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Original Article

Conventional type 2 lung dendritic cells are potent inducers of follicular helper T cells in the asthmatic lung

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Abbreviations:

cDC1, conventional type 1 dendritic cells;
 cDC2, conventional type 2 dendritic cells;
 DC, dendritic cell; GCB cell: germinal center
 B cell, ICOS: inducible T cell co-
 stimulator, ICOS-L: inducible T cell co-
 stimulator ligand, IRF4: interferon
 regulatory factor 4, IRF8: interferon
 regulatory factor 8, LDC: lung dendritic
 cell, MLN: mediastinal lymph node, PD-1:
 programmed cell death 1, Tfh cell: follicular
 helper T cell

ABSTRACT

Background: Follicular helper T (Tfh) cells represent a unique subset of helper CD4⁺ T cells in lymphoid follicles. Recently, Tfh cells were shown to play an important role in asthma through B cell differentiation. Conventional lung DCs are classified into two major subsets: conventional type 1 (cDC1) and type 2 (cDC2). Although the two subsets are different in driving particular T cell responses, the subset that induces Tfh cells in the asthmatic lung primarily has yet to be fully elucidated.

Methods: We evaluated Tfh cells, defined by the expression of CD4 and CXCR5, in HDM-challenged mice. Next, we characterized cDC1 and cDC2 purified from antigen-primed lung and examined their Tfh cell-inducing capacity. Additionally, the ability of lung DC-induced Tfh cells to cause germinal center B (GCB) cells to produce antigen-specific IgE was assessed.

Results: In HDM-challenged mice, Bcl-6-expressing Tfh cells were significantly increased in the mediastinal lymph nodes. Lung cDC2, but not lung cDC1, increased after HDM priming, and cDC2 secreted larger amounts of IL-6 with higher ICOS-L expression than cDC1. In the co-cultures with OVA-specific naïve CD4⁺ T cells, cDC2 from OVA-primed lung induced Bcl-6-expressing Tfh cells more efficiently, together with larger amounts of IL-6 and IL-21, than cDC1. Blockage of IL-6 or ICOS-L significantly reduced Tfh cell induction. Finally, cDC2-induced Tfh cells enabled GCB cells to produce OVA-specific IgE.

Conclusions: In asthmatic lung, cDC2 is the primary DC subset responsible for Tfh cell differentiation and plays an important role in humoral immunity in asthma by inducing Tfh cells.

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Introduction

Follicular helper T (Tfh) cells are a unique subset of helper CD4⁺ T cells that are located in lymphoid follicles of secondary lymphoid organs. Tfh cells provide help to B cell differentiation in the germinal center.¹ Indeed, they are essential for immunoglobulin

class switching and promote high-affinity antibody production by B cells.² Tfh cells express the chemokine receptor CXCR5 to enable homing to B cell follicles, as well as having costimulatory receptors, such as programmed cell death 1 (PD-1). The B cell lymphoma 6 protein (Bcl-6) is the master transcription factor, emerging during Tfh cell differentiation after an immune response. Additionally, Tfh cells mainly produce IL-21 and IL-4 for humoral immunity, such as immunoglobulin class switching.^{3,4} In humans, circulating Tfh cells have been shown to increase in patients with autoimmune diseases and are correlated with autoantibody titers.^{5–10} Notably, IL-21-secreting Tfh-like cells have been reported to play a pivotal role in the induction of Th2-mediated immune responses.^{11,12} In the

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HDM-sensitized asthma model, IL-21-secreting Tfh-like cells accumulate in mediastinal lymph nodes (MLNs) and potentially promote Th2 immunity to HDM.¹¹ In asthmatic patients, the frequency of circulating Tfh cells is elevated, and has been correlated with serum total IgE levels.¹² Collectively, these observations indicate that Tfh cells play an important role in asthma immunology through the activation and differentiation of B cells. However, no data are currently available regarding the induction of Tfh cells in the asthmatic lung.

Dendritic cells (DCs) are classified into subsets based on their lineage commitment, discrete profiles of cytokine production, and capacity to induce different T cell responses. In general, they are divided into two subsets: conventional DCs, with strong antigen-presenting ability, and plasmacytoid DCs, producing type 1 interferon in the immune response.^{13–15} Conventional DCs can be further classified into two major subsets: conventional type 1 (cDC1) and type 2 (cDC2). These two conventional DC subsets play different roles in driving particular T cell responses. cDC1 is mainly characterized by the cross-presentation of intracellular antigens to CD8⁺ T cells and the direct presentation of antigens to CD4⁺ T cells, leading to a Th1 response as cellular immunity. On the other hand, cDC2 is efficient in the direct presentation of extracellular antigens to CD4⁺ T cells and induces Th2 and Th17 immune responses as humoral immunity.^{13–18} In the lung, we previously demonstrated that lung cDC1 predominantly elicited Th1 and Th17 responses, whereas lung cDC2 primarily provoked a Th2 response with high IgA-producing capacity.^{19,20} However, among these two subsets, whether cDC1 or cDC2 is primarily responsible for inducing the differentiation of Tfh cells in peripheral tissue and specific organs, such as lungs, has yet to be fully elucidated. Recently, several studies have demonstrated that different conventional DC subsets induce Tfh cells in mucosal immunity. For example, human tonsil cDC2 was found to be a potent inducer of Tfh cells.²¹ Additionally, spleen Notch2-dependent cDC2 has been found to be necessary and sufficient for Tfh cell induction in animal experiments.²² On the other hand, cDC1 in the skin promotes Tfh cell differentiation under both steady state and inflammatory conditions,²³ while cDC1 was found to efficiently induce Tfh cells by antigen via C-type lectin²⁴. Therefore, the capacity of conventional DC subsets to induce Tfh cell differentiation is likely to depend on their different locations and conditions, such as inflammation. However, it remains unclear which subset of lung DCs (LDCs) elicits Tfh cell differentiation, particularly under certain pathological conditions, such as asthma, in the lung.

In the present study, the subsets of LDCs primarily responsible for inducing Tfh cells in the asthmatic lung were elucidated. Using an HDM-eliciting mouse asthma model, we observed a marked increase in Tfh cells in MLNs. In addition, cDC2 isolated from antigen-primed lung was found to induce Tfh cell differentiation from naïve CD4⁺ T cells more efficiently than cDC1. Furthermore, cDC2-induced Tfh cells, which express Bcl-6, potentially elicited antigen-specific IgE production from germinal center B (GCB) cells. These data suggest that lung cDC2 is a primary DC subset that induces Tfh cell differentiation in the asthmatic lung.

Methods

Mice

Experiments were performed on 8–12-week-old male C57BL/6 mice (Nippon-SLC, Shizuoka, Japan) and OVA-specific T cell receptor (TCR) transgenic mice (OT-II) from the Center for Animal Resources and Development, Kumamoto University, Japan. All experimental protocols were approved by the Animal Care and Use Committee of Hamamatsu University School of Medicine (28-02).

All experiments were conducted according to the guidelines of this committee.

Allergen sensitization of mice

Dried *Der f* extract as an HDM allergen was provided by Torii Pharmaceutical, Tokyo, Japan. C57BL/6 mice were anesthetized with a 3% isoflurane oxygen mixture and intranasally sensitized with 50 µg of HDM extract in 25 µL PBS on day 0, and subsequently challenged with 10 µg of HDM extract on days 16–18. Mice were sacrificed on day 21, and their serum, BAL fluid, lung, and MLN were collected (Supplementary Fig. 1a). In some experiments, the OVA protein was used as an antigen. C57BL/6 mice were immunized with 50 µg of OVA (Sigma–Aldrich, St. Louis, MO) and 2 mg of alum (Thermo Fisher Scientific, Waltham, MA) adjuvant dissolved in 200 µL of PBS via intraperitoneal injection on days 0 and 8. Subsequently, the mice were intranasally challenged with 50 µg of OVA in 25 µL of PBS on days 16–18.

Flow cytometry

Cells were stained in flow cytometry buffer (PBS containing 1% fetal bovine serum and 2 mmol/L EDTA). Cells were extracellularly stained in antibody cocktails for 30 min at 4 °C. To detect intranuclear transcription factors, cells were fixed and permeabilized using the Intracellular Fixation & Permeabilization Buffer Set (eBioscience, Vienna, AT), according to the manufacturer's instructions. Purified anti-mouse CD16/32 was used for staining. Live/dead cells were differentiated by near IR or aqua (Molecular Probes, Eugene, OR), according to the manufacturer's instructions. Flow cytometric analysis was performed using Gallios (Beckman Coulter, Brea, CA) and analyzed using FlowJo 10 (Tree Star, Ashland, OR). The antibodies used for flow cytometry are listed in Supplementary Table 1.

