

ORIGINAL ARTICLE

Basic Mechanisms in Allergic Disease

Sex-based differences in CD103⁺ dendritic cells promote female-predominant Th2 cytokine production during allergic asthma

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Summary

Background: Gender disparities in adult patients with asthma regarding its prevalence and severity are mainly due to enhanced type 2 T-helper (Th2) cytokine production in female patients compared to that in male patients. However, the pathways mediating this effect remain unclear.

Objective: We aimed to determine the roles of two major subsets of dendritic cells (DCs) in females, specifically those displaying CD11b or CD103, during enhanced Th2 priming after allergen exposure, using an ovalbumin-induced asthma mouse model.

Methods: Sex-based differences in the number of DCs at inflamed sites, costimulatory molecule expression on DCs, and the ability of DCs to differentiate naïve CD4⁺ T cells into Th2 population were evaluated after allergen exposure in asthmatic mice. In addition, we assessed the role of 17 β -oestradiol in CD103⁺ DC function during Th2 priming in vitro.

Results: The number of CD11b^{high} DCs and CD103⁺ DCs in the lung and bronchial lymph node (BLN) was increased to a greater extent in female mice than in male mice at 16 to 20 hours after ovalbumin (OVA) inhalation. In BLNs, CD86 and I-A/I-E expression levels and antigen uptake ability in CD103⁺ DCs, but not in CD11b^{high} DCs, were greater in female mice than in male mice. Furthermore, CD4⁺ T cells cultured with CD103⁺ DCs from female mice produced higher levels of interleukin (IL)-4, IL-5, and IL-13, compared with CD4⁺ T cells cultured with CD103⁺ DCs from male mice. The 17 β -oestradiol-oriented enhancement of CD86 expression on CD103⁺ DCs after allergen exposure induced the enhanced IL-5 production from CD4⁺ T cells.

Conclusions and Clinical Relevance: These findings suggest that with regard to asthma, enhanced Th2 cytokine production in females might be attributed to 17 β -oestradiol-mediated Th2-oriented CD103⁺ DCs in the BLN.

KEYWORDS

bronchial asthma, CD103⁺ dendritic cells, oestradiol, sex-based differences, Th2

1 | INTRODUCTION

In medicine, sex has been recognized as a biological characteristic that defines cellular, molecular, and immunological differences, and is an important modulator of disease severity and response to therapeutic intervention.^{1,2} Additionally, sex is deeply associated with the prevalence and severity of asthma, effectiveness of asthma treatment and adverse reactions to asthma treatment.³ The relapse rate after discharge from the emergency department is evidently higher for female patients than for male patients.⁴ Moreover, sex was found to be significantly associated with bronchial hyperresponsiveness in systemic inflammatory conditions.⁵ Meanwhile, the prevalence and severity of asthma are greater in boys than in girls; however, this difference is reversed after puberty due to age-related changes, particularly, alterations in sex hormone levels.⁶⁻⁸ In addition, postmenopausal use of oestrogen alone was associated with an increased rate of newly diagnosed asthma in menopausal women.^{9,10} Gender-based epidemiological differences⁴⁻⁸ are frequently associated with the specific overproduction of type 2 T-helper (Th2) cytokines in females. Actually, more interleukin (IL)-13-producing T cells are observed in female patients with atopic asthma than in male patients.¹¹ Thus, increasing evidence has indicated that symptom-based features of asthma in female patients are based on the differences in pathogenic features, including enhanced Th2-type immune responses.¹²

Animal studies have verified the influence of sex on the pathogenesis of asthma. Using an ovalbumin (OVA)-induced asthma mouse model, an increased number of CD4⁺ T cells and eosinophils, higher Th2 cytokine content in broncho-alveolar lavage (BAL) fluid and higher Th2 cytokine production by the spleen and bronchial lymph node (BLN) cells were noted in female asthmatic mice compared to those in male asthmatic mice.¹³⁻¹⁵ Furthermore, ovariectomy before OVA inhalation significantly inhibited lung eosinophil infiltration. Moreover, oestradiol treatment in ovariectomized mice partially restored the number of eosinophils in the BAL fluids of asthmatic mice.¹⁶

Dendritic cells (DCs), the most proficient antigen-presenting cells, bridge the gap between innate and adaptive immune responses by priming Th cells, including Th1, Th2, and Th17 cells, to respiratory allergens, which is a critical step for the development and exacerbation of allergic asthma.¹⁷ In patients with asthma, circulating myeloid DCs extravasate from the circulation within 4-5 hours after allergen inhalation to the bronchial wall during the first 24 hours,^{18,19} facilitating acquired immunity to the inhaled allergen. In humans, BDCA1⁺ myeloid (m) DCs accumulate to a greater extent in the airway epithelia of patients with Th2-high asthma, compared to that in patients with Th2-low asthma or healthy controls.²⁰ Mature BDCA1⁺ DCs from patients with atopic dermatitis induce fewer interferon (IFN)- γ -producing and more IL-4-producing helper T cells, compared with DCs from healthy controls.²¹ In patients with allergic rhinitis, local allergen challenge resulted in an increase in mDC and plasmacytoid DC numbers in the nasal mucosa and skin.²² Meanwhile,

allergen challenge increases the BDCA3 on mDC levels in the sputum of subjects with asthma, and the frequency of BDCA3⁺ mDCs and extent of allergy to house dust mites are reportedly positively correlated,²³ suggesting both BDCA1⁺ and BDCA3⁺ DC subsets are involved in asthmatic immune responses. However, sex-related differences in DC subsets responsible for enhanced-Th2 cytokine production in female patients with asthma remain unclear.

