunized

and unchallenged WT and CD1d^{-/-} mice were similar ($P > 0.05$). However, the numbers of LDCs from WT and CD1d^{-/-} mice immunized and challenged with OVA were markedly higher than those from unimmunized and unchallenged WT and CD1d^{-/-} mice ($P < 0.05$ or $P < 0.01$; Fig. 1, A and B). Furthermore, LDCs from CD1d^{-/-} mice immunized and chal-

lenged with OVA showed decreased expression of MHC-II, CD80, CD86, and CD40 compared with similarly treated WT mice ($P < 0.05$ or $P < 0.01$; Fig. 1, C and D). LDCs from WT and CD1d^{-/-} mice immunized and challenged with OVA exhibited increased expression of CD80, CD86, and CD40 compared with unimmunized and unchallenged WT and



CD1d^{-/-} mice ($P < 0.05$ or $P < 0.01$; Fig. 1, C and D). Interestingly, the expression of surface maturation markers on LDCs was not significantly different between CD1d^{-/-} and WT mice unimmunized and unchallenged with OVA ($P > 0.05$; Fig. 1, C and D).

Functionally mature DCs can produce large amounts of IL-12, IL-6, and TNF α , which are considered proinflammatory or immunostimulatory cytokines (33), but do not produce negligible quantities of IL-10, a typical tolerogenic cytokine (17). To investigate the modulatory effect of iNKT cells on the functional maturation of LDCs, LDCs were isolated from CD1d^{-/-} and WT mice immunized and challenged with OVA. After the final OVA challenge (24 h), cells were cultured for 72 h, and supernatants were collected afterward to measure the spontaneous production of IL-12p40, IL-12p70, IL-10, IL-6, and TNF α . The concentrations of IL-12p40, IL-12p70, IL-6, and TNF α were lower in the culture supernatant of LDCs from CD1d^{-/-} mice than those in WT mice ($P < 0.05$ or $P < 0.01$; Fig. 1E). In contrast, the concentration of IL-10 was higher in the culture supernatant of LDCs from CD1d^{-/-} mice than that from WT mice ($P < 0.05$; Fig. 1E). However, the concentrations of IL-12p40, IL-12p70, IL-6, IL-10, and TNF α in the culture supernatant of LDCs from unimmunized and unchallenged WT and CD1d^{-/-} mice were negligible (Fig. 1E). Taken together, these results indicate that iNKT cell deficiency can reduce cell numbers, the expression of surface maturation markers, and proinflammatory cytokine secretion of LDCs from mice immunized and challenged with OVA.

Elevated activation of iNKT cells by α -GalCer enhances surface maturation markers and proinflammatory cytokine secretion of LDCs from WT mice immunized and challenged with OVA. α -GalCer, a prototypic glycolipid derived originally from marine sponges, is a specific ligand that stimulates iNKT cell activation and is readily used to examine the role of iNKT cells in asthma, cancer, and autoimmunity (10, 40, 52). To further assess whether iNKT cells influence the phenotypic and functional maturation of LDCs, we measured surface maturation marker expression and spontaneous cytokine production of LDCs from asthmatic mice using an approach to promote the activation of iNKT cells through the intraperitoneal injection of 2 μ g α -GalCer, 1 h before challenge on day 25. The number and activity of iNKT cells in the lung, as well as maturation-associated surface marker expression and spontaneous cytokine production of LDCs, were determined 24 h after the final challenge. As shown in Fig. 2, A and B, the number of iNKT cells in the lung was significantly higher in OVA-induced asthmatic mice treated with α -GalCer than in OVA-induced asthmatic mice treated with PBS ($P < 0.01$). Moreover, using

OVA-induced asthmatic mice, α -GalCer administration enhanced the secretion of IFN- γ and IL-4 by lung iNKT cells compared with that similar cells treated with PBS ($P < 0.01$; Fig. 2, C and D). The expression of MHC-II, CD80, CD86, and CD40 of LDCs from OVA-induced asthmatic mice treated with α -GalCer was significantly higher than that in OVA-induced asthmatic mice treated with PBS ($P < 0.05$ or $P < 0.01$; Fig. 2, E and F). Furthermore, in OVA-induced asthmatic mice, the concentrations of IL-12p40, IL-12p70, IL-6, and TNF α were higher in the culture supernatant of LDCs treated with α -GalCer than those control (PBS-treated) LDCs ($P < 0.05$ or $P < 0.01$; Fig. 2G). However, the concentration of IL-10 in the culture supernatant of LDCs from OVA-induced asthmatic mice treated with α -GalCer or PBS was similar ($P > 0.05$). Collectively, our data suggest that elevated activation of iNKT cells by α -GalCer can enhance the expression of surface maturation markers and proinflammatory cytokine secretion of LDCs from WT mice immunized and challenged with OVA.

Adoptive transfer of iNKT cells augments surface molecule expression and proinflammatory cytokine secretion of LDCs from CD1d^{-/-} mice immunized and challenged with OVA. We further confirmed that iNKT cells specifically enhance the expression of maturation-associated surface markers of LDCs as well as proinflammatory cytokine production from mice immunized and challenged with OVA. iNKT cells in the spleen were isolated and purified from WT mice immunized and challenged with OVA; the purity of iNKT cells (specifically, PBS-57/mCD1d tetramer⁺ TCR- β ⁺ cells) was >95% (Fig. 3A). Subsequently, iNKT cells were adoptively transferred into CD1d^{-/-} mice via tail vein injections 1 h before OVA challenge on day 25. Adoptive transfer of iNKT cells significantly promoted the expression of MHC-II, CD80, CD86, and CD40 on LDCs from CD1d^{-/-} mice immunized and challenged with OVA compared with those treated with PBS ($P < 0.05$ or $P < 0.01$; Fig. 3, B and C). Furthermore, the concentrations of IL-12p40, IL-12p70, IL-6, and TNF α were significantly higher in the culture supernatant of LDCs from CD1d^{-/-} mice immunized and challenged with OVA and subjected to adoptive transfer of iNKT cells compared with LDCs from CD1d^{-/-} mice immunized and challenged with OVA but treated with PBS ($P < 0.05$ or $P < 0.01$; Fig. 3D). However, in the culture supernatant of LDCs from CD1d^{-/-} mice immunized and challenged with OVA, the concentration of IL-10 was significantly lower with adoptive transfer of iNKT cells ($P < 0.05$; Fig. 3D). Thus, adoptive transfer of iNKT cells can augment the production of maturation-associated surface markers and proinflammatory cytokines of LDCs from CD1d^{-/-} mice immunized and challenged with OVA.

Fig. 1. Reduced number of lung dendritic cells (LDCs) and downregulation of surface maturation markers, and proinflammatory cytokine secretion of LDCs from CD1d^{-/-} mice immunized and challenged with ovalbumin (OVA) CD1d^{-/-} and wild-type (WT) mice immunized and challenged with OVA that were euthanized 24 h after the final challenge, and then LDCs (CD11c⁺F4/80⁺) were isolated using a combination of magnetic microbead selection and flow cytometry. LDCs were quantified by CD11c and F4/80 staining using flow cytometry. A: representative dot plots of LDCs in lung mononuclear cell (MNC) populations from CD1d^{-/-} and WT mice immunized and challenged with OVA or PBS. B: no. of LDCs from CD1d^{-/-} and WT mice immunized and challenged with OVA or PBS. C: cells were stained for maturation-associated surface markers and detected by flow cytometry. Expression of MHC II, CD80, CD86, and CD40 was assessed in CD11c⁺F4/80⁺ cells (shaded histograms), and isotype controls (line) from CD1d^{-/-} and WT mice immunized and challenged with OVA or PBS. Percentages are those of positive cells. D: expression levels of nonpolymorphic major histocompatibility complex (MHC) II, CD80, CD86, and CD40 in CD11c⁺F4/80⁺ cells from the lungs of CD1d^{-/-} and WT mice immunized and challenged with OVA or PBS. E: LDCs from CD1d^{-/-} and WT mice immunized and challenged with OVA or PBS were cultured in vitro for 72 h; the concentrations of IL-12p70, IL-12p40, IL-6, IL-10, and TNF α in the culture supernatants were determined by ELISA. Data are represented as means $\pm$ SD from 3 independent experiments ($n = 18$), and 1 representative experiment is indicated. * $P < 0.05$; ** $P < 0.01$. NS, not significant.