BAL analysis

BAL fluid was collected through 3 × 800 µL instillations of PBS into the trachea of cannulated mice, followed by blood collection through cardiac puncture. The BAL cells were stained and differentiated by flow cytometry with antibodies against CD45, CD3e, CD4, CD11b, CD11c, CD19, Gr-1, Siglec-F, and Ly-6G. The leukocyte fraction was evaluated using Gallios.

Measurement of immunoglobulins

Blood was collected directly by cardiac puncture, while serum was collected after centrifugation and stored at –80 °C until analysis. Total serum IgE was measured by ELISA, according to the manufacturer's instructions (BD Biosciences, Franklin Lakes, NJ). HDM-specific IgE was measured using flat-bottomed 96-well plates (Thermo Fisher Scientific) coated with 5 µg/mL of HDM extract in carbonate buffer (0.1 M sodium carbonate, pH 9.5). Samples were diluted 1:2 in assay diluent (PBS with 10% fetal bovine serum, pH 7.0) and incubated overnight at 4 °C. Detection was performed with biotinylated rat anti-mouse IgE (LO-ME-3) antibody (5 µg/mL) (AbD Serotec, Oxford, UK), avidin alkaline phosphatase (Sigma–Aldrich), and *p*-nitrophenyl phosphate (Sigma–Aldrich). OVA-specific IgE in the culture supernatant was measured by ELISA, according to the manufacturer's instructions (Chondrex, Redmond, WA).

Measurement of cytokine production

The levels of IL-2, IL-4, IL-6, IL-10, IL-17A, IFN-γ, IL-12p70, and TNF in the culture supernatants were measured using cytometric

bead array (CBA) kits (BD Biosciences, San Jose, CA), and the levels of IL-21 were determined using ELISA kits (RayBiotech, Peachtree Corners, GA), according to the manufacturer's instructions.

Identification of Tfh cells

Tfh cells were collected from the spleen and MLN of an asthma mouse model. The cells were stained by flow cytometry with antibodies. Tfh cells were identified as CD45⁺ CD4⁺ CXCR5⁺ cells. Unstained CD45⁺ CD4⁺ cells with anti-CXCR5 antibody were used as the negative controls. In addition, we assessed the expression of PD-1, an inducible T cell co-stimulator (ICOS), and Bcl-6 which is a transcriptional factor of Tfh cells.

Preparation of cDC1 and cDC2 and naïve CD4⁺ T cells

Conventional LDC subtypes were isolated as previously described,^{14,25,26} with some modifications. Lungs were excised and enzymatically digested for 40 min at 37 °C with 200 IU/mL collagenase type II (Worthington Biochemical, NJ) and 100 IU/mL DNase (Worthington Biochemical). The digested lungs were mechanically dispersed using a GentleMACS Dissociator (Miltenyi Biotec, Bergish-Gladbach, Germany) for 30 s. Single-cell suspensions were percolated through a 70-µm cell strainer. The total number of CD11c⁺ cells was determined using magnetic cell sorting (MACS) (Miltenyi Biotec, Auburn, CA) with anti-CD11c mAb-conjugated magnetic microbeads (Miltenyi Biotec), according to the manufacturer's protocol. CD45⁺ CD64[−] F4/80[−] lineage[−] MHCII⁺ CD11c⁺ XCR1⁺ CD172a[−] LDCs (cDC1) and CD45⁺ CD64[−] F4/80[−] lineage[−] MHCII⁺ CD11c⁺ XCR1[−] CD172a⁺ LDCs (cDC2) were isolated using a MoFlo Astrios EQ (Beckman Coulter, Brea, CA). Lineage negative cells were identified as CD3e[−] CD4[−] CD8[−] NK1.1[−] CD19[−] CD45R[−] cells. In addition, the intracellular transcription factors interferon regulatory factor 4 (IRF4) and interferon regulatory factor 8 (IRF8), on conventional LDC subtypes, were stained and analyzed 7 days after priming (Supplementary Fig. 1b). Naïve CD4⁺ T cells were obtained from the spleen of OT-II mice, which were excised and suspended by passing through a 70-µm cell strainer. CD4⁺ Va2⁺ Vb5⁺ T cells were isolated using a MoFlo Astrios EQ. The purified conventional LDC subtypes and naïve T cell populations routinely contained 95–98% cells.

Characteristics of cDC1 and cDC2 by HDM extract sensitization

Purified cDC1 and cDC2 from HDM extract-primed lungs were seeded into 96-well plates at 1×10^5 cells/mL in RPMI-1640 complete medium, supplemented with 10% heat-inactivated fetal calf serum, 1 mM sodium pyruvate (Gibco, Gaithersburg, MD), 200 U/mL penicillin, and 100 µg/mL streptomycin. They were stimulated with 1 µg/mL HDM extract. After 24 h, the cells and supernatants were collected for analysis. The surface markers on conventional LDC subsets were analyzed using FACS, and the levels of cytokines produced by conventional LDC subsets were analyzed using CBA and ELISA kits.

Priming CD4⁺ T cells with cDC1 and cDC2

Naïve CD4⁺ T cells from OT-II mice (1×10^5 cells/well) were co-cultured with cDC1 and cDC2 from the lungs of OVA-primed mice (1×10^4 cells/well) in the presence of OVA_{323–339} peptide (ISQV-HAAHAEINEAGR, 10 µg/mL, Invitrogen, Carlsbad, CA) for 7 days in RPMI-1640 complete medium. Then, the priming CD4⁺ T cells and supernatant were collected (Supplementary Fig. 1c). The differentiation of naïve CD4⁺ T cells into Tfh cells was evaluated by FACS, and the levels of cytokine production in the supernatant were

measured using CBA and ELISA kits. To inhibit IL-6 or ICOS-L signaling, naïve CD4⁺ T cells were co-cultured with cDC2 as described above, and anti-IL-6 antibody (10 µg/mL) (MP5-20F3, BioXCell, Lebanon, NH) or anti-ICOS-L (CD275) antibody (10 µg/mL) (HK5.3, BioXcell) was added into the culture for 7 days (Supplementary Fig. 1d). The differentiation of naïve CD4⁺ T cells into Tfh cells was assessed by FACS.

Co-culture with naïve GCB cells and induced Tfh cells and cDC2

Purified Tfh cells were isolated from MLNs of an OVA asthma mouse model using MoFlo Astrios EQ. Naïve GCB cells from the spleen were isolated using MACS with anti-PNA mAb-conjugated magnetic microbeads (Miltenyi Biotec). Then, PNA⁺ CD19⁺ CD3e[−] B220⁺ CD38[−] IgM[−] IgD[−] IgE[−] GL7⁺ FAS⁺ cells were isolated using a MoFlo Astrios EQ. Tfh cells (1×10^5 cells/well) were co-cultured with naïve GCB cells (1×10^5 cells/well) in RPMI-1640 complete medium in the presence of 10 µg/mL OVA for 7 days (Supplementary Fig. 1e). In addition, naïve CD4⁺ T cells from OT-II mice (1×10^5 cells/well) were co-cultured with cDC1 and cDC2 from lungs of OVA-primed mice (1×10^4 cells/well) in the presence of 10 µg/mL OVA for 7 days in RPMI-1640 complete medium, and subsequently naïve GCB cells (1×10^5 cells/well) were added to the culture for an additional 7 days (Supplementary Fig. 1f). The supernatants were collected and OVA-specific IgE levels were measured by ELISA.

Statistical analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software, San Diego, CA). Data were analyzed using the Wilcoxon/Kruskal–Wallis test for comparison between two groups, and Tukey's test was used for multiple group comparisons. Data are expressed as the mean ± SEM. Statistical significance was set at $p < 0.05$.