Recent studies in mice have shown that the CD11b⁺ CD103⁻ (CD11b⁺) and CD11b⁻ CD103⁺ (CD103⁺) DCs, homologs of human CD1c (BDCA1)⁺ and CD141 (BDCA3)⁺ DCs, respectively, play crucial roles in priming the Th2 response after allergen encounter.²⁴⁻²⁶ Upon allergen inhalation, conditional depletion of total DCs abolished Th2 cytokine production by lymph node cells and accumulation of airway eosinophils in a murine asthma model.²⁷ Moreover, the selective depletion of either CD11b⁺ DCs or CD103⁺ DCs significantly reduced these asthmatic responses.^{28,29} Therefore, both DC subsets are required to induce a complete immune response to inhaled allergen.

In this study, we aimed to clarify the role of DCs in sex-related enhanced Th2 cytokine production upon allergen inhalation using a mouse model.

2 | MATERIALS AND METHODS

2.1 | Mice

Male and female C57BL/6 (wild-type; WT) mice, and OT-I and OT-II transgenic (Tg) mice were obtained from CLEA Japan (Osaka, Japan) and the Jackson Laboratory (Bar Harbor, ME, USA), respectively. All mice were maintained under specific pathogen-free conditions at the Institute for Animal Experimentation, Tohoku Medical and Pharmaceutical University (Sendai, Japan). All experimental procedures involving animals were approved by the Committee of Animal Experiments at Tohoku Medical and Pharmaceutical University (approval numbers: 14002-cn, 15001-cn, and 16002-cn). We took utmost care to alleviate any pain and suffering of the mice.

2.2 | Sensitization and antigen challenge

Mice, 6-8 weeks old, were sensitized through intraperitoneal injection of 8 μ g OVA (Grade V; Sigma-Aldrich, St Louis, MO, USA) adsorbed with 4 mg aluminium hydroxide (Wako Pure Chemical Industries, Osaka, Japan) in 500 μ L saline, on days 0 and 5. On day 26, mice were challenged with aerosolized OVA (0.5% in saline) for 1 hour on two occasions 4 hour apart.^{15,30}

2.3 | Preparation of the lung white blood cells

Mice were sacrificed before or at various time intervals after OVA challenge. Pulmonary leucocytes were prepared as previously described.³¹ Briefly, the lung vascular bed was flushed by injecting 5 mL chilled saline into the right ventricle. The entire lungs were

teased with a 40- μ m cell strainer (BD Falcon, Bedford, MA, USA) and incubated in RPMI 1640 medium (Nakarai-Tesque, Kyoto, Japan) with 10% foetal calf serum (FCS; Thermo Fisher Scientific, Waltham, MA, USA), 100 U/mL penicillin G, 100 μ g/mL streptomycin, 10 mmol/L HEPES and 2 mmol/L L-glutamine, containing 20 U/mL collagenase D and 1 μ g/mL DNase I (Roche Diagnostics GmbH, Mannheim, Germany). After incubation for 60 minutes at 37°C with vigorous shaking, cells were resuspended in 4 mL 40% (v/v) Percoll (Pharmacia, Uppsala, Sweden) and layered onto 4 mL 80% (v/v) Percoll. After centrifugation at 600 $\times$ g for 20 minutes at 15°C, cells at the interface were collected. For some experiments, the cells were centrifuged and mounted onto a glass slide, stained with Diff-Quick (Sysmex Corp., Kobe, Japan), and then observed under a microscope. The number of leucocyte fractions was estimated by multiplying the total leucocyte number by the proportion of each fraction, for 200 cells.

2.4 | Preparation of the BAL fluids

BAL fluids were prepared as previously described.¹⁵ Briefly, BAL samples collected on day 3 or 5 after OVA inhalation with 2 $\times$ 0.25 mL chilled PBS through a cannula inserted in the trachea were centrifuged at 450 $\times$ g for 10 minutes at 4°C, and the supernatants were stored at -80°C for the cytokine assay. Cells (2 $\times$ 10⁵) were stained with Diff-Quick solution, and cell differential percentage was determined by counting a minimum of 200 cells under light microscopy. The number of effector Th2 cells in BAL fluids was determined by flow cytometry.

2.5 | Preparation of DCs

Entire lungs and BLNs were extracted from sensitized and challenged WT mice 16 or 20 hours after OVA inhalation, respectively. Pulmonary leucocytes and BLN cells were prepared as previously described.¹⁴ The number of each DC subset per lung and BLN was calculated based on the total number of leucocytes, and percentage of each DC subset in those populations was estimated using a BD ARIAII flow cytometer (BD Biosciences, San Jose, CA, USA). In another experiment, CD11c⁺ cells were enriched using an autoMACS Separator (Miltenyi Biotec, Bergish Gladbach, Germany) and anti-mouse Pan DC MicroBeads (Miltenyi Biotec). SSC^{low} CD11c^{hi} I-A/I-E^{hi} CD11b^{hi} CD103⁻ DCs and SSC^{low} CD11c^{hi} I-A/I-E^{hi} CD103⁺ DCs were purified by cell sorting using a BD ARIAII flow cytometer. The proportion of CD11c^{hi} I-A/I-E^{hi} cells in the sorted cells was consistently 95%-98%. Furthermore, the proportion of CD11b^{hi} and CD103⁺ cells in DCs was consistently 96%-98%. Following centrifugation, morphologies were assessed using a microscope by mounting cells onto a glass slide and subsequent staining with Diff-Quick. To assess antigen uptake ability of lung DCs and migration into the BLN, Alexa Fluor 647-conjugated OVA (100 μ g; Thermo Fisher Scientific) and LPS (0.1 μ g; Sigma-Aldrich) or non-labelled endotoxin-free OVA (Hyglos GmbH, Bernried am Starnberger See, Germany) and LPS in 50 μ L saline were injected intratracheally into sensitized WT mice.