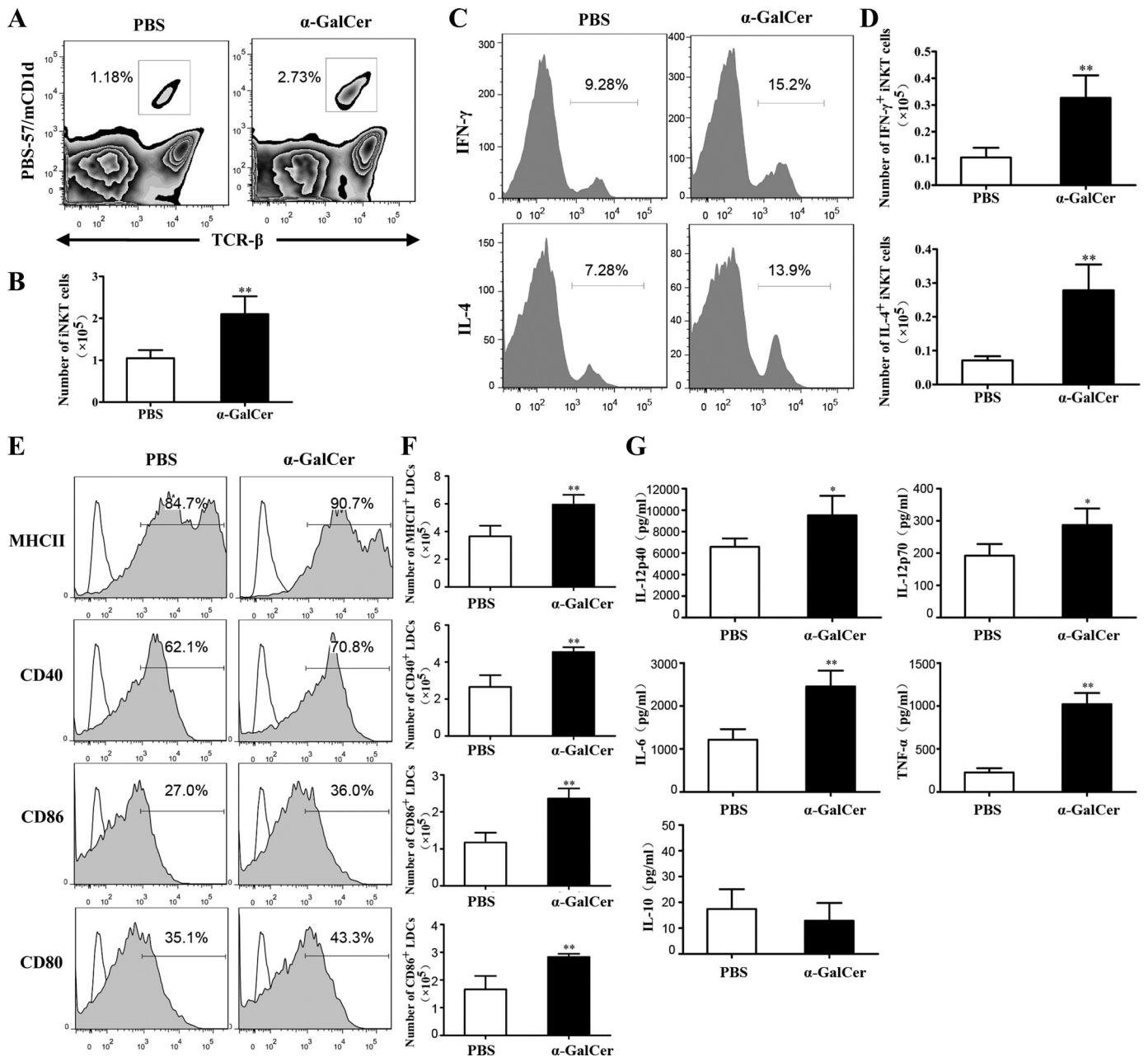


Fig. 2. Activation of invariant natural killer T (iNKT) cells by α -galactosylceramide (α -GalCer) enhances surface maturation markers, and proinflammatory cytokine secretion of LDCs from WT mice immunized and challenged with OVA (administered an intraperitoneal injection of 2 μ g of α -GalCer 1 h before challenge on day 25). **A:** flow cytometry of lung iNKT cells from OVA-induced asthmatic mice after treatment with PBS or α -GalCer. The corresponding percentages (adjacent to outline areas) indicate the frequency of lung iNKT cells. **B:** percentage of iNKT cells in a population of lung mononuclear cells (MNCs). **C:** IFN- γ (top) and IL-4 (bottom) production in lung iNKT cells from OVA-induced asthmatic mice treated with α -GalCer or PBS were determined. **D:** percentage of IFN- γ - and IL-4-producing lung iNKT cells. **E:** expression of MHC II, CD80, CD86, and CD40 on CD11c⁺F4/80⁻ cells (shaded histograms) and isotype controls (line) are shown for cells isolated from the lungs of OVA-induced asthmatic mice treated with α -GalCer or PBS. Percentages are those of positive cells. **F:** expression levels of MHC II, CD80, CD86, and CD40 on CD11c⁺F4/80⁻ cells from the lungs of WT mice immunized and challenged with OVA and treated with α -GalCer or PBS. **G:** LDCs were isolated from the OVA-induced asthmatic mice treated with α -GalCer or PBS and cultured in vitro. After 72 h, the concentrations of IL-12p70, IL-12p40, IL-6, IL-10, and TNF- α in the culture supernatants were analyzed by ELISA. Data are shown as means $\pm$ SD of 3 independent experiments ($n = 18$), and 1 representative experiment is indicated. * $P < 0.05$; ** $P < 0.01$.

Adoptive transfer of iNKT cells promotes Th2 inflammatory response and immunogenic maturation of LDCs from CD1d^{-/-} mice immunized and challenged with HDM. House dust mite (HDM), one of the commonest aeroallergens in asthmatic patient living environment, is closely associated with the development of asthma (16). Therefore, we further inves-

tigated whether iNKT cells specifically promote phenotypic and functional maturation of DCs in a mouse model of asthma induced by HDM. Thus, iNKT cells from WT mice immunized and challenged with HDM were adoptively transferred into CD1d^{-/-} mice via tail vein injections 1 h before HDM challenge on day 7. Adoptive transfer of iNKT cells strongly

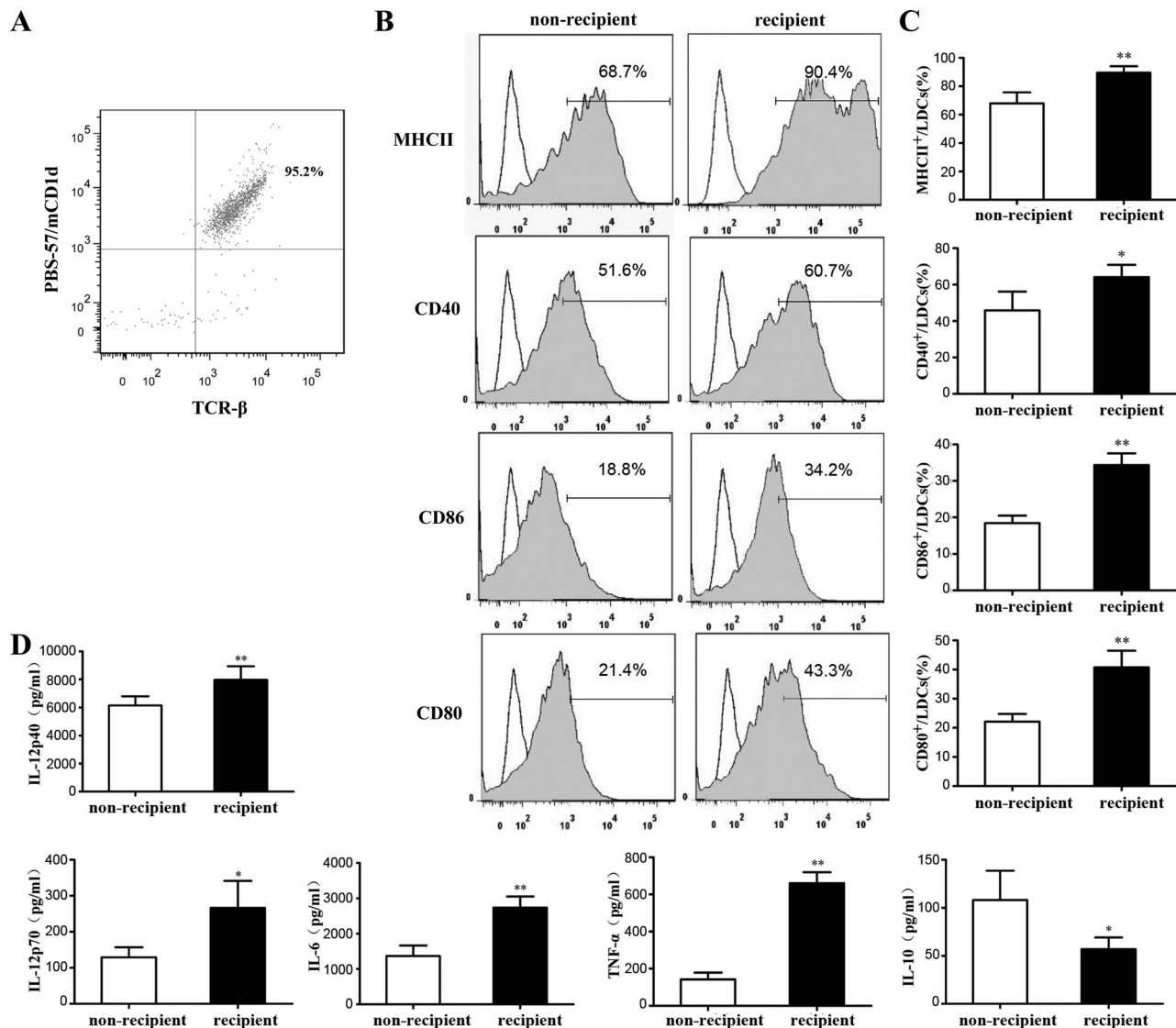


Fig. 3. Adoptive transfer of iNKT cells augments surface maturation markers, and proinflammatory cytokine secretion of LDCs from CD1d^{-/-} mice immunized and challenged with OVA iNKT cells (1×10^6 /mouse) from WT mice and adoptively transferred, via tail vein injection, to CD1d^{-/-} mice that were immunized and challenged with OVA 1 h before OVA challenge on day 25. **A**: flow cytometry verified the purity of the adoptively transferred cell population, which was stained with both PBS-57/mCD1d tetramers and a monoclonal antibody against TCR- β , (i.e., the proportion of iNKT cells was >95%). **B**: expression of MHC II, CD80, CD86, and CD40 on CD11c⁺F4/80⁻ cells (shaded histograms) and isotype controls (line) was assessed in cells from the lungs of CD1d^{-/-} mice immunized and challenged with OVA and adoptively transferred with iNKT cells or PBS. **C**: expression levels of MHC II, CD80, CD86, and CD40 on CD11c⁺F4/80⁻ cells from the lungs of CD1d^{-/-} mice immunized and challenged with OVA adoptively transferred with iNKT cells or PBS. **D**: LDCs were obtained from CD1d^{-/-} mice immunized and challenged with OVA and adoptively transferred with iNKT cells or PBS and then cultured in vitro. After 72 h, the concentrations of IL-12p70, IL-12p40, IL-6, IL-10, and TNF α in the culture supernatants were assessed by ELISA. Data are expressed as means $\pm$ SD of 3 independent experiments ($n = 18$), and 1 representative experiment is indicated. * $P < 0.05$; ** $P < 0.01$.

promoted inflammatory cell infiltration in the lung (Fig. 4A) and a rise in the number of eosinophils, macrophages, and total cells in the BALF ($P < 0.05$ or $P < 0.01$; Fig. 4B) and PAS-positive cells in the epithelium of the airway ($P < 0.01$; Fig. 4C) as well as enhanced HDM-specific IgE and IgG1 concentrations in the serum from the HDM-induced asthmatic mice treated with adoptive transfer of iNKT cells compared with PBS treatment ($P < 0.05$ or $P < 0.01$; Fig. 4D). Furthermore, compared with PBS treatment, the concentrations of IL-4, IL-5, and IL-13 in the BALF were significantly enhanced in the HDM-induced asthmatic mice treated with adoptive transfer of iNKT cells ($P < 0.05$ or $P < 0.01$; Fig. 4E). However, the concentration of IFN γ in the BALF was not

significantly different between the two groups ($P > 0.05$; Fig. 4E). We further found that adoptive transfer of iNKT cells markedly enhanced the expression of MHC-II, CD86, and CD40 on LDCs from CD1d^{-/-} mice immunized and challenged with HDM, compared with those treated with PBS ($P < 0.05$ or $P < 0.01$; Fig. 5, A and B). In addition, the levels of IL-12p40, IL-12p70, IL-6, and TNF α were significantly higher in the culture supernatant of LDCs from CD1d^{-/-} mice immunized and challenged with HDM and subjected to adoptive transfer of iNKT cells compared with in LDCs from CD1d^{-/-} mice immunized and challenged with HDM but treated with PBS ($P < 0.05$ or $P < 0.01$; Fig. 5C). In contrast, in the culture supernatant of LDCs from CD1d^{-/-} mice immunized and