Results

HDM extract challenge induced Th2 response in lung with accumulation of Tfh cells in MLNs

In HDM-challenged mice (HDM group), the number of total cells, eosinophils (CD45⁺ CD3e[−] CD11b[−] CD11c[−] CD19[−] Siglec-F⁺ Gr-1 or Ly-6G[−]), and neutrophils (CD45⁺ CD3e[−] CD11b[−] CD11c[−] CD19[−] Siglec-F[−] Gr-1 or Ly-6G⁺) in BAL fluid was significantly increased compared with that in non-sensitized mice (Ctrl group) (Fig. 1a, Supplementary Fig. 1a). The HDM groups showed inflammatory cell infiltration around airways and blood vessels, as well as mucus production in lung sections, as demonstrated by HE and PAS staining, respectively (Fig. 1b). In addition, the total IgE and HDM-specific IgE levels in the serum of the HDM group were significantly higher than those of the Ctrl group (Fig. 1c). Next, we identified and enumerated Tfh cells (CD45⁺ CD4⁺ CXCR5⁺ cells) in MLNs and spleens in HDM-challenged mice (Fig. 1d). The HDM group had a significantly higher proportion of Tfh cells among CD4⁺ T cells in the MLN than the Ctrl group (HDM $9.38 \pm 0.8\%$ vs. Ctrl $1.80 \pm 0.7\%$, $p < 0.001$) (Fig. 1e). However, no significant difference was found in the proportion of Tfh cells in spleen cells between the HDM and Ctrl groups ($11.45 \pm 1.58\%$ vs. $11.26 \pm 1.15\%$, $p = 0.874$). In addition, we examined the expression of PD-1, ICOS, and Bcl-6, a Tfh cell transcription factor, in MLN cells (Fig. 1f). In the MLN cells of the HDM group, CD4⁺ CXCR5⁺ cells showed high levels of Bcl-6 expression and moderate expression of PD-1.

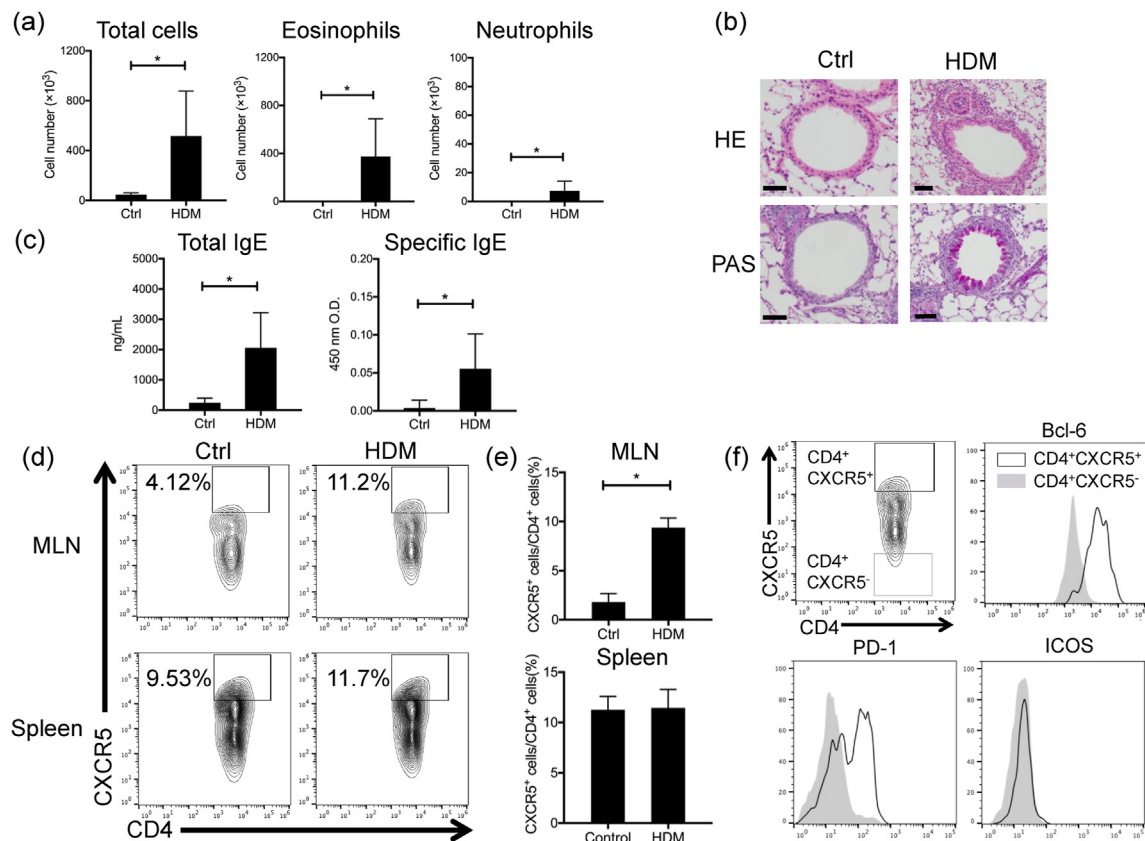


Fig. 1. HDM extract challenge induced Th2 response in lung with accumulation of follicular helper T (Tfh) cells in mediastinal lymph node (MLN). C57/BL6 mice were sensitized intranasally with 50 μ g HDM extract in 25 μ L of PBS on day 0, challenged with 10 μ g HDM extract on day 16–18, and sacrificed 3 days after the last challenge (HDM group). The control (Ctrl) group was administered PBS on the same schedule. Differential cell counts in BAL fluid (a), representative images for hematoxylin and eosin (HE) and periodic acid-Schiff (PAS) stained lung tissue sections (scale black bar; 50 μ m) (b), total IgE, and HDM-specific IgE levels in serum (c). Tfh cells were identified in the MLN and spleen of HDM extract-challenged mice. The cells were stained and analyzed by FACS using antibodies, as described in the Materials and Methods. Representative Tfh cells gating of MLN and spleen cells are shown (d). Numbers indicate the percentage of gated cells in the panel. Percentage of CXCR5 positive CD4⁺ T cells in MLN and spleen (e). Numbers indicate the percentage of CXCR5 positive cells on CD4⁺ T cells in percentage graphs. The expression of B cell lymphoma-6 (Bcl-6), Programmed cell death-1 (PD-1), and inducible T-cell co-stimulator (ICOS) are compared between CXCR5 positive CD4⁺ cells (upper gating) and CXCR5 negative CD4⁺ cells (lower gating) using FACS. CXCR5 positive CD4⁺ cells (black line, white area) and CXCR5 negative CD4⁺ cells (shaded area) are shown on histograms (f). Data are presented as the mean $\pm$ SEM of five mice/group/experiment (n=5). **p* < 0.05.

Identification of two conventional LDC subsets, and kinetics of each conventional LDC subset number after HDM priming

In HDM-primed mice, conventional LDCs were identified in enzymatically digested lungs using MACS with anti-CD11c mAb-conjugated magnetic beads, and presorted by FACS as CD45⁺ CD64⁺ F4/80⁺ lineage⁺ (CD3e CD4 CD8 NK1.1 CD19 CD45R) MHCII⁺ CD11c⁺ cells (Fig. 2a–e). When gating on CD45⁺ CD64⁺ F4/80⁺ lineage⁺ MHCII⁺ CD11c⁺ cells, two distinct subpopulations of conventional LDCs were observed, in terms of their expression of XCR1 and CD172a (Fig. 2f). XCR1⁺ CD172a⁺ LDCs (cDC1) and XCR1⁺ CD172a⁺ LDCs (cDC2) comprised 33.4 $\pm$ 1.8% and 65.2 $\pm$ 2.9% of CD45⁺ CD64⁺ F4/80⁺ lineage⁺ MHCII⁺ CD11c⁺ cells 7 days after HDM priming, respectively (Supplementary Fig. 1b). These two conventional LDC subsets were purified using a MoFlo Astrios EQ flow cytometer, and yielding 3.5–4.8 $\times$ 10⁴ and 5.7–12.5 $\times$ 10⁴ cells of cDC1 and cDC2 per HDM-primed mouse, respectively. In addition, we assessed the expression of IRF4 and IRF8 (Fig. 2g). IRF4 expression was slightly higher in lung cDC2 than in lung cDC1, while IRF8 expression was much higher in lung cDC1 than in lung cDC2.

Next, we examined the numbers of each conventional LDC subset at various time points after HDM priming (Fig. 2h). Total LDCs increased from 3 days, with the highest number of LDCs

obtained on day 7 after priming. The number of lung cDC2 significantly increased (more than 2-fold) 7 days after priming, whereas the number of lung cDC1 did not change over time (at 7 days after the priming; cDC1 (40,319 $\pm$ 2829 cells) vs. cDC2 (93,096 $\pm$ 16,274 cells) per sensitized mouse, *p* < 0.05).

Expression of costimulatory molecules and cytokine production by each conventional LDC

At 7 days after HDM priming, lung cDC1 and cDC2 were analyzed for their expression of surface markers by FACS (Fig. 3a). PD-L1 was moderately expressed in lung cDC1, but not in lung cDC2. On the other hand, the expression of CD86 and ICOS-L was higher in lung cDC2 than in lung cDC1. Both lung cDC1 and cDC2 showed low levels of CD80 expression, and no expression of CD40 or PD-L2.