2.6 | Preparation of the lung and BLN homogenate

For RT-PCR, the entire lungs were excised from WT mice before and 3 and 16 hours after OVA challenge. BLNs were excised from WT mice before and 16 and 20 hours after OVA inhalation. These tissues were homogenized in buffer RLT (Qiagen, Valencia, CA, USA) supplemented with 1% 2-mercaptoethanol. For cytokine assay, the entire lungs were excised 1 day after OVA inhalation and homogenized in chilled 0.1% Triton-X PBS with 1% protease inhibitor (Sigma-Aldrich). After centrifugation at 15 000 $\times$ g for 15 minutes at 4°C, the supernatants were stored at -80°C.¹⁵

2.7 | Culture of DCs with T cells

CD4⁺ T cells or CD8⁺ T cells were isolated from the spleen cell population of naïve male and female OT-II or OT-I Tg mice using an auto MACS Separator (Miltenyi Biotec) and anti-mouse CD4 or CD8 Micro Beads (Miltenyi Biotec), respectively. Furthermore, CD3⁺ CD4⁺ CD11c⁻ and CD3⁺ CD8⁺ CD11c⁻ cells were purified from CD4⁺ and CD8⁺ T cell population by cell sorting using a BD ARIAII flow cytometer. The proportion of CD4⁺ T cells or CD8⁺ T cells in the sorted cell population was >99%. For the culture experiment of DCs with T cells, T cell population was prepared as a mixture of T cells from both male and female mice at the ratio of 1:1 to exclude the influence of sex-related differences in the ability of T cells, based on our previous observation that the potential of CD4⁺ T cells to produce Th2 cytokine is different between male and female mice.³² CD4⁺ T cells from OT-II mice or CD8⁺ T cells from OT-I mice were cultured with each DC subset at a ratio of 3:1 in the presence of OVA₃₂₃₋₃₃₉ or OVA₂₅₇₋₂₆₄ peptide, respectively, for 4 days in RPMI 1640 medium supplemented with 10% FCS, 100 U/mL penicillin G, 100 μ g/mL streptomycin, 2 mmol/L L-glutamine, and 50 μ mol/L 2-mercaptoethanol. Following primary culture, T cells were further cultured in microwells coated with 1 μ g/mL purified anti-CD3 ϵ (Clone 145-2C11; BioLegend, San Diego, CA, USA) and 5 μ g/mL anti-CD28 (Clone 37.51; BioLegend). For some experiments, CD4⁺ T cells were labelled with 1 μ mol/L 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE; Dojindo Laboratories, Kumamoto, Japan) for 10 minutes before coculture with CD103⁺ DCs for 2 or 4 days.

2.8 | CD103⁺ bone marrow-derived DCs

CD103⁺ bone marrow-derived DCs (BM-DCs) were prepared as described previously by Mayer et al.³³ In brief, BM-derived cells from WT male mice were cultured at a density of 1.5 $\times$ 10⁶ cells/mL in 10 mL RPMI 1640 medium without phenol red supplemented with 10% FCS, 100 U/mL penicillin G, 100 μ g/mL streptomycin, 2 mmol/L L-glutamine, and 50 μ mol/L 2-mercaptoethanol, and containing 3 ng/mL murine granulocyte-macrophage colony-stimulating factor (GM-CSF; Wako pure Chemical Industries, Ltd., Osaka, Japan), and 200 ng/mL murine FMS-like tyrosine kinase 3 ligand (Flt3L; PeproTech Inc., Rocky Hill, NJ, USA) in the presence of vehicle, 17 β -oestradiol (1 $\times$ 10⁻⁷ M; Sigma-Aldrich), or 17 β -oestradiol

plus ICI182,780 (Tocris Bioscience, Ellisville, MO, USA). ICI182,780 is an oestrogen receptor antagonist. On day 9, the non-adherent cells were harvested and replated at a density of 3×10^6 cells/mL in 10 mL complete medium supplemented with GM-CSF and Flt3L in the presence of the vehicle, 17 β -oestradiol, or 17 β -oestradiol plus ICI182,780, as on day 0. On day 15, non-adherent cells were collected and used as CD103⁺ BM-DCs. CD103⁺ BM-DCs, at a density of 5×10^5 cells/mL, were stimulated for 24 hours with OVA and/or murine recombinant IL-33 (R&D Systems, Inc., Minneapolis, MN, USA) in the presence of vehicle, 17 β -oestradiol, or 17 β -oestradiol plus ICI182,780. For some experiments, 0.25 μ g/mL recombinant anti-B7-2/CD86 antibody (R&D Systems, Inc.) or isotype-matched control antibody was used to block CD86/CD28 costimulatory signals.

2.9 | Measurement of cytokine and chemokine concentration

Levels of IL-4, IL-5, IL-13, IL-17A, IL-33, and IFN- γ were assayed using enzyme-linked immunosorbent assay (ELISA) kits (eBioscience, San Diego, CA, USA). The detection limits were 4 pg/mL for IL-4, IL-5, IL-13, and IL-17A; 25 pg/mL for IL-33; and 15 pg/mL for IFN- γ . The CCL20 content in the lung homogenate was measured using ELISA kit (R&D Systems, Inc.). The limit of detection was 0.30 pg/mL. Total protein levels of the lung homogenates were assayed using a detergent-compatible protein assay kit (Bio-Rad Laboratory, Hercules, CA, USA). The cytokine and chemokine concentrations in the lung were adjusted for the protein levels of each lung.¹⁵