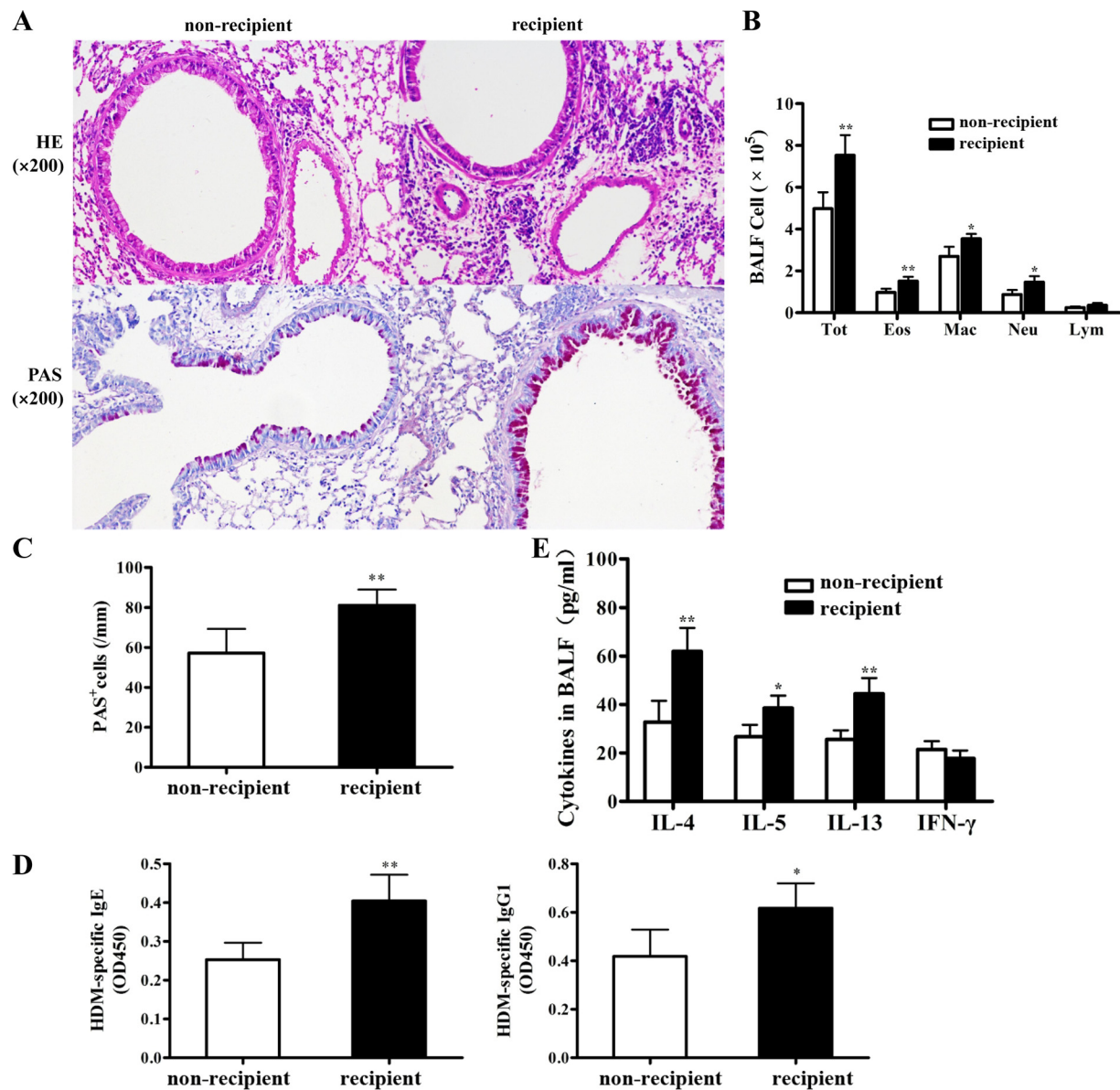


Fig. 4. Adoptive transfer of iNKT cells promotes Th2 inflammatory response in CD1d^{-/-} mice immunized and challenged with HDM iNKT cells (1×10^6 /mouse) from WT mice immunized and challenged with HDM that were adoptively transferred via tail vein injection to CD1d^{-/-} mice that had been immunized and challenged with HDM 1 h before HDM challenge on day 7. **A**: histopathologic analysis of lung tissue from CD1d^{-/-} mice adoptively transferred with iNKT cells or PBS, using hematoxylin and eosin (HE) and periodic acid-Schiff (PAS) staining. **B**: total and differential cell counting in from bronchoalveolar lavage fluid (BALF) of CD1d^{-/-} mice treated with adoptive transfer of iNKT cells or PBS. Tot, total cell counts; Eos, eosinophils; Neu, neutrophils; Mac, macrophages; Lym, lymphocytes. **C**: Lung goblet cell hyperplasia is indicated as the no. of PAS-positive cells per unit of length (mm) of the basement membrane. **D**: HDM-specific IgE and IgG1 concentrations in the serum of mice adoptively transferred with iNKT cells or PBS. **E**: BALF was harvested 72 h after the final challenge, and the concentrations of IL-4, IL-5, IL-13, and IFN γ from mice treated by the adoptive transfer of iNKT cells or PBS were analyzed by ELISA. Data are expressed as means $\pm$ SD of 2 independent experiments ($n = 12$), and 1 representative experiment is indicated. * $P < 0.05$; ** $P < 0.01$.

challenged with HDM, the concentration of IL-10 was significantly lower with adoptive transfer of iNKT cells ($P < 0.05$; Fig. 5C). Collectively, adoptive transfer of iNKT cells can augment Th2 inflammatory response and the production of maturation-associated surface markers and proinflammatory cytokines in LDCs from CD1d^{-/-} mice immunized and challenged with HDM.

iNKT cells from the OVA-induced asthmatic mice together with OVA induce immunogenic DC maturation in vitro. To assess whether iNKT cells together with OVA induce phenotypic and functional maturation of DCs, coculture experiments

were performed with purified iNKT cells from WT mice immunized and challenged with OVA and immature BMDCs in the presence or absence of OVA (100 μ g/ml), and immature BMDCs were cultured with OVA (100 μ g/ml) or PBS for 20 h. DCs cocultured with iNKT cells in the presence of OVA showed high expression of MHC-II, CD80, CD86, and CD40 (Fig. 6A), and their culture supernatant exhibited elevated levels of proinflammatory cytokines such as IL-12p40, IL-12p70, IL-6, and TNF α ($P < 0.05$ or $P < 0.01$; Fig. 6B). However, the expression of MHC-II, CD80, CD86, and CD40 on DCs cocultured with iNKT cells in the absence of OVA was