To evaluate the production of cytokines by the two LDC subsets, cDC1 and cDC2 isolated from the lungs of HDM-primed mice were stimulated by 1 μ g/mL HDM extract for 24 h *in vitro*. The cytokine levels in the culture supernatants were measured. IL-6, IL-10, and IL-21 were detected in the culture supernatants of both lung cDC1 and cDC2. The levels of IL-6 were significantly higher in the cultures of lung cDC2 than in those of lung cDC1 (cDC1 0.56 $\pm$ 0.01 pg/mL vs. cDC2 17.34 $\pm$ 1.25 pg/mL, *p* < 0.001) (Fig. 3b). No significant

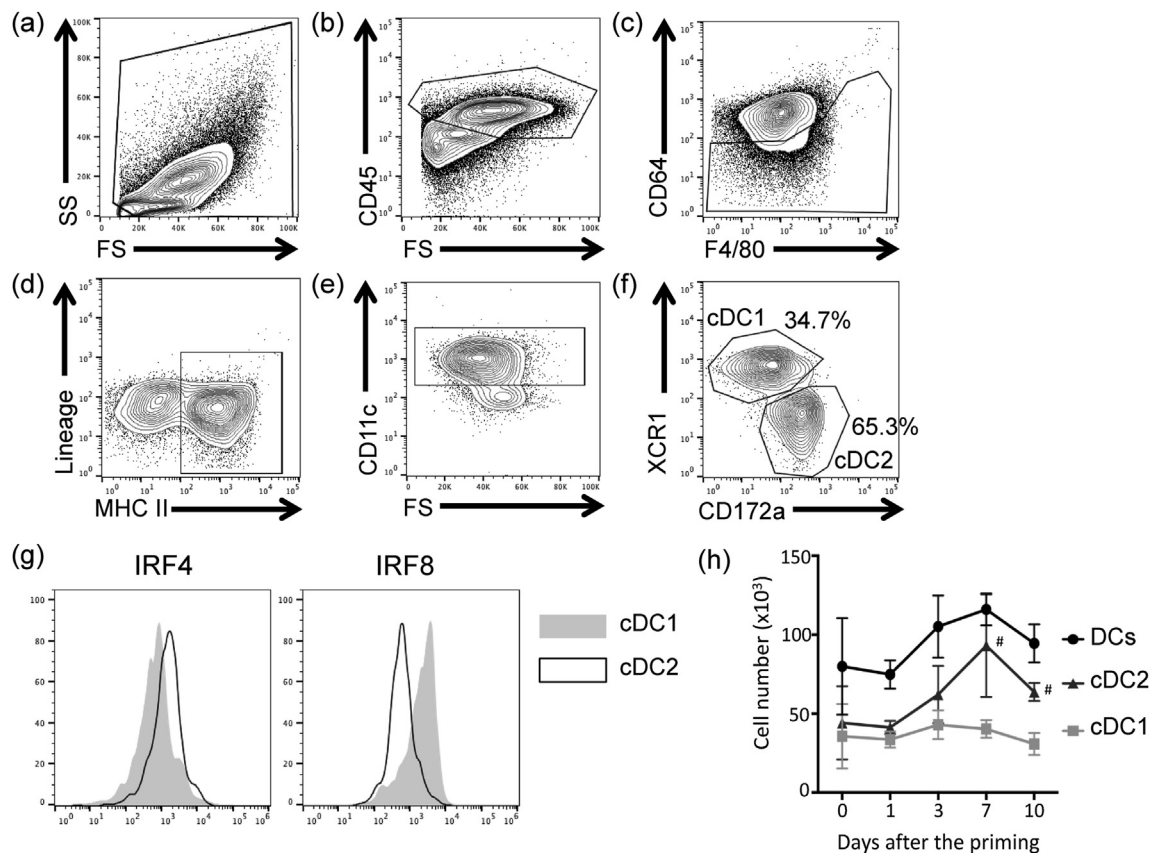


Fig. 2. Identification of two conventional lung dendritic cell (LDC) subsets, conventional type 1 (cDC1) and type 2 (cDC2), and the kinetics of each conventional LDC subset number after HDM priming. Each LDC subset was purified from the enzymatically digested lungs of C57BL/6 mice without HDM extract priming using magnetic and flow cytometric cell sorting, as described in the Materials and Methods. A representative gating strategy for conventional LDC subtypes are shown 7 days after HDM extract priming. Cells in the gated area in (a) were analyzed for CD45, CD64, F4/80, MHC class II, CD11c (b-e). XCR1 and CD172a LDC populations were sorted from CD45⁺ CD64⁺ F4/80⁺ MHC class II⁺ CD11c⁺ cells using a MoFlo Astrios EQ fluorescence activated cell sorter (f). cDC1 is gating CD45⁺ CD64⁺ F4/80⁺ lineage⁺ MHCII⁺ CD11c⁺ XCR1⁺ CD172a⁺ cells. On the other hand, cDC2 is CD45⁺ CD64⁺ F4/80⁺ lineage⁺ MHCII⁺ CD11c⁺ XCR1⁺ CD172a⁺ cells. The expression of transcription factor interferon regulatory factor 4 (IRF4) and interferon regulatory factor 8 (IRF8) are compared between cDC1 (shaded area) and cDC2 (black line, white area) on the histogram (g). The cell number of total LDCs and two conventional LDC subsets after HDM extract priming is also shown (h). Circles indicate total LDCs, triangles cDC2, and squares cDC1. Data are presented as the mean $\pm$ SEM of over five independent experiments (four mice/group/experiment). Statistical analysis was performed at each time point. # $p < 0.05$; cDC1 vs. cDC2.

differences were found in the levels of IL-10 or IL-21 or those of IL-2, IL-4, IL-TNF, or IFN- γ (data not shown) between lung cDC1 and cDC2. The levels of IL-17A and IL-12p70 were below the detection limit in both culture supernatants.

Lung cDC2 induced Tfh cells more efficiently than lung cDC1 in OVA-primed mice in vitro

Using another asthma model with different antigens, we evaluated the number of conventional LDC subsets at various time points after antigen priming. Similar to the HDM-primed asthma model, the number of lung cDC2, but not lung cDC1, increased after OVA priming and reached a maximum on day 7 (Supplementary Fig. 2). Next, we examined the capacity of each lung cDC subset to induce Tfh cells in the co-culture with OVA-specific naive CD4⁺ T cells obtained from OT-II mice in the presence of OVA₃₂₃₋₃₃₉ peptide (Supplementary Fig. 1c). In these co-cultures, cDC2 from lungs 7 days after OVA priming induced a significantly higher volume of Tfh cells than cDC1 from the same lungs (cDC1 0.77 $\pm$ 0.23% vs. cDC2 3.85 $\pm$ 1.08%, $p = 0.023$) (Fig. 4a, b).

Furthermore, we characterized the induced Tfh cells in the co-cultures phenotypically and functionally. FACS analysis showed that the induced CD4⁺ T cells were divided into three gating populations by CXCR5 expression (Fig. 5a). CD4⁺ CXCR5⁺ T cells

had strong expression of Bcl-6, which is a transcriptional factor characteristic of Tfh cells, PD-1, and ICOS, while non-gating CD4⁺ T cells and CD4⁺ CXCR5⁺ T cells did not. Thus, the induced CD4⁺ CXCR5⁺ T cells were phenotypically considered to be Tfh cells. Additionally, the levels of IL-6 were significantly higher in the co-culture supernatants with lung cDC2 than in those with lung cDC1 (cDC1 5.16 $\pm$ 1.58 pg/mL vs. cDC2 26.55 $\pm$ 9.54 pg/mL, $p = 0.039$) (Fig. 5b). High levels of IL-21 were also found in the co-culture supernatants with lung cDC2, but not in those with lung cDC1 (cDC1 6.13 $\pm$ 6.13 pg/mL vs. cDC2 384.2 $\pm$ 136.6 pg/mL, $p = 0.019$).

To investigate the role of IL-6 production and ICOS-L expression on lung cDC2 in Tfh cell induction, we added neutralizing antibodies against IL-6 or ICOS-L to the co-culture with lung cDC2 and OVA-specific naive CD4⁺ T cells in the presence of OVA₃₂₃₋₃₃₉ peptide (Supplementary Fig. 1d). In these co-cultures, the addition of anti-IL-6 or anti-ICOS-L antibody significantly reduced the number of induced Tfh cells compared to the absence of these antibodies (cDC2 4.85 $\pm$ 1.88%, cDC2+ α -IL-6 1.19 $\pm$ 1.04%, and cDC2+ α -ICOS-L 1.17 $\pm$ 1.34%) (cDC2 vs. cDC2+ α -IL-6, $p = 0.015$; cDC2 vs. cDC2+ α -ICOS-L, $p = 0.015$) (Supplementary Fig. 3a, b).