2.10 | Flow cytometric analysis

Cells were diluted to attain a density of 1×10^6 /100 μ L, and pre-incubated with anti-Fc γ RII and III mAb (Clone 93; BioLegend) on ice for 15 minutes in PBS containing 1% FCS and 0.1% sodium azide. To identify DC subsets, the lung cells and BLN cells were stained with FITC-conjugated anti-I-A/I-E (Clone M5/114.15.2; BioLegend), PE-conjugated anti-CD11c (Clone N418; BioLegend), PerCP-conjugated anti-CD11b (Clone M1/70; BioLegend), and PE/Cy7-conjugated anti-CD103 (Clone 2E7; BioLegend). In addition, to evaluate surface molecule expression levels on DCs, APC-conjugated anti-CD40 (Clone 3/23; BioLegend), CD80 (Clone 16-10A1; BioLegend), CD86 (Clone GL-1; BioLegend), or Alexa Fluor 700-conjugated anti-CCR7 (Clone 4B12; eBioscience, San Diego, CA, USA) were used. In another experiment, to evaluate ST2 expression levels on DCs, they were stained with FITC-conjugated anti-ST2 (Clone DJ8; MD Biosciences, Oakdale, MN, USA), PE-conjugated anti-CD11c, PerCP-conjugated anti-CD11b, PE/Cy7-conjugated anti-CD103, and APC/Cy7-conjugated anti-I-A/I-E (Clone M5/114.15.2; BioLegend). In the in vitro experiments, to evaluate CD86 and I-A/I-E expression levels on CD103⁺ BM-DCs, they were stained with FITC-conjugated anti-I-A/I-E and APC-conjugated anti-CD86. Furthermore, to isolate CD4⁺ T cells used in coculture experiments, the spleen cells from OT-II Tg mice were stained with FITC-conjugated anti-CD4 (Clone GK1.5;

BioLegend), PE-conjugated anti-CD11c, and PerCP-conjugated anti-CD3 ϵ (Clone 145-2C11; BioLegend). In the isolation of CD4⁺ T cells for division-tracking experiments with CFSE, the spleen cells from OT-II Tg mice were stained with PE-conjugated anti-CD11c, APC/Cy7-conjugated anti-CD4 (Clone GK1.5; BioLegend), and PerCP-conjugated anti-CD3 ϵ or APC-conjugated anti-CD3 ϵ (Clone 145-2C11; BioLegend). In addition, to isolate CD8⁺ T cells for coculture experiments, the spleen cells from OT-I Tg mice were stained with FITC-conjugated anti-CD8 α (Clone 53-6.7; BioLegend), PE-conjugated anti-CD11c, and PerCP-conjugated anti-CD3 ϵ . Furthermore, to evaluate ST2 expression on BLN CD4⁺ T cells after the culture with CD103⁺ BM-DCs, cells were stained with FITC-conjugated anti-ST2 and APC-conjugated anti-CD3 ϵ . Cells in the BAL fluid were stained with FITC-conjugated anti-CD3 ϵ (Clone 145-2C11; BioLegend) or APC/Cy7-conjugated anti-CD3 (Clone 17A2; BioLegend), PE-conjugated anti-CD44 (Clone IM7; BioLegend), PerCP-conjugated anti-CD62L (Clone MEL-14; BioLegend), APC-conjugated anti-ST2 (Clone DIH9; BioLegend), and Alexa Fluor 700-conjugated anti-CD4 (Clone RM4-5; BD Biosciences) to identify effector Th2 cells. The positive populations or mean values of the fluorescence intensity are defined based on isotype-matched control IgG for each Ab or fluorescence minus one (FMO) controls. To distinguish false-positive signals from real positive ones, delta mean fluorescence intensity (MFI), defined as the ratio of MFI of sample histogram to MFI of control histogram, was used in evaluation of the results in some experiments. Dead cells, stained with the LIVE/DEAD fixable blue dead cell stain kit (Thermo Fisher Scientific), were excluded from analysis. Data showed that 95%-99% of the lung leucocytes were viable. Data from these studies were analysed using Diva (BD Biosciences) and FlowJo (Tree Star Inc., Ashland, OR, USA) software. The number of DCs was estimated by multiplying the leucocyte number, calculated as mentioned previously, by the proportion of each subset. However, the interpretation of flow cytometry data presents limitations owing to various factors, such as the types of antibodies included and the gating strategy.

2.11 | Real-time RT-PCR analysis

Total RNA was extracted from DCs, entire lung homogenates, and BLN homogenates using a ReliaPrep RNA cell miniprep system (Promega Corporation, WI, USA) or an RNeasy micro kit (Qiagen, Valencia, CA, USA). First-strand cDNA was synthesized using the PrimeScript RT reagent kit with gDNA Eraser (TaKaRa Bio Inc., Otsu, Japan). Real-time reverse transcription (RT)-PCR was performed using gene-specific primers and Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and a Step One Plus Real-Time PCR system (Applied Biosystems).¹⁵ The primer sequences used for amplification are shown in Table S1. The expression levels of target genes and hypoxanthine-guanine phosphoribosyltransferase (Hprt) as a reference gene were calculated for each sample using the reaction efficiency, as determined by performing amplifications using standards.

2.12 | Confocal microscopic analysis

DCs were prepared from the BLN of male and female mice sensitized with non-labelled OVA and then intratracheally administered with Alexa Fluor 647-conjugated OVA. Cells were stained with Cell Mask green plasma membrane stain (Thermo Fisher Scientific) and SYTO 82 orange fluorescent nucleic acid stain (Thermo Fisher Scientific) according to manufacturer's instructions. After incubation, the cells were diluted to a density of $1 \times 10^5/200 \mu\text{L}$ in Opti-MEM (Thermo Fisher Scientific) containing 10% FCS, 0.1% sodium azide, and 10 mmol/L HEPES and then seeded in 35-mm glass bottom dishes (Matsunami Glass Ind., Ltd, Osaka, Japan). Confocal studies were performed using an oil immersion objective ($60 \times$ UPLSAPO, numerical aperture 1.35) and Fluoview FV1000 confocal laser scanning microscopy (Olympus, Tokyo, Japan). FV1000 software version 4.2 (Olympus) was used to acquire and process the confocal images. Tri-colour images were acquired using a sequential acquisition mode to avoid cross-excitation.