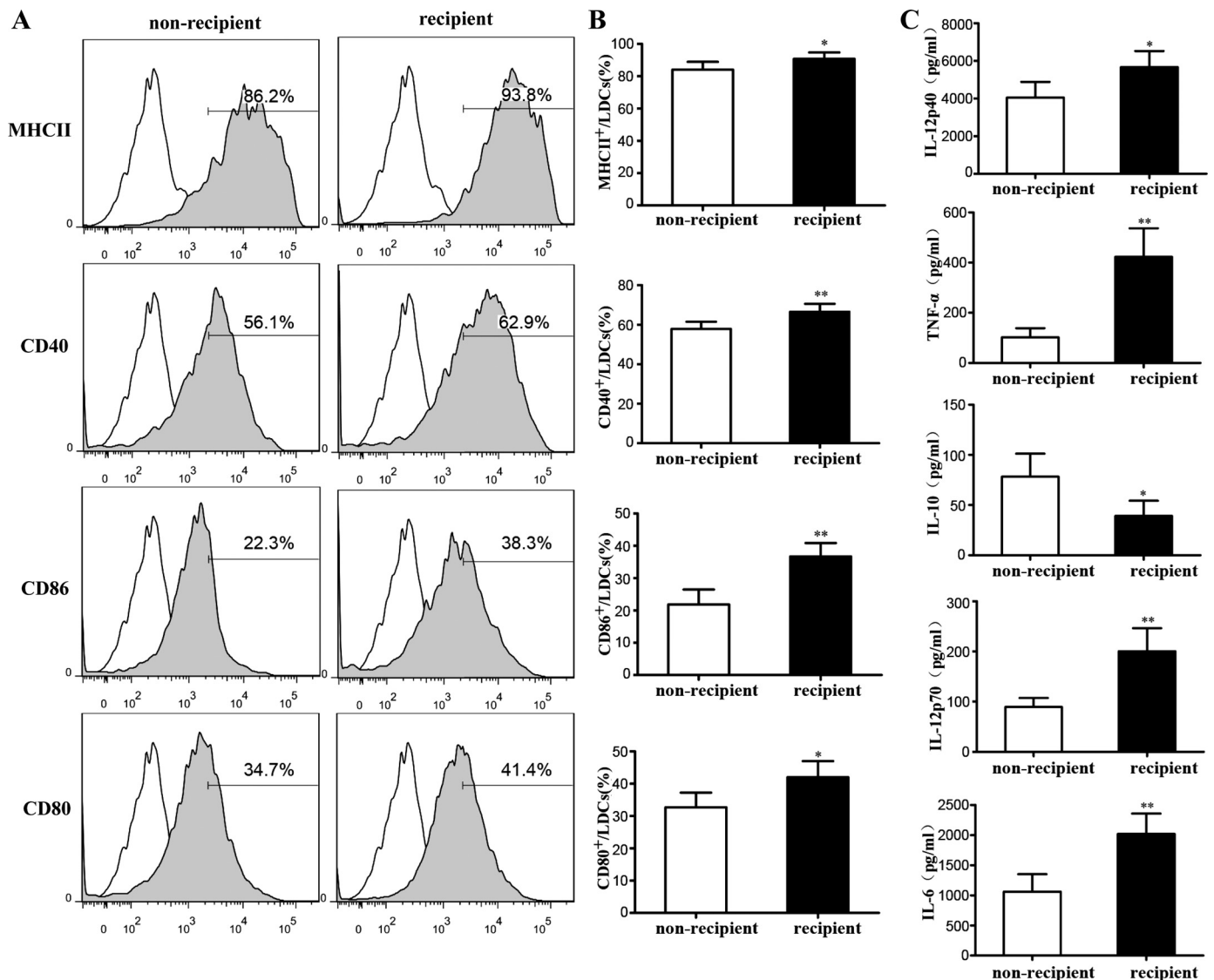


Fig. 5. Adoptive transfer of iNKT cells promotes surface molecule expression and proinflammatory cytokine production of LDCs from CD1d^{-/-} mice immunized and challenged with HDM iNKT cells (1×10^6 /mouse) from WT mice immunized and challenged with HDM that were adoptively transferred via tail vein injection to CD1d^{-/-} mice that were immunized and challenged with HDM 1 h before HDM challenge on day 7. **A**: expression of MHC II, CD80, CD86, and CD40 on CD11c⁺F4/80⁻ cells (shaded histograms) and isotype controls (line) was assessed in cells from the lungs of CD1d^{-/-} mice immunized and challenged with HDM and adoptively transferred with iNKT cells or PBS. **B**: expression levels of MHC II, CD80, CD86, and CD40 on CD11c⁺F4/80⁻ cells from the lungs of CD1d^{-/-} mice immunized and challenged with OVA adoptively transferred with iNKT cells or PBS. **C**: LDCs were obtained from CD1d^{-/-} mice immunized and challenged with HDM and adoptively transferred with iNKT cells or PBS and then cultured in vitro. After 72 h, the concentrations of IL-12p70, IL-12p40, IL-6, IL-10, and TNFα in the culture supernatants were assessed by ELISA. Data are expressed as means $\pm$ SD of 3 independent experiments ($n = 18$), and 1 representative experiment is indicated. * $P < 0.05$; ** $P < 0.01$.

intermediate between that of immature DCs cultured in the presence of OVA or PBS and that of DCs cocultured with iNKT cells in the presence of OVA (Fig. 6A). Furthermore, culture supernatants of DCs cocultured with iNKT cells in the absence of OVA showed enhanced concentrations of IL-10 ($P < 0.01$; Fig. 6B). To further assess the capacity of DCs cocultured with iNKT cells alone to instruct cytokine secretion by CD4⁺ T cells, DO11.10 CD4⁺ T cells were cocultured for 48 h with BMDCs cocultured with iNKT cells alone, BMDCs treated with PBS, or BMDCs cocultured with iNKT cells in combination with OVA in the presence of OVA (100 μg/ml), and then the concentrations of IL-4 and IFNγ in culture supernatants were measured. Our results showed that no dif-

ference was observed in the concentrations of IL-4 and IFNγ in culture supernatants from DO11.10 CD4⁺ T cells cocultured with BMDCs cocultured with iNKT cells alone and PBS ($P > 0.05$; Fig. 6C). By contrast, there was significantly elevated production of IL-4 by DO11.10 CD4⁺ T cells cocultured with BMDCs cocultured with iNKT cells in combination with OVA ($P < 0.01$; Fig. 6C). To eliminate the possibility that iNKT cells were responsible for the elevated IL-10 concentrations measured in the culture supernatant from the iNKT cells-BMDCs coculture, iNKT cells from WT mice immunized and challenged with OVA in the presence of OVA were cultured. The result showed that IL-10 secretion was very limited in the culture supernatant, indicating that DCs strongly upregulated

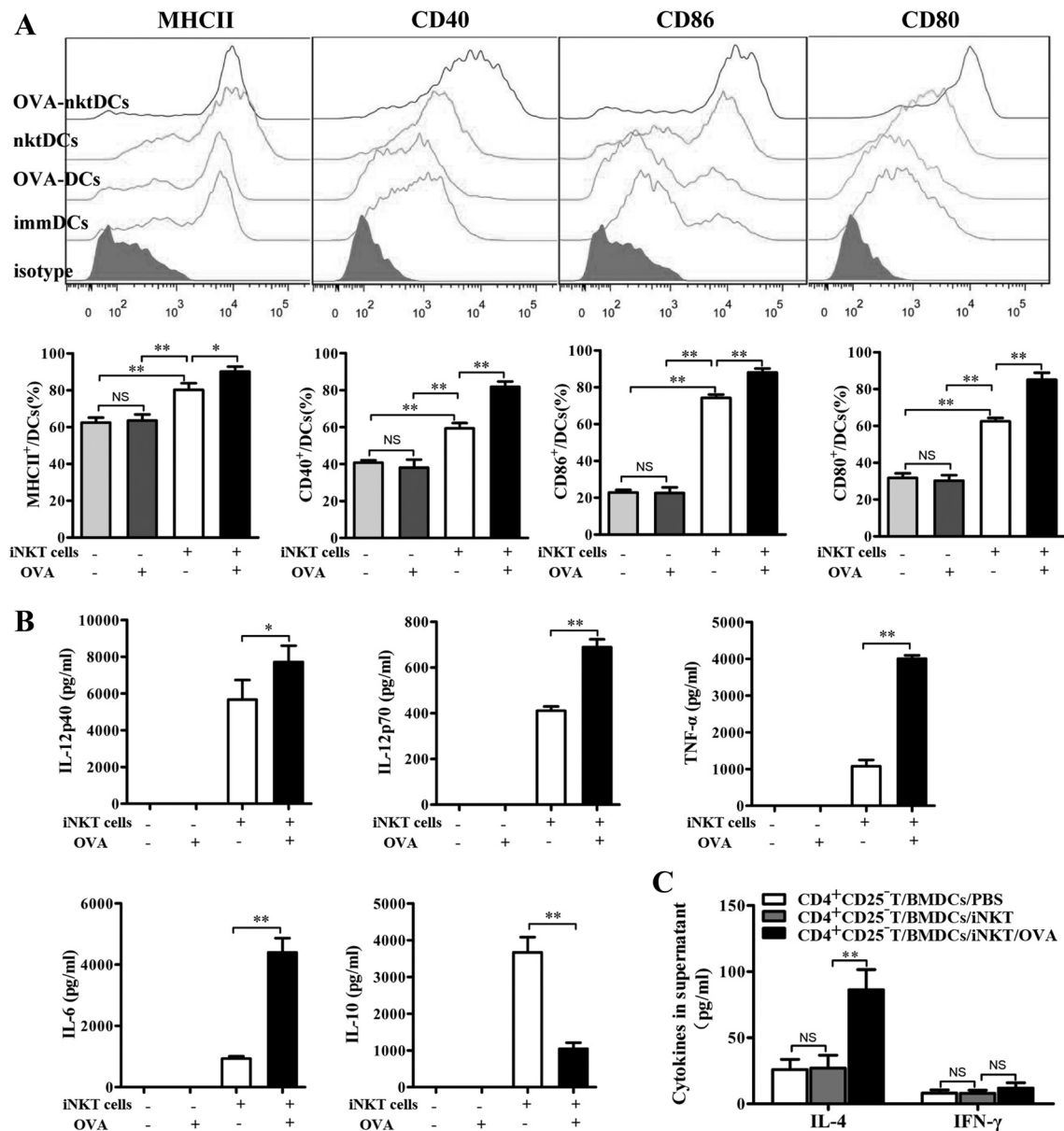


Fig. 6. iNKT cells from the OVA-induced asthmatic mice together with OVA induce immunogenic dendritic cell (DC) maturation in vitro. Immature bone marrow-derived DCs (BMDCs) were cocultured with iNKT cells isolated from OVA-induced asthmatic mice (BMDC/iNKT cell ratio, 1:2) with or without OVA (100 μ g/ml); immature BMDCs cultured in the presence of OVA (100 μ g/ml) or PBS for 20 h were used as controls. DO11.10 CD4⁺ T cells were obtained from the spleens of naive DO11.10 OVA-specific T cell receptor (TCR)-transgenic mice, followed by coculture with isolated BMDCs cocultured with iNKT cells alone or with OVA or BMDCs treated with PBS (untreated BMDCs) in the presence of OVA (100 μ g/ml) for 48 h. A: the expression of MHC II, CD80, CD86, and CD40 on DCs (CD11c⁺ cells) and isotype controls is shown from different DC specimens, measured by flow cytometry as indicated. B: culture supernatants were obtained from different DCs cocultured with iNKT cells with or without OVA or cultured with OVA or PBS after 20 h, and the levels of IL-12p70, IL-12p40, IL-6, IL-10, and TNF α were examined by ELISA. C: culture supernatants were collected from DO11.10 CD4⁺ T cells cocultured with different DCs in the presence of OVA to measure the concentrations of IL-4 and IFN γ by ELISA after 48 h. Data are expressed as means $\pm$ SD of 3 independent experiments ($n = 18$), and 1 representative experiment is indicated. * $P < 0.05$; ** $P < 0.01$.

IL-10 production in the culture supernatant from the iNKT cells-BMDCs coculture (data not shown). Collectively, these data demonstrate that immature DCs undergo immunogenic maturation upon interaction with iNKT cells in the presence of OVA, whereas immature DCs adopt a tolerogenic maturation status upon interaction with iNKT cells alone.