Functionally, we examined the capacity of the induced Tfh cells to produce specific IgE from GCB cells (PNA⁺ CD19⁺ CD3e⁺ B220⁺ CD38⁺ IgM⁺ IgD⁺ IgE⁺ GL7⁺ FAS⁺). GCB cells from the spleen were

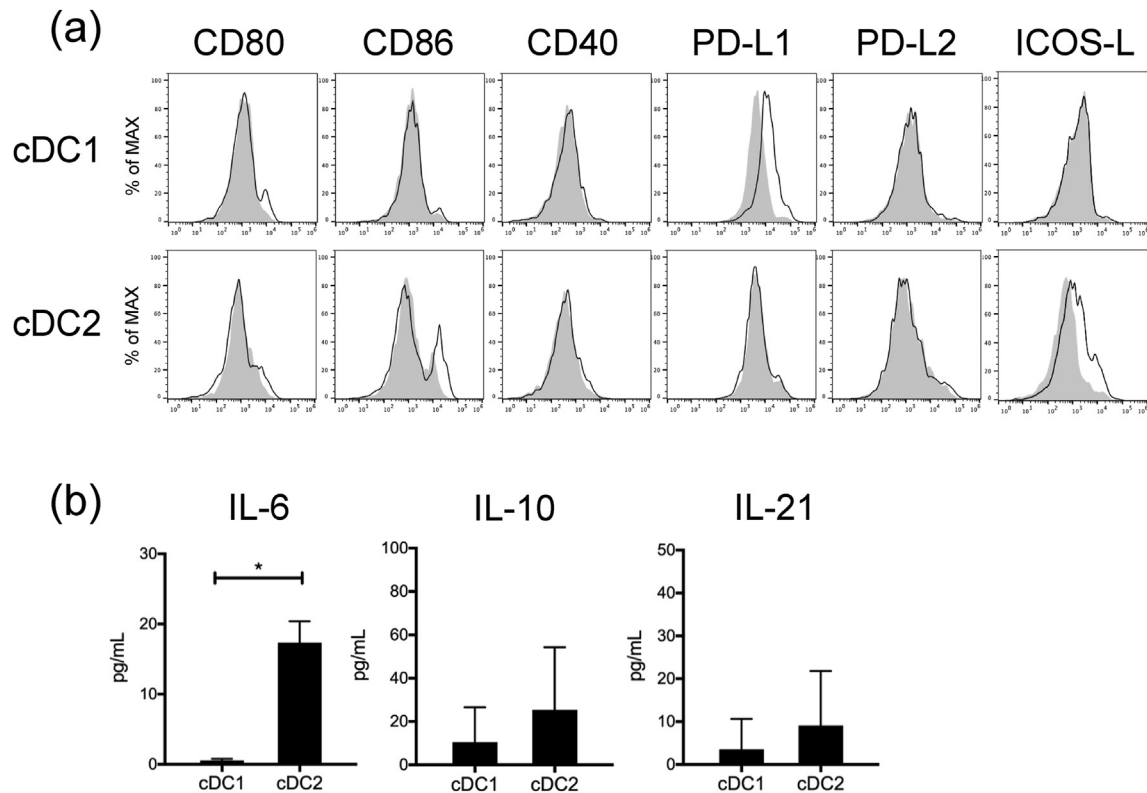


Fig. 3. Expression of costimulatory molecules and cytokine production by each conventional lung dendritic cell (LDC) subset. The isolated conventional type 1 LDC (cDC1) and type 2 LDC (cDC2) from HDM-primed mouse were stimulated with 1 μ g/mL HDM extract for 24 h *in vitro*. The expression levels of costimulatory molecules on cDC1 (top) and cDC2 (bottom) were analyzed by FACS using the indicated monoclonal antibodies (a). Histograms show the expression levels of the cell markers on stimulated conventional LDC subsets (solid line, white area) and unstimulated (shaded area). These results are representative of more than 6 independent experiments. The cytokine levels in the supernatants were measured using cytometric bead arrays and ELISA (b). Data are presented as the mean $\pm$ SEM of six independent experiments (four mice/group/experiment). * $p < 0.05$.

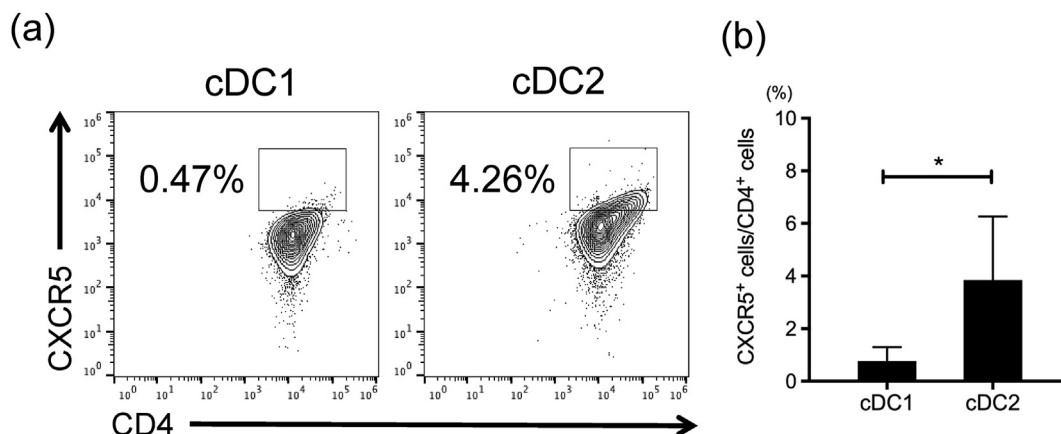


Fig. 4. Conventional type 2 lung dendritic cells (cDC2) more strongly induce follicular helper T (Tfh) cells than do conventional type 1 lung dendritic cells (cDC1) in OVA-primed mice. Naïve CD4⁺ T cells from OVA-specific T cell receptor transgenic (OT-II) mice were co-cultured with cDC1 and cDC2 from lungs of OVA-primed mice for 7 days. Representative follicular helper T (Tfh) cells gating of induced CD4⁺ T cells after co-cultured are shown (a). Numbers indicate the percentage of gated cells in the panel. The percentage of induced CXCR5 positive cells on CD4⁺ T cells were evaluated in percentage graphs (b). Data are presented as the mean $\pm$ SEM of 3 independent experiments (four OVA-primed mice and one OT-II mouse/experiment). * $p < 0.05$.

co-cultured with either Tfh cells from MLN of OVA-challenged mice (Supplementary Fig. 1e) or with the naïve OT-II CD4⁺ T cells plus LDCs from the lungs of OVA-primed mice in the presence of 10 μ g/mL OVA for 7 days (Supplementary Fig. 1f). The supernatants were collected, and OVA-specific IgE levels were measured. Tfh cells from MLN of OVA-challenged mice efficiently induced GCB cells to produce a large amount of OVA-specific IgE (GCB cells alone

22.2 $\pm$ 13.71 pg/mL vs. GCB cells + Tfh cells 124.0 $\pm$ 37.73 pg/mL, $p = 0.035$) (Fig. 5c). Similarly, GCB cells that had been co-cultured with naïve OT-II CD4⁺ T cells plus the lung cDC2, but not lung cDC1, secreted abundant OVA-specific IgE (cDC1 + naïve T cells + GCB cells 8.25 $\pm$ 7.28 pg/mL vs. cDC2 + naïve T cells + GCB cells 218.3 $\pm$ 185.9 pg/mL, $p = 0.037$) (Fig. 5d). These data indicate that Tfh cells induced by the cDC2 from lungs of OVA-primed mice