2.13 | Measurement of airway responsiveness

Airway responsiveness was measured as previously described.^{34,35} In brief, to measure airway responsiveness, lung resistance at the baseline and in response to aerosolized saline and acetyl- β -methylcholine (methacholine; Sigma-Aldrich) was measured for 3 minutes under each condition using Buxco whole body plethysmography (Buxco Electronics, Sharon, CT, USA).

2.14 | Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA). Differences between the two groups were tested using a two-tailed analysis and an unpaired Student's *t* test. Differences among the three groups or more were tested using ANOVA with a post hoc analysis (Turkey's multiple comparison test). A *P*-value of $<.05$ was considered significant.

3 | RESULTS

3.1 | Sex-based differences in the accumulation of the lung DCs

To investigate sex-associated differences during the initial pulmonary cell responses within 24 hours of allergen inhalation in asthmatic mice, the number of lung inflammatory cells between male and female mice was compared. The number of total cells and mononuclear cells, such as monocytes, macrophages, and DCs, in the lungs 16 hours after OVA inhalation was higher in female mice than in male mice (Figure 1A,B). A similar tendency was also observed with respect to the number of eosinophils and lymphocytes, although the differences between male and female mice did not reach significance at the time points examined (Figure 1B). Meanwhile, the number of lung leucocytes 20 hours after OVA inhalation was not significantly different between male and female mice. Furthermore, the number

of total cells 24 hours after OVA inhalation in female mice was lower than that in male mice, although there was no statistical significance (Figure 1A). In this model, increased accumulation of eosinophils (Figure 1C) and effector Th2 cells (Figure 1D) in the BAL fluids on day 5, and higher IL-4, IL-5, and IL-13 production (Figure 1E) in the BAL fluids on day 3 post-OVA inhalation were observed in female mice compared with those in male mice. In addition, the lung resistance in response to inhaled methacholine increased in a dose-dependent manner in both male and female mice. Moreover, the lung resistance was significantly enhanced in female mice compared to that in male mice, 1 day after OVA inhalation (Figure 1F).

Next, to address sex-specific differences in the number of DCs in response to inhaled allergens, two major DC subsets from alveolar macrophages (CD11c^{hi} I-A/I-E^{low} SSC^{hi} cells) and monocytes (CD11c^+ I-A/I-E⁺ cells) were distinguished using internal complexity [side scatter (SSC) parameters], and the expression levels of I-A/I-E on CD11c^+ cells, followed by the expression levels of CD11b and CD103, were determined (Figure 2A). The number of total DCs and CD11b^{hi} DCs in male mice significantly increased 20 hours after allergen inhalation and was maintained up to 24 hours after inhalation, whereas the number of these cells in female mice was significantly elevated, peaked 20 hours after allergen inhalation, and decreased thereafter. The number of total DCs and CD11b^{hi} DCs was significantly higher in female mice than in male mice at 16 hours, but was not significantly different at 20 and 24 hours post-OVA inhalation. In contrast, the number of CD103^+ DCs in the lung was not significantly changed within 24 hours of OVA inhalation in both male and female mice, although the number at 16 hours post-OVA inhalation was significantly higher in female mice than in male mice (Figure 2B,C). These results suggest that initial pulmonary cell responses after allergen exposure, particularly, the increase in DCs occurring within 20 hours after allergen inhalation, are enhanced in the lungs of female mice compared to that in male mice.

To further address this possibility, we next examined the mRNA expression of CCL2, CCL7, CCL19, CCL20, and CCL21 in the lung, which attract monocytes, immature DCs, and precursors of DCs to the inflammatory sites.³⁶ As shown in Figure 3A, CCL20 mRNA levels in the lung at 3 and 16 hours post-OVA inhalation were significantly higher in female mice than in male mice, although other chemokine levels in the lung were not different between the sexes. Furthermore, mRNA expression of Flt3L and IL-33, cytokines that are associated with DC differentiation and activation in the lung during an asthma attack,^{24,37} was significantly increased in the lung of female mice compared to that in male mice at 3 or 16 hours post-OVA inhalation (Figure 3B). In addition, CCL20 and IL-33 protein levels in the lung at 16 hours post-OVA inhalation were also significantly higher in female mice than in male mice (Figure 3C).

3.2 | Sex-based differences in DC migration into BLN

To define sex-associated differences in the number of DCs in the BLN after allergen inhalation, the number of CD11b^{hi} and CD103^+