Enhancing the effect of iNKT cell activation on surface maturation markers and proinflammatory cytokine secretion by LDC involves in CD40L expression. It has been shown that iNKT cells express CD40 ligand (CD40L or CD154) when

activated (46) and may thereby induce activation and maturation of DCs via interaction with CD40 (27, 50). To investigate the potential mechanism through which iNKT cells promote the maturation of LDCs, the expression of CD40L by iNKT cells was examined from WT mice or OVA-induced asthmatic mice administered with the intraperitoneal injection of 2 μ g α -GalCer or identical volume of PBS 1 h before challenge on day 25. As outlined in Fig. 7, A and B, CD40L expression of the lung iNKT cells was significantly upregulated in the OVA-induced asthmatic mice ($P < 0.05$), and α -GalCer administra-

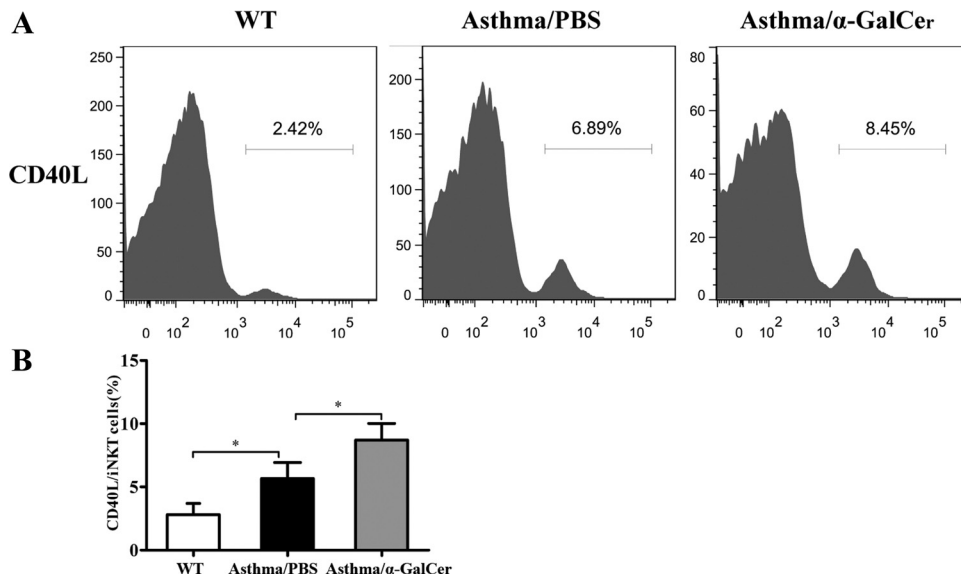


Fig. 7. α -GalCer treatment increases CD40L expression in iNKT cells from WT mice immunized and challenged with OVA and WT mice immunized and challenged with OVA that were treated an intraperitoneal injection of 2 μ g of α -GalCer 1 h before challenge on day 25, and CD40L expression on iNKT cells was assessed by flow cytometry. **A:** flow cytometry of CD40L⁺ iNKT cells in the lung from WT mice and OVA-induced asthmatic mice after treatment with PBS or α -GalCer. The corresponding percentages indicate the frequency of lung CD40L⁺ iNKT cells. **B:** expression levels of CD40L on lung iNKT cells from WT mice and mice immunized and challenged with OVA and treated with α -GalCer or PBS. Data are expressed as means $\pm$ SD of 2 independent experiments ($n = 12$), and 1 representative experiment is indicated. * $P < 0.05$.

tion markedly promotes CD40L expression of the lung iNKT cells ($P < 0.05$).

To further assess the modulatory effect of CD40L expression on LDC maturation, freshly isolated LDCs from WT mice were cocultured in the presence of OVA (100 μ g/ml) with iNKT cells from asthmatic mice in the presence or absence of anti-CD40L (50 μ g/ml) for 48 h, and then surface maturation markers of LDCs and proinflammatory cytokine secretion in culture supernatant were analyzed. Our data showed that blockade of CD40L markedly suppressed expression of MHCII, CD80, CD86, and CD40 of LDCs ($P < 0.05$ or $P < 0.01$; Fig. 8, A and B) and levels of proinflammatory cytokines like IL-12p70, IL-6, and TNF α in the culture supernatant ($P < 0.01$; Fig. 8C). Thus, these preliminary data suggest that it is possible that the enhancing influence of iNKT cell activation on LDC maturation involves CD40L expression.

LDCs isolated from WT mice, but not CD1d^{-/-} mice, immunized and challenged with OVA have enhanced capacity to prime Th2 response *in vitro*. To directly investigate the modulatory effect of iNKT cells on LDC function in priming OVA-specific T cell responses, CD4⁺CD25⁻ T cells, of which purity was >98% (Fig. 9A), were isolated from OVA-immunized mice. These cells were cocultured with LDCs from WT mice or CD1d^{-/-} mice immunized and challenged with OVA in the presence of OVA (100 μ g/ml) for 48 h. We showed significantly increased frequency of IL-4-positive CD4⁺ T cells in CD4⁺ T cells cocultured with LDCs from WT mice compared with LDCs from CD1d^{-/-} mice immunized and challenged with OVA ($P < 0.01$; Fig. 9, B and C). However, no difference was observed in the proportion of IFN γ -producing CD4⁺ T cells during coculture with LDCs from WT and CD1d^{-/-} mice immunized and challenged with OVA ($P > 0.05$; Fig. 9B and 9C). In addition, using CD4⁺CD25⁻ T cells from mice immunized and challenged with OVA, IL-4, IL-5, and IL-13 levels were significantly higher in the culture supernatant of T cells cocultured with LDCs from WT mice than in that of T cells cocultured with LDCs from CD1d^{-/-} mice ($P < 0.01$; Fig. 9D). In contrast, the concentration of IFN γ was not significantly different between the two groups ($P > 0.05$; Fig.

9D). Furthermore, we found that culture supernatants of CD4⁺CD25⁻ T cells cocultured with LDCs from WT mice immunized and challenged with OVA showed markedly enhanced concentrations of Th2 cytokines such as IL-4, IL-5, and IL-13, whereas the levels of IL-5 and IFN γ produced by CD4⁺CD25⁻ T cells cocultured with LDCs from WT mice unimmunized and unchallenged with OVA were significantly reduced compared with CD4⁺CD25⁻ T cells cultured with PBS ($P < 0.01$; Fig. 9D). Also, our data showed that no significant difference was found in the frequency of IL-17A⁺CD4⁺ T cells during coculture between CD4⁺CD25⁻ T cells with LDCs from WT and CD1d^{-/-} mice immunized and challenged with OVA in the presence of OVA (100 μ g/ml, $P > 0.05$; Fig. 9, B and C). Moreover, IL-17A levels were not significantly different in the culture supernatant of CD4⁺CD25⁻ T cells from mice immunized with OVA cocultured with LDCs from WT mice compared with that of T cells cocultured with LDCs from CD1d^{-/-} mice. ($P > 0.05$; Fig. 9D). Taken together, these data suggest that after immunization and challenge with OVA, LDCs from WT mice, compared with those from CD1d^{-/-} mice, markedly promote Th2-immune responses *in vitro*.

Adoptive transfer of LDCs from WT mice, but not CD1d^{-/-} mice, immunized and challenged with OVA enhances Th2 responses in OVA-induced asthmatic mice. To further assess the influence of iNKT cells on LDC immunogenic maturation *in vivo*, LDCs from WT and CD1d^{-/-} mice immunized and challenged with OVA were adoptively transferred into the OVA-induced asthmatic mouse model via intranasal injection 1 h before OVA challenge on day 25 to determine their ability to induce Th2 responses. The protocol of LDC isolation is shown in Fig. 10A. Adoptive transfer of LDCs, of which purity is >99%, from WT mice immunized and challenged with OVA significantly enhanced Th2 responses in the OVA-induced asthmatic mice. Specifically, elevated inflammatory cell infiltration in the lung (Fig. 10B), significant increases in the number of eosinophils, macrophages, and total cells in the BALF ($P < 0.05$ or $P < 0.01$; Fig. 10C), enhanced PAS-positive goblet cells in the airway epithelium ($P < 0.05$; Fig. 10D), and elevated OVA-specific IgE and IgG1 concentrations