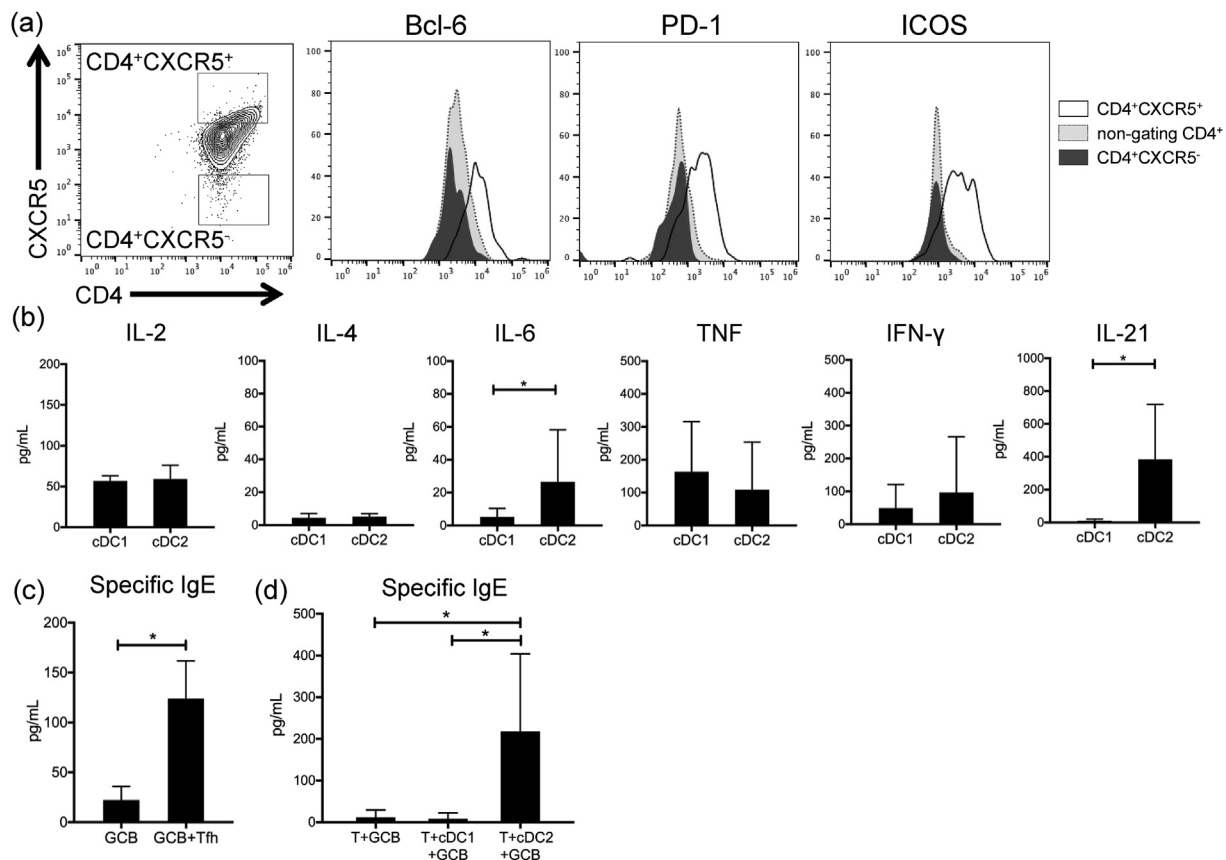


Fig. 5. Follicular helper T (Tfh) cells induced by each conventional lung dendritic cell (LDC) subset from OVA-primed mouse have distinct phenotypal and functional characteristics. The expression of the proteins B cell lymphoma 6 (Bcl-6), programmed cell death 1 (PD-1), and inducible T cell co-stimulator (ICOS) was compared among induced CD4⁺CXCR5⁺ T cells (upper gating), non-gating CD4⁺ T cells, and CD4⁺CXCR5⁻ T cells (lower gating) after co-culture with naïve CD4⁺ T cells from OVA-specific T cell receptor transgenic (OT-II) mice and conventional type 2 LDCs (cDC2) from lungs of OVA-primed mice using FACS. CD4⁺CXCR5⁺ T cells (black line, white area), non-gating CD4⁺ T cells (dot line, shaded area), and CD4⁺CXCR5⁻ T cells (black area) are shown on histograms (a). The cytokine levels in the supernatants of co-cultured with naïve CD4⁺ T cells and conventional LDC subset were measured by cytometric bead arrays and ELISA (b). Data are presented as mean $\pm$ SEM of 4 independent experiments (four OVA-primed mice and one OT-II mouse/experiment). * p < 0.05. The isolated Tfh cells from mediastinal lymph node (MLN) of OVA-primed mice were co-cultured with isolated naïve germinal center B (GCB) cells from spleen with RPMI-1640 complete medium in the presence of OVA for 7 days. OVA-specific IgE levels in the supernatant were measured using ELISA (c). The naïve CD4⁺ T cells from OT-II mice and conventional LDC subsets from lungs of OVA-primed mice were co-cultured in the presence of OVA for 7 days. Isolated naïve GCB cells from spleen were added to the culture for additional 7 days. OVA-specific IgE levels in the supernatant were measured using ELISA (d). Data are presented as mean $\pm$ SEM of 4-6 independent experiments (four OVA-primed mice, one OT-II mouse, and one C57BL/6 mouse/experiment). * p < 0.05.

showed the phenotypal and functional characteristics of typical Tfh cells.

Discussion

In the present study, we demonstrated that Tfh cells, defined by expressing CD45, CD4, and CXCR5, increased in the MLN of HDM-challenged mice, following an increase in lung cDC2 after priming. Furthermore, cDC2, but not cDC1, obtained from the lungs of antigen-primed mice, efficiently induced Tfh cell differentiation from naïve T cells *in vitro*. Indeed, the lung cDC2-induced Tfh cells expressed Bcl-6, a transcription factor of Tfh cells, and caused GCB cells to produce a large amount of antigen-specific IgE, all of which are characteristic features of typical Tfh cells. These results suggest that lung cDC2 plays an important role in the humoral immunity of asthma by inducing Tfh cells.

Tfh cells are a specialized subset of CD4⁺ T cells that promote survival, affinity maturation, and class switch recombination of B cells. Recently, growing evidence has indicated that Tfh cells are essential for eliciting Th2 immune responses, such as asthma. Coquet *et al.* reported that intranasal allergen challenge induced IL-21-producing Tfh-like cells in lung-draining lymph nodes.¹¹ In

addition, Uwadiae *et al.* demonstrated that HDM inhalation exposure induced marked accumulation of Tfh cells in the lung-draining MLN, while the number of Tfh cells in the spleen and the blood remained unchanged or increased slightly.²⁷ Consistent with these observations, the present study showed that HDM challenge significantly increased the frequency of Tfh cell in MLN, but not in spleen. MLN is a local draining lymph node in response to allergen exposure into the lung. Therefore, a Tfh cell response is thought to be induced primarily in MLN, and the response in the spleen and the blood may not, or if it does, be weak. Although Tfh cells are considered to be mainly generated from naïve CD4⁺ T cells, the precise mechanism of Tfh cell induction remains unclear, especially under pathological conditions. DCs are thought to play a critical role in the early phase of Tfh cell differentiation. However, it is unknown which DC subsets control Tfh cell differentiation. In particular, few studies have been conducted on LDC subsets that primarily induce Tfh cells in the asthmatic lung. In a recent study by Krishnaswamy *et al.*, different conventional DC subset-specific deletion mice were immunized in OVA/LPS or influenza A/PR/8/34 via the subcutaneous or intranasal route, which were then evaluated for their Tfh response and specific antibody production after immunization. The authors concluded that cDC2 was necessary and sufficient for Tfh

cell priming to inhaled antigens and specific antibody production.²⁸ Although this study did not directly demonstrate that particular subsets of LDCs are capable of efficiently inducing Tfh cells *in vitro*, these results are consistent with our observations. Extending the findings of Krishnaswamy's study, we clearly indicated that cDC2 from OVA-primed mouse lungs induced a markedly larger number of Tfh cells than cDC1 in the co-cultures with naïve CD4⁺ T cells from OT-II mouse spleens. In addition, after antigen priming, the number of cDC2, but not cDC1, increased in the lung over time, suggesting that the increased DC2 in the primed lung further augmented Tfh cell generation. Taken together, these results suggest that cDC2 in the lung is a primary DC subset responsible for Tfh cell differentiation in the asthmatic lung. On the other hand, cDC2 also induced CD4⁺ CXCR5⁺ T cells and non-gating CD4⁺ T cells (Fig. 5a). These cells did not express Bcl-6, PD-1, or ICOS. Our previous study showed that CD11b⁺ LDCs (lung cDC2) primarily induced CD4⁺ CXCR4⁺ T cells producing IL-4 and IL-10, but not IFN- γ or IL-17A in the same co-culture assay.¹⁹ Thus, the induced CD4⁺ CXCR5⁺ T cells and non-gating CD4⁺ T cells may belong to Th2 populations.