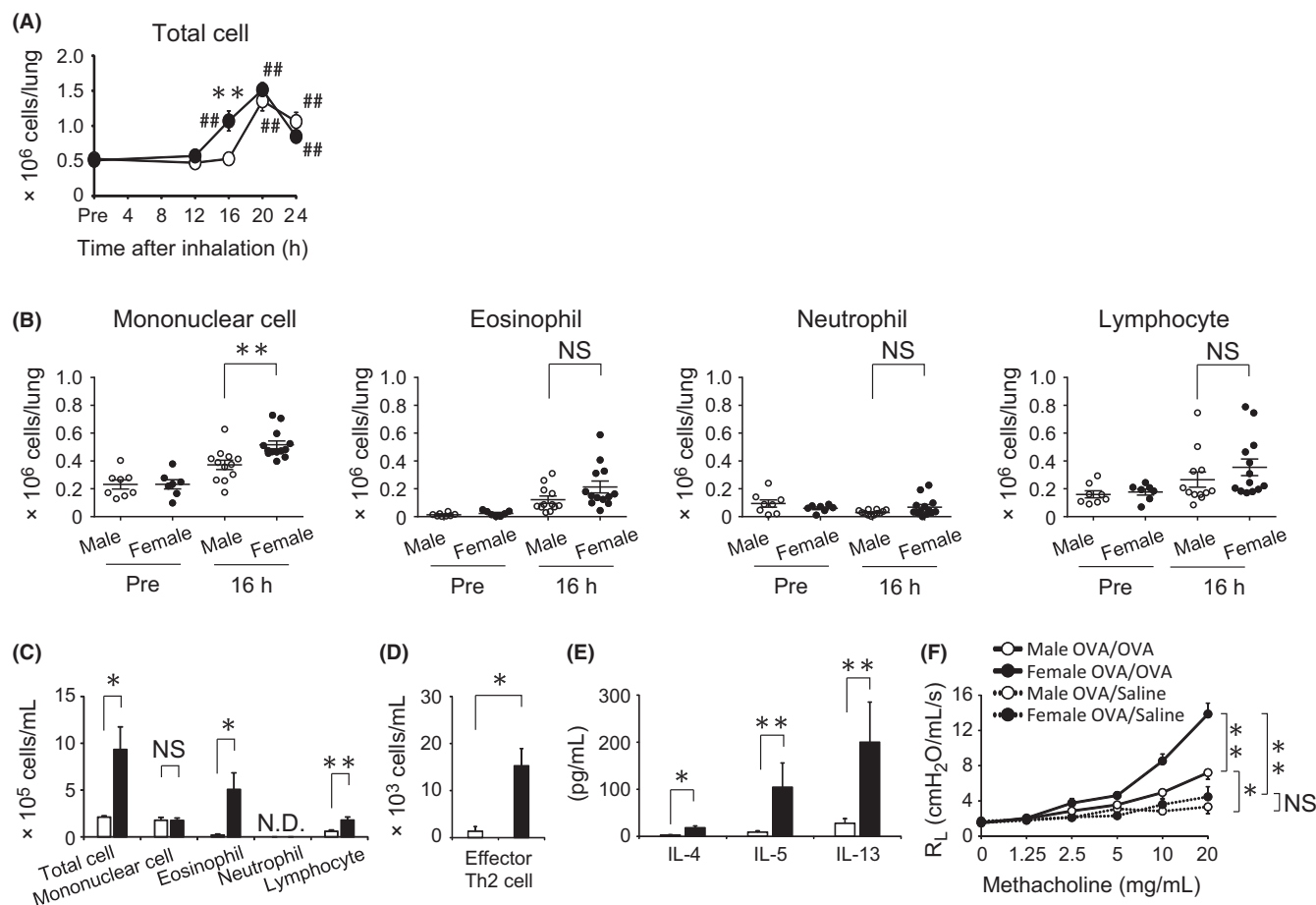


FIGURE 1 Sex-based differences in inflammatory cell infiltration in the lung. (A) The number of white blood cells in the mice lungs was counted before (Pre) and 12, 16, 20, and 24 h after (post) ovalbumin (OVA) challenge. (B) The lung white blood cells pre- and 16 h post-OVA challenge were stained with Diff-Quick and then observed using a microscope. The number of leucocyte fractions was estimated by multiplying the total leucocyte number by the proportion of each fraction. (C) The amount of cells in each leucocyte fraction and (D) the number of effector Th2 cells in the BAL fluids 5 days post-OVA challenge were measured. (E) IL-4, IL-5, and IL-13 levels in the BAL fluids 3 days post-OVA challenge were measured by ELISA. (F) Airway responsiveness in male and female sensitized mice were measured 1 day after OVA (OVA/OVA) or saline (OVA/Saline) challenge using whole body plethysmography. Data are shown as the mean $\pm$ SEM based on at least two independent experiments ($n = 7$ –13). Open bar and circles, male mice; closed bar and circles, female mice. * $P < .05$ compared to male mice; ** $P < .01$ compared to male mice; ## $P < .01$ compared to the level before OVA inhalation; NS, not significant

DCs in the BLN was initially examined, as shown in Figure 4A. Female mice exhibited an increasingly robust accumulation of DCs from 16 to 20 hours after OVA inhalation compared to that in male mice (Figure 4B,C). The number of total DCs, CD11b^{hi} DCs, and CD103⁺ DCs in BLN had reached to peak levels 20 to 24 hours after OVA inhalation and then further decreased to basal levels at 72 hours after OVA inhalation in both male and female mice. The number of CD11b^{hi} DCs at 16 hours post-OVA inhalation was significantly higher in female mice than in male mice (Figure 4C), similar to the time-dependent change that was observed in the lung (Figure 2C). In contrast, the number of CD103⁺ DCs in the BLN 16 to 20 hours after OVA inhalation was significantly higher in female mice than in male mice (Figure 4C); nevertheless, the number of the cells in the lung was not altered in both male and female mice after allergen inhalation (Figure 2C). To address the discrepancy in the distribution of CD103⁺ DCs after allergen exposure, we next examined

sex-associated differences in the migratory properties of each DC subset. The percentage of CCR7-expressing cells in CD103⁺ DCs was significantly increased in the lungs of female mice, but not in male mice, after OVA inhalation (Figure 5A). However, such sex-associated differences were not observed in the case of CD11b^{hi} DCs (Figure 5A). Meanwhile, total number of CCR7⁺ CD11b^{hi} DCs and CCR7⁺ CD103⁺ DCs in the lung 16 hours after OVA inhalation was significantly higher in female mice than in male mice (Figure 5B). In the BLN, the mRNA expression levels of ligands for CCR7, including CCL19 and CCL21, were not statistically different between male and female mice either before or 16 and 20 hours after OVA inhalation (Figure 5C). These results suggest that increase in the number and proportion of CCR7⁺ DCs together results in the sex difference in the kinetics of each DC subsets in the BLN; DCs from the female mice responded earlier and resolved sooner than those from the male mice did in the lungs of asthmatic mice.