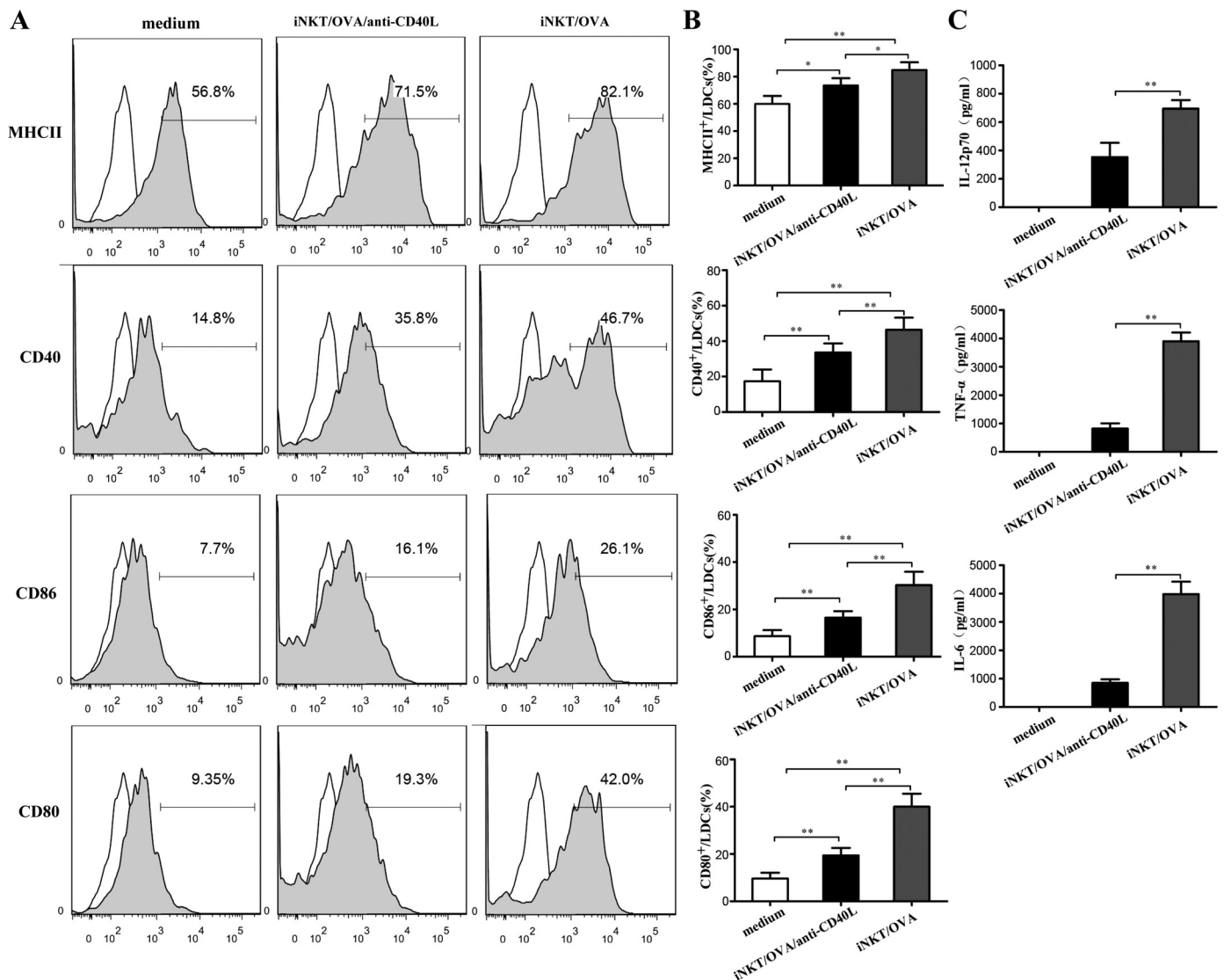


Fig. 8. Elevated surface maturation markers and proinflammatory cytokine production in LDCs involves in CD40L expression. Freshly isolated LDCs from WT mice were cocultured in the presence of OVA with iNKT cells from asthmatic mice in the presence or absence of anti-CD40L for 48 h, and then surface maturation markers of LDCs and proinflammatory cytokine secretion in the culture supernatant were analyzed. A: flow cytometry of MHC II, CD80, CD86, and CD40 in LDCs (CD11c⁺ cells; shaded histograms) and isotype controls (line) cocultured in the presence of OVA with iNKT cells in the presence or absence of anti-CD40L. Percentages are those of positive cells. B: expression levels of MHC II, CD80, CD86, and CD40 on LDCs (CD11c⁺ cells) cocultured with iNKT cells in the presence or absence of anti-CD40L. C: LDCs were cocultured with iNKT cells in the presence of OVA with iNKT cells in the presence or absence of anti-CD40L for 48 h; the concentrations of IL-12p70, IL-6, and TNF α in the culture supernatants were determined by ELISA. Data are presented as means $\pm$ SD from 3 independent experiments ($n = 18$), and 1 representative experiment is indicated. * $P < 0.05$; ** $P < 0.01$.

in the serum ($P < 0.05$ or $P < 0.01$; Fig. 10E) were observed in asthmatic mouse adoptively transferred with LDCs from WT mice immunized and challenged with OVA. Furthermore, the levels of IL-4, IL-5, IL-13, and IL-17A in the BALF and MLN cell culture supernatant were significantly higher in asthmatic mice treated with adoptive transfer of LDCs from WT mice immunized and challenged with OVA ($P < 0.05$ or $P < 0.01$; Fig. 10, F and G). By contrast, the concentration of IFN γ did not change significantly between the two groups ($P > 0.05$; Fig. 10, F and G). Finally, AHR was determined by direct measurement of RL and Cdyn. RL and Cdyn to increasing concentrations of methacholine (Mch), ranging from 3.12 to 50 mg/ml in PBS, was examined. Elevated RL to Mch was observed in OVA-induced asthmatic mice after adoptive trans-

fer of LDCs from WT mice immunized and challenged with OVA compared with receiving adoptive transfer of LDCs from CD1d^{-/-} mice immunized and challenged with OVA ($P < 0.05$ or $P < 0.01$; Fig. 10H). However, no difference was found in terms of Cdyn between the two groups ($P > 0.05$; Fig. 10H). Collectively, these data show that LDCs from WT mice, in contrast to those from CD1d^{-/-} mice immunized and challenged with OVA, can significantly enhance Th2-immune responses in vivo.

iNKT cell induces LDC expression of some markers of alternative activation in a mouse model of asthma induced by OVA. DCs can upregulate multiple markers associated with alternatively activated macrophage such as CD206, i.e., mannose receptor (MR), resistin-like molecule- α (RELM- α), and

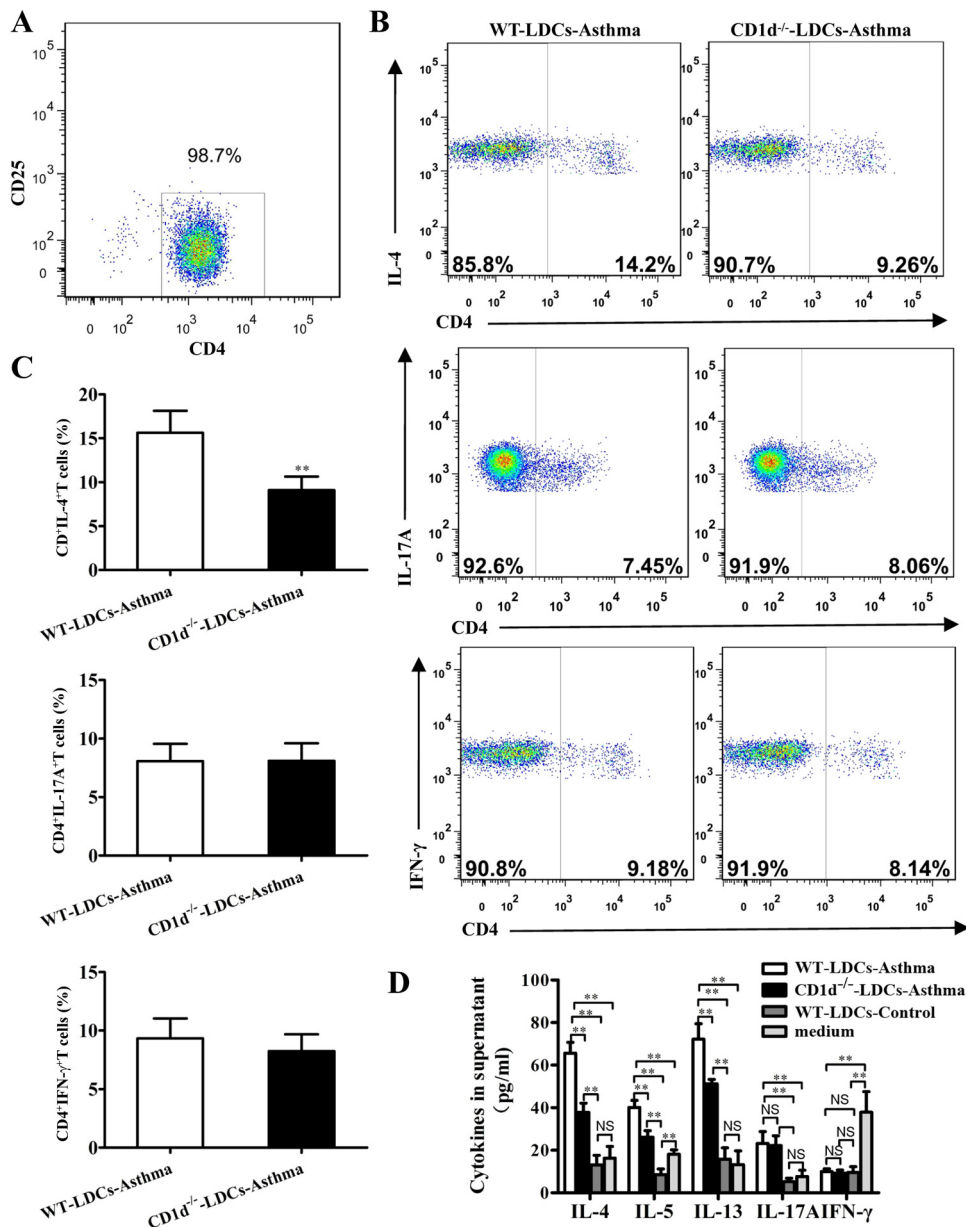


Fig. 9. Enhanced Th2 priming capacity in LDCs isolated from WT mice, but not CD1d^{-/-} mice, immunized and challenged with OVA and CD4⁺CD25⁺ T cells isolated from the spleens of WT mice immunized with OVA were cocultured with LDCs from WT mice or CD1d^{-/-} mice immunized and challenged with OVA, WT mice unimmunized and unchallenged with OVA, or PBS in the presence of OVA for 48 h. **A**: flow cytometric analysis shows the purity of isolated CD4⁺CD25⁺ T cells from the spleens of OVA-immunized mice. **B**: intracellular cytokine staining to analyze OVA-specific cytokine secretion by CD4⁺CD25⁺ T cells cocultured with LDCs from WT or CD1d^{-/-} mice immunized and challenged with OVA, in the presence of OVA, using flow cytometric analysis. **C**: the proportion of IL-4⁺, IL-17A⁺, or IFN-γ-producing CD4⁺CD25⁺ T cells upon coculture with LDCs from WT or CD1d^{-/-} mice immunized and challenged with OVA in the presence of OVA. **D**: the concentrations of IL-4, IL-5, IL-13, IL-17A, and IFN-γ in the culture supernatants as analyzed by ELISA. Data are expressed as means ± SD of 3 independent experiments ($n = 18$), and 1 representative experiment is indicated. ** $P < 0.01$.

arginase 1 (Arg1) in Th2 inflammation settings to induce Th2 response (9, 23). A notable feature of iNKT cells is their capacity to quickly produce large amounts of Th2 cytokines after activation, including IL-4, IL-13, and IL-5 (5). Therefore, we investigated whether iNKT cell could induce LDC expression of some markers of alternative activation in an OVA-induced mouse model of asthma. The frequency of CD206⁺CD11c⁺F4/80⁻ cells (CD206⁺DCs) in the lung and the expression of RELM-α and Arg1 mRNA of LDCs were measured from WT mice and asthmatic mice treated with the intraperitoneal injection of 2 μg of α-GalCer or an identical volume of PBS 1 h before challenge on day 25. Our data showed that the frequency of CD206⁺CD11c⁺F4/80⁻ cells in the lung was markedly elevated in the OVA-induced asthmatic mice treated with α-GalCer compared with the OVA-induced asthmatic mice treated with PBS and WT mice ($P < 0.01$; Fig. 11, A and B). Moreover, α-GalCer treatment significantly

enhanced the expression of RELM-α mRNA of LDCs isolated from the OVA-induced asthmatic mice compared with the OVA-induced asthmatic mice treated with PBS and WT mice ($P < 0.05$ or $P < 0.01$; Fig. 11C). However, the expression of Arg1 mRNA was not significantly increased in LDCs from the OVA-induced asthmatic mice treated with α-GalCer compared with PBS ($P > 0.05$; Fig. 11C). To further examine the effect of iNKT cells on LDC expression of some markers of alternative activation, iNKT cells from WT mice immunized and challenged with OVA were adoptively transferred into CD1d^{-/-} mice via tail vein injections 1 h before OVA challenge on day 25, and then the frequency of CD206⁺CD11c⁺F4/80⁻ cells in the lung and the expression of RELM-α and Arg1 mRNA of LDCs isolated from the lung were measured. Our data indicated that, compared with treatment with PBS, adoptive transfer of iNKT cells significantly facilitated the frequency of CD206⁺CD11c⁺F4/80⁻ cells in