Several factors are involved in Tfh cell differentiation and maintenance, including cytokines and costimulatory molecules. Among them, IL-6 has been shown to be critical for inducing naïve CD4⁺ T cells to differentiate Tfh cells.⁴ Indeed, IL-6 induces Bcl-6 expression by activating CD4⁺ T cells at the DC priming phase, which leads to the induction of CXCR5⁺ CD4⁺ T cells for Tfh cell differentiation.^{2,29,30} We previously reported that CD11b⁺ LDCs (lung cDC2) secreted significantly higher amounts of IL-6 than CD103⁺ LDCs (lung cDC1) when stimulated with Toll-like receptor ligands (LPS, PAM₃CSK₄, and CPG-ODN) or co-stimulation molecules, such as anti-CD40 mAb.^{19,20} In the present study, we confirmed that lung cDC2 produced markedly higher amounts of IL-6 than lung cDC1 in response to HDM. In addition, neutralizing IL-6 with anti-IL-6 antibody significantly reduced the number of the induced Tfh cells in the co-cultures of lung cDC2. Collectively, these observations suggest that lung cDC2 has the ability to produce a large amount of IL-6 in response to various stimuli, which leads to more efficient Tfh cell differentiation than lung cDC1. Additionally, ICOS/ICOS-L signaling is required for the optimal differentiation and maintenance of Tfh cells.^{2,27,29–31} In humans and mouse models with ICOS deficiency, Tfh cells cannot be induced with severe impairment of GC formation.³² Moreover, the blockade of ICOS/ICOS-L signaling after allergen exposure reduces the induction of Tfh differentiation.²⁷ In the present study, we found greater ICOS-L expression in lung cDC2 than in lung cDC1. In fact, lung cDC1 had almost no ICOS-L expression. Additionally, blockade of ICOS signaling with anti-ICOS-L antibody resulted in a significant reduction of the number of the induced Tfh cells in the co-cultures of lung cDC2. Thus, the ICOS-L expression in lung cDC2 was likely to be associated with their potent capacity to induce Tfh cells. On the other hand, recent studies have shown that PD-1/PD-L1 signaling negatively regulates Tfh cell differentiation.^{2,29,30,33} The present study demonstrated that lung cDC1 showed moderate PD-L1 expression, while lung cDC2 did not. Thus, PD-L1 expression in lung cDC1 may be related to the poor inducibility of Tfh cells. Taken together, these observations suggest that several factors of lung cDC2, including high ICOS-L expression, no PD-L1 expression, and potent IL-6 producing ability, are attributable to their Tfh cell-inducing capacity.

The present study also showed that the lung cDC2-induced Tfh cells efficiently elicited antigen-specific IgE production from GCB cells *in vitro* with abundant amounts of IL-21 in the co-cultures. IL-21 is a Tfh cell-derived cytokine that is important for Tfh cell maintenance as well as B cell differentiation.^{34,35} In particular, IL-21 produced by Tfh cells is critical for the initiation and maintenance

of GC and extra-follicular antibody responses.^{29,34,35} Thus, our observations suggest that the lung cDC2-induced Tfh cells have the capacity to produce high levels of IL-21, which contributes to maintaining Tfh cells, as well as producing antigen-specific immunoglobulins from GCB cells.

In conclusion, the present study demonstrated that lung cDC2, but not lung cDC1, efficiently induced Tfh cells in the asthmatic lung, and the induced Tfh cells could support the production of antigen-specific immunoglobulins from GCB cells. Our findings suggest that targeting lung cDC2 is a promising approach to regulate antibody response through Tfh cell differentiation in asthma.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.alit.2021.01.008>.

Conflict of interest

The authors have no conflict of interest to declare.

Authors' contributions

SS and KF designed the study, performed the experimental work, and collected the data. RH and FN contributed to the laboratory work. All authors performed data analysis and interpretation of the results. SS, KF, and TS wrote the manuscript. All authors read and approved the final manuscript.

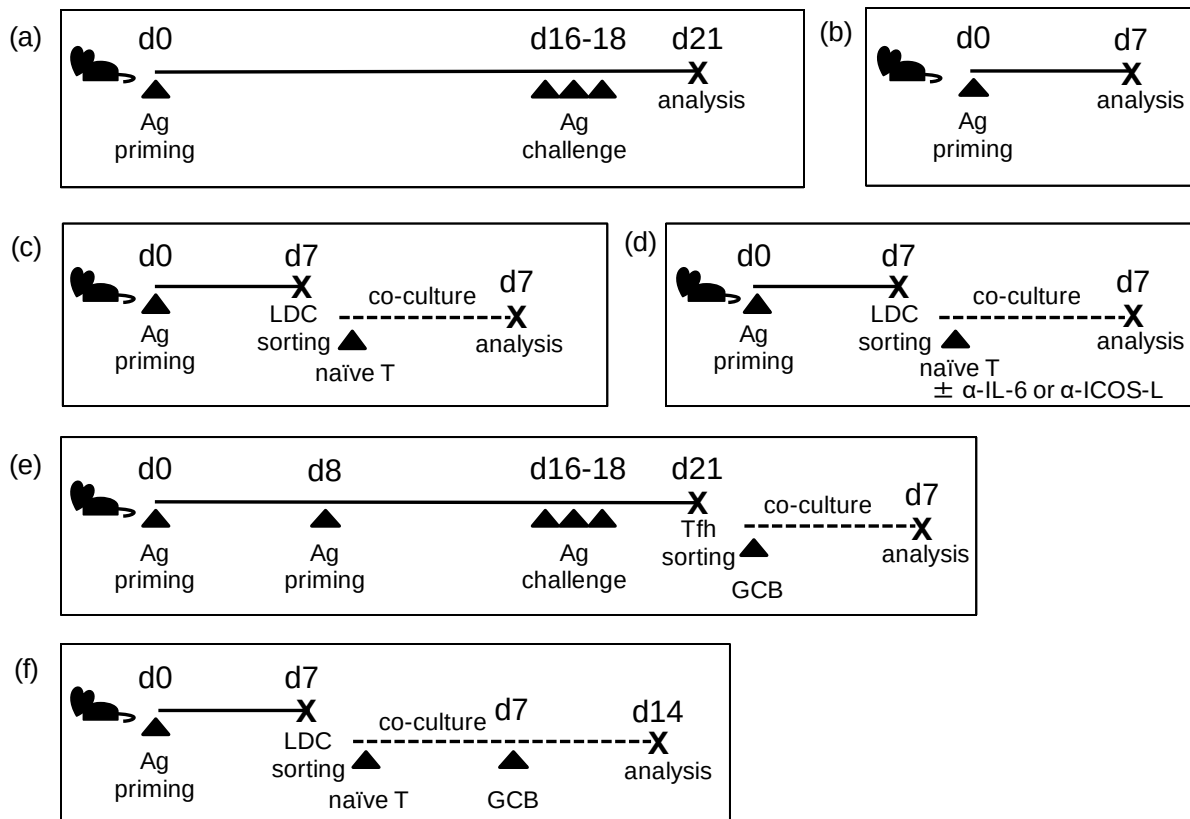
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Supplementary file

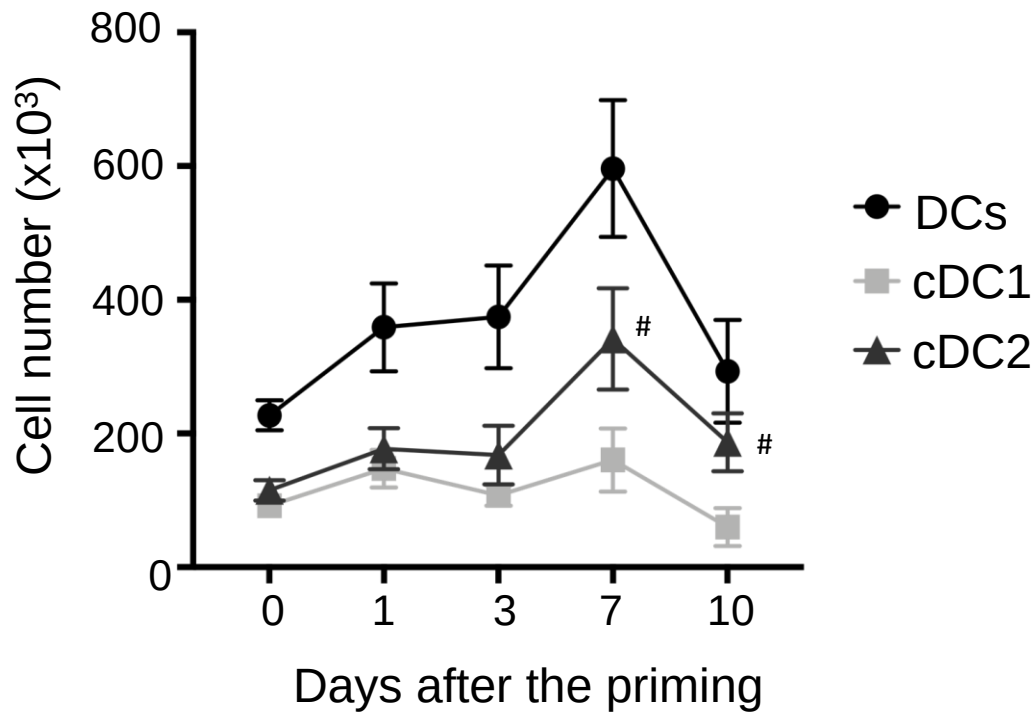
Supplementary Figure 1.