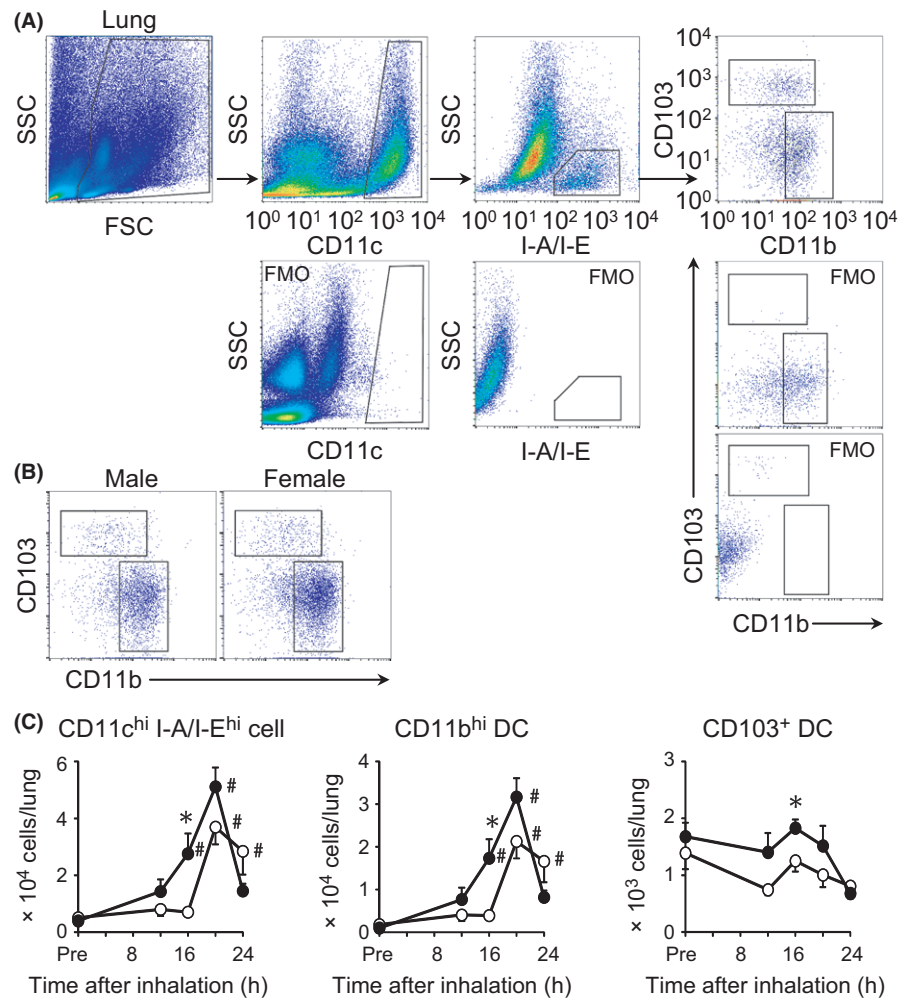


FIGURE 2 Sex-based differences in the number of dendritic cells in the lung. (A) Flow cytometric gating for SSC^{low} $CD11c^{\text{hi}}$ $I-A/I-E^{\text{hi}}$ dendritic cells (DCs) from the mouse lungs. Positive populations are defined based on fluorescence minus one (FMO) controls. (B) Representative profile of the expression of CD11b and CD103 on DCs in the lung of male and female mice 16 h after OVA challenge. (C) Total DCs, $CD11b^{\text{hi}}$ DCs, and $CD103^+$ DCs in the lung were counted pre- and 12, 16, 20 and 24 h post-OVA challenge. Data are shown as the mean $\pm$ SEM based on at least three independent experiments ($n = 8-12$). Open circle, male mice; closed circle, female mice. * $P < .05$ compared to male mice; # $P < .05$ compared to the level before OVA inhalation

3.3 | Sex-based differences in the antigen-presenting potential of BLN DCs

The pathogenesis of asthma is directed by allergen-specific Th2 cells that are generated in the secondary lymphoid organs, such as the BLN, where DC antigen presentation and signalling through costimulatory molecules are critical for the generation of Th2 cells. Therefore, to test sex-associated differences in the ability of BLN DCs to act as antigen-presenting cells, we initially examined the antigen uptake ability. As shown in Figure S1, OVA was actually taken up intracellularly by DCs of both male and female mice. Furthermore, a high frequency of OVA-positive DCs was observed in migrated DCs (male mice vs. female mice: $74.6 \pm 2.2\%$ vs. $84.2 \pm 3.2\%$; $P = .054$), but low levels were found in resident DCs in the BLNs from both male and female mice (Figure 6A). In the BLN, $81.3 \pm 2.8\%$ of DCs in male mice and $79.2 \pm 5.6\%$ of DCs in female mice were migratory (Figure 6B). Moreover, fluorescent OVA signals were more intense in migrated $CD103^+$ DCs from female mice than those from male mice, although such sex-associated differences were not observed in migrated $CD11b^{\text{hi}}$ DCs (Figure 6C). Next, the analysis of costimulatory molecule expression on BLN DCs revealed enhanced expression of CD86 and I-A/I-E, but not CD40 and CD80, in $CD103^+$ DCs from

female mice, compared to those in $CD103^+$ DCs from male mice, although sex-associated differences were not observed for costimulatory molecule expression on $CD11b^{\text{hi}}$ DCs (Figure 6D). Furthermore, IL-33 receptor expression on BLN DCs was significantly enhanced in $CD103^+$ DCs compared to that in $CD11b^{\text{hi}}$ DCs in female mice, whereas such differences were not observed between $CD11b^{\text{hi}}$ and $CD103^+$ DCs in male mice (Figure 6E). These results suggest that enhanced activation and antigen uptake in BLN $CD103^+$ DCs from female mice, as compared to those in corresponding DCs from male mice, are responsible for sex-based differences in Th2 cytokine production during asthma.