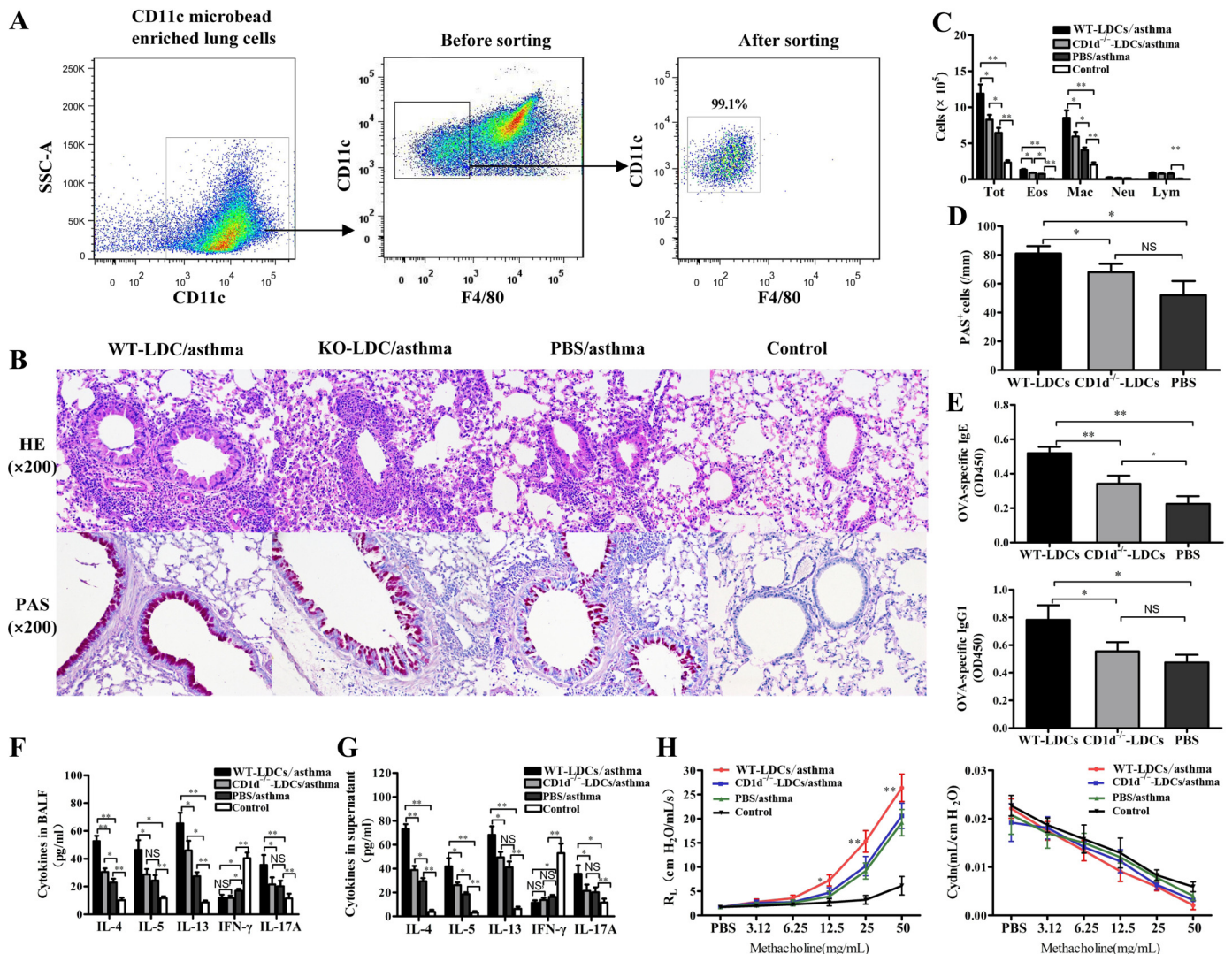


Fig. 10. Adoptive transfer of LDCs from WT mice, but not CD1d^{-/-} mice, immunized and challenged with OVA enhances the Th2 responses in OVA-induced asthmatic mice. LDCs (2×10^5) obtained from WT and CD1d^{-/-} mice immunized and challenged with OVA into OVA-induced asthmatic mice via intranasal injection 1 h before the OVA challenge on day 25; mice were euthanized 24 h after the final OVA challenge for further analysis. **A**: the protocol of LDC isolation and the purity of the adoptively transferred LDC populations. **B**: histopathological analysis of lung tissue from mice adoptively transferred with LDCs or PBS using hematoxylin and eosin (HE) staining and periodic acid-Schiff (PAS) staining. **C**: total cell and differential cell counting in the BALF of mice treated with adoptive transfer of iNKT cells or PBS. Tot, total cell counts; Eos, eosinophils; Neu, neutrophils; Mac, macrophages; Lym, lymphocytes. **D**: lung goblet cell hyperplasia is shown as the no. of PAS-positive cells per unit of length (mm) of the basement membrane. **E**: OVA-specific IgE and IgG1 concentrations in the serum of mice adoptively transferred with LDCs or PBS. **F**: BALF was obtained 24 h after the final challenge, and the levels of IL-4, IL-5, IL-13, IL-17A, and IFN-γ from mice treated with the adoptive transfer of LDCs or PBS were measured by ELISA. **G**: MLN cells were collected from mice adoptively transferred with LDCs or PBS and cultured in the presence of OVA in vitro. After 72 h, culture supernatants were obtained and the concentrations of IL-4, IL-5, IL-13, IL-17A, and IFN-γ measured by ELISA. Data are expressed as means $\pm$ SD of 3 independent experiments ($n = 18$), and 1 representative experiment is indicated. **H**: airway response to increasing concentrations of methacholine was examined. Data are expressed as means $\pm$ SD ($n = 6$) of 3 independent experiments ($n = 6$), and 1 representative experiment is indicated. Significant differences between OVA-induced asthmatic mice adoptively transferred with LDCs from WT and CD1d^{-/-} mice immunized and challenged with OVA are shown as * $P < 0.05$ and ** $P < 0.01$.

the lung (Fig. 11, *D* and *E*) and the expression of RELM- α mRNA of LDCs (Fig. 11*F*) from CD1d^{-/-} mice immunized and challenged with OVA compared with those treated with PBS ($P < 0.01$). However, the expression of Arg1 mRNA was similar in LDCs from CD1d^{-/-} mice immunized and challenged with OVA treated with adoptive transfer of iNKT cells and PBS ($P > 0.05$; Fig. 11*F*). Taken together, iNKT cells can induce expression of CD206 and RELM- α of LDCs, reflecting alternative activation of LDCs in an OVA-induced mouse model of asthma.

DISCUSSION

In the present study, our results showed downregulation of maturation-associated surface markers and proinflammatory cytokines in LDCs when iNKT cells were deficient in a mouse model of asthma. In contrast, surface maturation markers and proinflammatory cytokine secretion were increased in LDCs from mouse models of asthma treated with α -GalCer or adoptively transferred iNKT cells. Furthermore, iNKT cells could enhance Th2-immune responses by modulating the function of