Supplementary Figure 1. Schematic protocols of asthma mouse model (priming and challenge) and *in vitro* co-culture experiments. In asthma mouse model, C57BL/6 mice were intranasally primed with 50 μ g of HDM extract in 25 μ L of PBS on day 0, and subsequently challenged with 10 μ g of HDM extract in 25 μ L of PBS on days 16-18. Mice were sacrificed on day 21 (a). Conventional lung dendritic cell (LDC) subsets were isolated from the lungs of C57BL/6 mice 7 days after HDM priming and analyzed (b). Conventional LDC subsets were isolated from the lungs of C57BL/6 mice 7 days after OVA priming. Naïve CD4⁺ T cells from the spleen of OVA-specific T cell receptor transgenic (OT-II) mice were co-cultured with

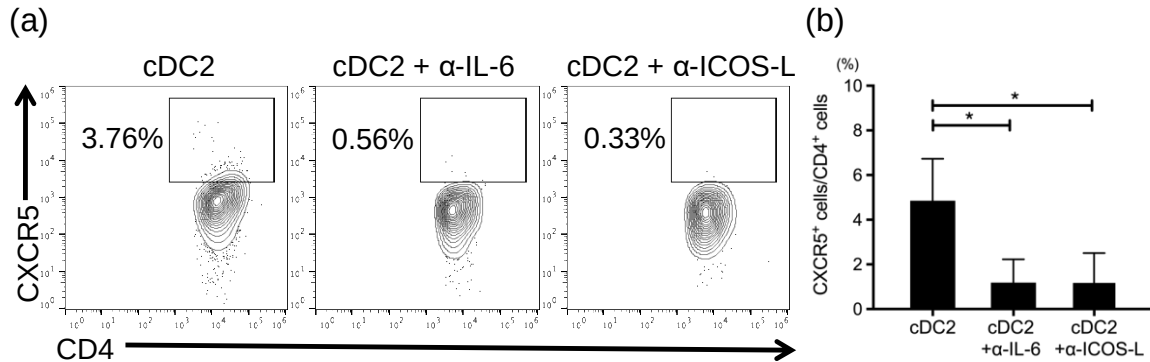
isolated conventional LDC subsets from the lungs of OVA-primed mice in the presence of 10 µg/mL OVA peptide for 7 days (c). Naïve CD4⁺ T cells were co-cultured with conventional type 2 lung dendritic cells (cDC2) as described above, and 10 µg/mL anti-IL-6 or 10 µg/mL anti-ICOS-L antibody was added into the culture for 7 days (d). C57BL/6 mice were immunized with 50 µg of OVA and 2 mg of alum adjuvant dissolved in 200 µL of PBS via intraperitoneal injection on days 0 and 8, and subsequently intranasally challenged with 50 µg of OVA in 25 µL of PBS on days 16–18. Purified follicular helper T (Tfh) cells isolated from mediastinal lymph nodes (MLNs) of the OVA-asthma mouse model were co-cultured with naïve germinal center B (GCB) cells from spleen of C57BL/6 mice in the presence of 10 µg/mL OVA for 7 days (e). Conventional LDC subsets were isolated from the lungs of C57BL/6 mice 7 days after OVA priming. Naïve CD4⁺ T cells from the spleen of OT-II mice were co-cultured with isolated conventional LDC subsets from the lungs of OVA-primed mice in the presence of 10 µg/mL OVA for 7 days, and subsequently naïve GCB cells were added to the culture for an additional 7 days (f). The culture supernatants were collected and OVA-specific IgE levels were measured. Ag, allergen; LDC, lung dendritic cell; naïve T, naïve CD4⁺ T cells; cDC2, conventional type 2 lung dendritic cells; α-IL-6, anti-IL-6 antibody; α-ICOS-L, anti-ICOS-L antibody; Tfh, follicular helper T cells; MLNs, mediastinal lymph nodes; GCB, germinal center B cells.

Supplementary Figure 2.



Supplementary Figure 2. The cell number of total lung dendritic cells (LDC) and two conventional LDC subtypes after OVA priming. Circles, total LDC; squares, conventional type 1 LDC (cDC1); and triangles, conventional type 2 LDC (cDC2). Data are presented as the mean $\pm$ SEM of over five independent experiments (four mice/group/experiment). Statistical analysis was performed at each time point. [#] $p < 0.05$: cDC1 vs. cDC2.

Supplementary Figure 3.



Supplementary Figure 3. The induction of follicular helper T (Tfh) cells in Conventional type 2 lung dendritic cells (cDC2) is inhibited by the addition of anti-IL-6 and anti-ICOSL antibodies. Naïve CD4⁺ T cells from OVA-specific T cell receptor transgenic (OT-II) mice were co-cultured with cDC2 from lungs of OVA-primed mice for 7 days. Representative Tfh cells gating of induced CD4⁺ T cells after co-cultured are shown (a). Numbers indicate the percentage of gated cells in the panel. The percentage of induced CXCR5 positive cells on CD4⁺ T cells were evaluated in percentage graphs (b). Data are presented as the mean $\pm$ SEM of 4 independent experiments (four OVA-primed mice and one OT-II mouse/experiment). * $p < 0.05$. cDC2, conventional type 2 lung dendritic cells; α -IL-6, anti-IL-6 antibody; α -ICOS-L, anti-ICOS-L antibody.

Supplementary Table 1. FACS antibodies used in this study

Target	Fluorochrome	Clone	Supplier	Concentration
B220	BV510	RA3-6B2	BioLegend	1:200
BCL-6	PE	IG191E/A8	BioLegend	1:125
CD3	PE/Cy7	17A2	BioLegend	1:500
CD3	PerCP/Cy5.5	17A2	BioLegend	1:250
CD3e	PE	145-2C11	eBioscience	1:200
CD4	AF700	GK1.5	BioLegend	1:500
CD4	APC	RB6-8C5	BioLegend	1:500
CD4	BV421	GK1.5	BioLegend	1:500
CD8 α	APC/Cy7	53-6.7	BioLegend	1:500
CD11b	BV421	M1/70	BioLegend	1:500
CD11c	APC/Cy7	N418	BioLegend	1:500
CD11c	FITC	N418	BioLegend	1:500
CD19	APC	6D5	BioLegend	1:200
CD19	FITC	MB19-1	BioLegend	1:500
CD19	PerCP/Cy5.5	6D5	BioLegend	1:250
CD38	Alexa700	90	eBioscience	1:200
CD40	BV421	3/23	BioLegend	1:250
CD45	BV510	30-F11	BioLegend	1:500
CD45	FITC	30-F11	BioLegend	1:500
CD45R	PerCP/Cy5.5	RA3-6B2	BioLegend	1:250
CD64	PE/Cy7	X54-5/7.1	BioLegend	1:250
CD80	BV421	16-10A1	BioLegend	1:250
CD86	BV421	GL-1	BioLegend	1:250
CD161	PerCP/Cy5.5	PK136	BioLegend	1:250
CD172a	APC	P84	BioLegend	1:150
CXCR5	PE/Cy7	L138D7	BioLegend	1:250
F4/80	APC/Cy7	BM8	BioLegend	1:250
FAS	PERCP-Cy5.5	15A7	BioLegend	1:100
GL7	FITC	GL7	BioLegend	1:100
Gr-1	APC	RB6-8C5	BioLegend	1:500
ICOS	BV510	C398.4A	BioLegend	1:250
ICOS-L	biotin	HK5.3	BioLegend	1:250
IgD	Pacific Blue	11-26c.2a	BioLegend	1:100
IgM	APC/CY7	RMM-1	BioLegend	1:50
IRF4	PE	IRF4.3E4	BioLegend	1:150
IRF8	PE	V3GYWCH	Thermo Fisher	1:150
Ly-6G	PE	RB6-8C5	BD Pharmingen	1:500
MHCII	Alexa Fluor 700	M5/114.15.2	BioLegend	1:500
PD-1	APC	RMP1-30	BioLegend	1:125
PD-L1	BV421	10F.9G2	BioLegend	1:250
PD-L2	BV421	TY25	BioLegend	1:250
Siglec-F	PE	E50-2440	BD Pharmingen	1:500
Straptavidin	BV421	-	BioLegend	1:250
Va2	FITC	B20.1	BD Pharmingen	1:500
Vb5	PE	MR9-4	BD Pharmingen	1:500
XCR1	PE	ZET	BioLegend	1:150