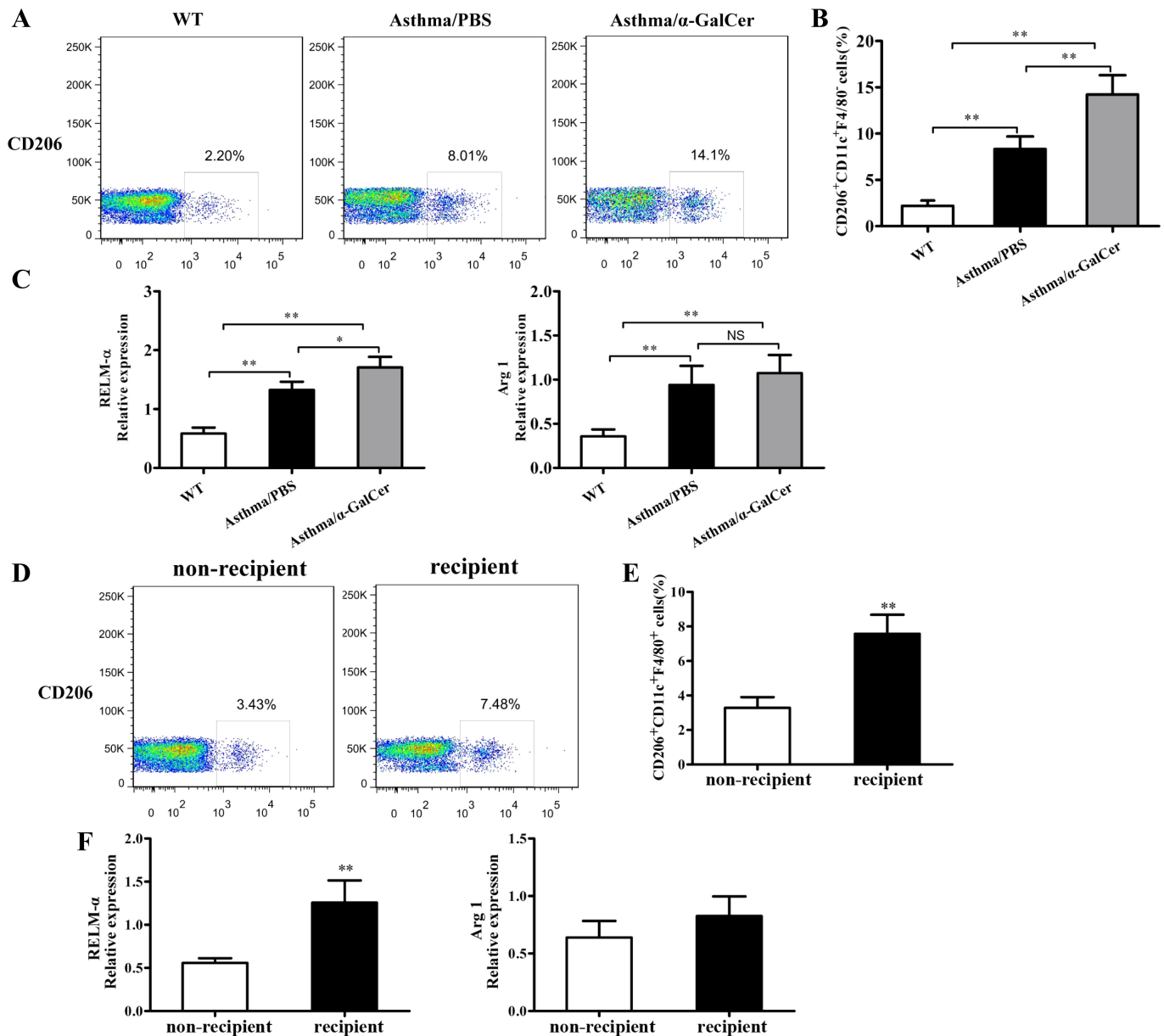


Fig. 11. iNKT cell induces LDC expression of some markers of alternative activation in a mouse model of asthma induced by OVA. LDCs were isolated from WT mice, asthmatic mice treated with α -GalCer or PBS, and CD1d^{-/-} mice with adoptive transfer of iNKT cells (1×10^6 /mouse) from WT mice immunized and challenged with OVA or PBS 1 h before challenge on day 25, and then the number of CD206⁺CD11c⁺F4/80⁺ cells (CD206⁺ DCs) in the lung was measured by flow cytometry, and the expression of RELM- α and Arg1 mRNA was detected by quantitative RT-PCR. **A**: flow cytometry of CD206⁺CD11c⁺F4/80⁺ cells in the lung from WT mice and OVA-induced asthmatic mice after treatment with PBS or α -GalCer. The corresponding percentages indicate the frequency of lung CD206⁺CD11c⁺F4/80⁺ cells (CD206⁺ DCs). **B**: expression levels of CD206 on LDCs from WT mice and OVA-induced asthmatic mice treated with α -GalCer or PBS. **C**: expression of resistin-like molecule- α (RELM- α) and arginase 1 (Arg1) mRNA of LDCs from WT mice and OVA-induced asthmatic mice treated with α -GalCer or PBS. **D**: expression of CD206 on CD11c⁺F4/80⁺ cells was assessed in cells from the lungs of CD1d^{-/-} mice immunized and challenged with OVA and adoptively transferred with iNKT cells or PBS. **E**: expression levels of CD206 on CD11c⁺F4/80⁺ cells from the lungs of CD1d^{-/-} mice immunized and challenged with OVA adoptively transferred with iNKT cells or PBS. **F**: expression of RELM- α and Arg1 mRNA of LDCs from the lungs of CD1d^{-/-} mice immunized and challenged with OVA adoptively transferred with iNKT cells or PBS. Data are expressed as means $\pm$ SD of 2 to 3 independent experiments ($n = 12-18$), and 1 representative experiment is indicated. * $P < 0.05$; ** $P < 0.01$.

LDCs in vivo and in vitro. In addition, immature BMDCs gained a proinflammatory mature status upon interaction with iNKT cells together with OVA, whereas immature BMDCs adopted a tolerogenic maturation status upon interaction with iNKT cells alone. In addition, iNKT cell can induce LDCs expression of CD206 and RELM- α , reflecting alternative activation of LDCs in a mouse model of asthma. α -Galactosyl-

ceramide treatment significantly enhanced expression of CD40L in lung iNKT cells of a mouse model of asthma, and the blockade of CD40L strongly suppressed surface maturation markers and proinflammatory cytokine production by LDCs. Therefore, our data indicated that iNKT cells can promote immunogenic maturation of LDCs to enhance Th2 response in mouse models of asthma.

DCs are presently divided into tolerogenic immature and immunogenic mature differentiation stages (33). In our present study, LDCs from WT mice unimmunized and unchallenged with OVA showed low expression of surface maturation markers, including costimulatory molecules CD80, CD86, and CD40, as well as MHC II, and negligible production of proinflammatory or immunostimulatory cytokines such as IL-12, IL-6, and TNF α and prevent not only Th2 but also Th1 cytokine production, thereby indicating that they were considered to be immature and tolerogenic DCs (11, 33). In contrast, LDCs from WT mice immunized and challenged with OVA were characterized by upregulation of surface maturation markers, high release of proinflammatory or immunostimulatory cytokines, and downregulation of IL-10 and significantly promoted Th2 cytokines, hence suggesting that they were referred to as mature and immunogenic DCs (11, 33). We further investigated the modulatory influence of iNKT cells on phenotype and function of LDCs. Our results showed that iNKT cell deficiency can reduce the number of LDCs as well as maturation-associated surface markers and proinflammatory cytokine secretion of CD1d^{-/-} mice immunized and challenged with OVA. The CD1d-KO mice lack the MHC class I restricting element required by iNKT cell and thereby lack iNKT cells (3). Therefore, the downregulation of MHC-II, CD80, CD86, and CD40 of LDCs from the CD1d-KO mice immunized and challenged with OVA may be due mostly to not having iNKT cells. Furthermore, maturation-associated surface markers and proinflammatory cytokine secretion of LDCs were increased in the OVA-induced asthmatic mice treated with α -GalCer or in the OVA- or HDM-induced asthmatic mice that received adoptive transfer of iNKT cells. Collectively, these *in vivo* and *in vitro* findings indicated that iNKT cells could comprehensively facilitate immunogenic maturation of LDCs in an OVA- or HDM-induced mouse model of allergic asthma.

Because of the relatively low counts of DCs *in vivo* in the steady state, many studies have been performed using *in vitro*-induced DCs with DC progenitors from bone marrow in mice (26). It has been reported that BMDCs possess inherent tolerogenic capacity (37, 45). In our present study, BMDCs cultured in the presence of PBS for 20 h showed low expression of MHC-II, CD80, CD86, and CD40 and the concentrations of IL-12p40, IL-12p70, IL-6, IL-10, and TNF α in the culture supernatant were negligible, indicating that BMDCs are immature. Also, our data showed that LDCs from WT mice unimmunized and unchallenged with OVA were considered to be immature and tolerogenic DCs. Therefore, BMDCs and LDCs in the steady state are immature, thereby sharing functional similarities. As such, coculture experiments were performed with purified iNKT cells from WT mice immunized and challenged with OVA and BMDCs in the presence of OVA to investigate the role of iNKT cells in modulating the function of LDCs. Our results showed that immature DCs became proinflammatory upon interaction with iNKT cells in the presence of OVA. Interestingly, our preliminary findings showed that immature BMDCs adopt a tolerogenic maturation status upon interaction with iNKT cells in the absence of OVA. However, one important question remains to be addressed. Although LDCs were long considered as a homogeneous population that shares functional similarities with BMDCs (21, 44), it is now obvious that different subsets of LDCs exist in

the airways (47). Therefore, it is necessary to thoroughly investigate the modulatory effect of iNKT cells on certain subsets of LDCs in future studies.

LDCs can instruct CD4⁺ T cells differentiate into Th2 effectors, initiating and maintaining maladaptive immunity after allergen exposure in asthma (21, 30). Our data showed that LDCs from WT mice, in contrast to those from CD1d^{-/-} mice immunized and challenged with OVA, markedly facilitated the Th2-immune response *in vitro*. Furthermore, adoptive transfer of LDCs from WT mice, compared with those from CD1d^{-/-} mice immunized and challenged with OVA, significantly enhanced Th2-immune responses *in vivo*. Taken together, the above *in vitro* and *in vivo* results suggested that iNKT cells could modulate the functional capacity of LDCs to promote Th2-immune responses in a mouse model of asthma. The hallmark of asthma is induction of CD4⁺ Th2 response, which is characterized by production of cytokines like IL-4, IL-5, and IL-13 (1). Our results showed that iNKT cells can promote proinflammatory cytokine secretion like IL-12 and TNF- by LDCs, which would be expected to play a critical role in the development of polarized Th1 response (12, 22, 34, 43). However, in addition to the regulated secretion of polarizing cytokines, DCs can upregulate multiple markers associated with alternatively activated macrophages such as CD206 and RELM- α in Th2 inflammation settings, which can play a key role in Th2 polarization (9, 23). In our present study, iNKT cells can induce alternative activation of LDCs in mouse models of asthma induced by OVA. Thus, it is possible that iNKT cells promote Th2 immunity through the upregulation of markers of alternative activation of LDCs in asthma.

CD40 is essential for the maturation and activation of DCs (25, 35). Our data indicated that iNKT cells could significantly enhance CD40 expression on LDCs from an OVA- or HDM-induced mouse model of allergic asthma. It has been reported that iNKT cells upregulate CD40L in the presence of optimal stimuli (46) and might thereby induce DC maturation and activation via interaction with CD40, which contributes to the development of both CD4⁺ and CD8⁺ T cell immunity (14, 27, 50). Our data showed obviously high CD40L expression on iNKT cells and indicated that α -GalCer treatment could enhance the expression of CD40L on iNKT cells from WT mice immunized and challenged with OVA. Furthermore, the coculture experiment of LDCs with iNKT cells showed that the blockade of CD40L strongly suppressed surface maturation markers and proinflammatory cytokine production by LDCs. Therefore, it is possible that iNKT cells promote the function of LDCs through the CD40-CD40L pathway in asthma, but further studies are required to confirm this.

The role of iNKT cells in human asthma has been controversial. Early reports indicated an increased number of iNKT cells in the lungs of patients with asthma compared with patients with sarcoidosis and healthy controls (2, 18, 41). However, these studies have recently been challenged by other researchers, who reported reduced iNKT cell counts (38, 49). Recent findings have highlighted the role of iNKT cells in human asthma because of a marked increase of iNKT cells in patients with asthma (28). HDM, one of the common aeroallergens in asthma patient-living environments, is closely associated with the development of asthma (16). Therefore, we used the HDM-induced mouse model of asthma as well as the OVA-induced mouse model of asthma to investigate the mod-

ulatory effects of iNKT cells on phenotype and function of LDCs, likely reflecting the role of iNKT cells in human asthma. However, it is critically important to investigate the findings from the study of human subjects before reaching any conclusions about the contributing role of iNKT cells in human asthma.

In summary, our study has demonstrated that iNKT cells can promote the immunogenic maturation of LDCs to enhance the Th2 response in mouse models of asthma.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Q.H., L.L., Q.Y., A.W., S.C., R.L., Y.H., G.Z., X.D., H.Y., S.H., and H.N. performed experiments; Q.H., L.L., Q.Y., A.W., R.L., Y.H., G.Z., X.D., H.Y., S.H., and H.N. analyzed data; Q.H. and H.N. interpreted results of experiments; Q.H. and S.C. prepared figures; Q.H. and H.N. drafted manuscript; Q.H. and H.N. edited and revised manuscript; Q.H. and H.N. approved final version of manuscript; H.N. conceived and designed research.

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