3.4 | Sex-based differences in BLN DC-induced T cell differentiation

To directly address the contribution of DCs to sex-related Th2 cytokine production, we initially purified $CD11b^{\text{hi}}$ and $CD103^+$ DCs from BLNs. These sorted populations were consisted of DCs, although there is the possibility of contamination with $CD3^+$ cells in these populations (Figure 7A). In addition, sex-associated differences in the morphology of each DC subset were not observed between male and female mice (Figure 7A). Next, we cultured each BLN DC subset

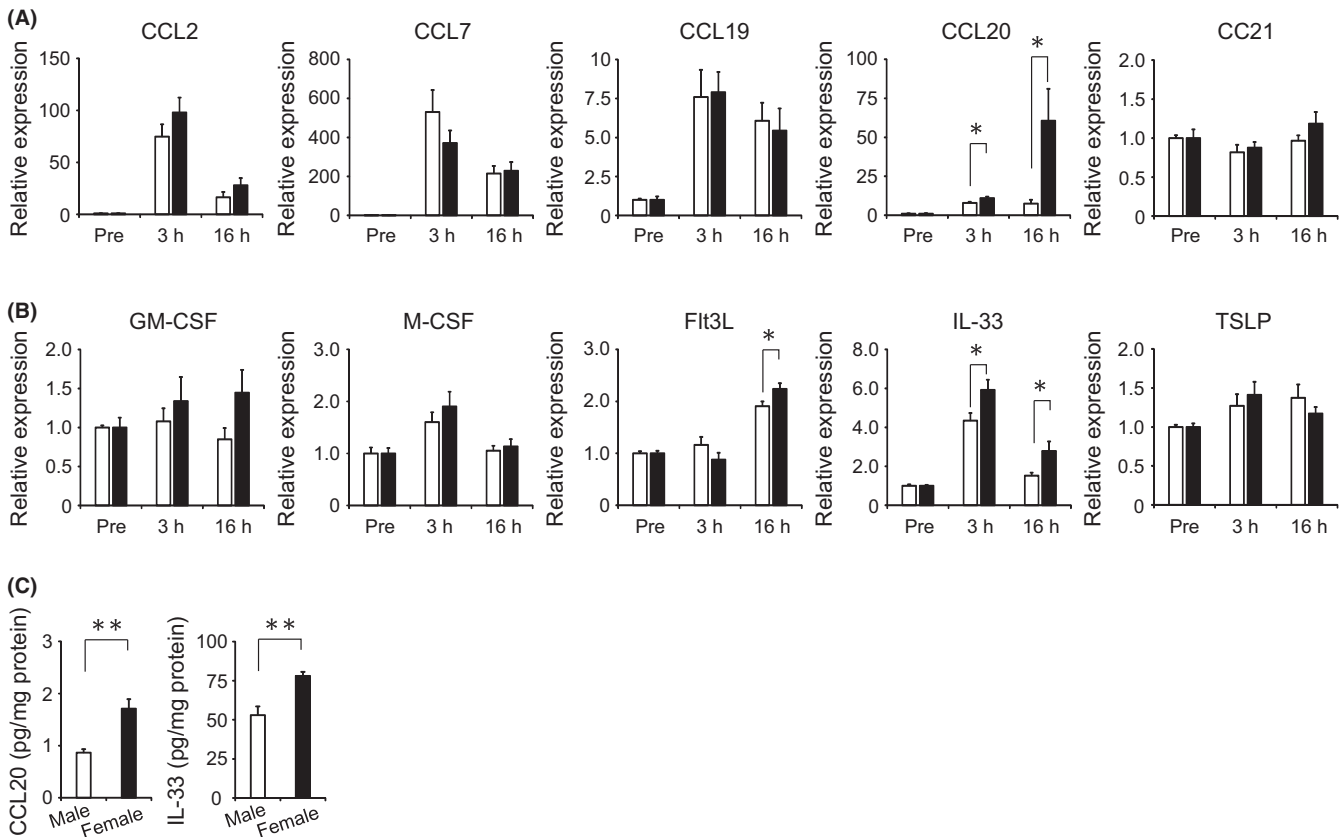


FIGURE 3 Sex-based differences in cytokine and chemokine expression in the lung. The mouse lungs were excised pre-, and 3 and 16 h post-OVA challenge. (A) Chemokine mRNA expression and (B) cytokine mRNA expression in the lungs were determined by real-time RT-PCR. (C) CCL20 and IL-33 protein levels in the lung 16 h post-OVA challenge were measured by ELISA. Data are shown as the mean $\pm$ SEM based on two to three independent experiments ($n = 6-14$). Open bar, male mice; closed bar, female mice. * $P < .05$ compared to male mice; ** $P < .01$ compared to male mice

with OVA peptide-specific T cell receptor-expressing CD4⁺ T cells. As shown in Figure 7B, IL-4, IL-5, and IL-13 levels in the supernatants were higher after culturing with CD103⁺ DCs than with CD11b^{hi} DCs in case of both male and female mice. In addition, CD103⁺ DCs from female mice induced higher IL-4, IL-5, and IL-13 production from CD4⁺ T cells than that by CD103⁺ DCs from male mice; however, sex-associated differences in Th2 cytokine production were not observed after culturing these cells with CD11b^{hi} DCs from male and female mice. IFN- γ was also produced from CD4⁺ T cells, the levels of which were 10- to 100-fold higher than those of Th2 cytokine in both male and female mice; however, no sex-associated differences were observed with either CD11b^{hi} or CD103⁺ DCs. Furthermore, CD11b^{hi} DCs and CD103⁺ DCs from male and female mice induced similar levels of IL-17 production from CD4⁺ T cells, which were 40- to 4000-fold lower than those of other cytokines. For cytokine production in CD8⁺ T cells, CD103⁺ DCs from female mice induced higher levels of IL-5, but not IFN- γ , production than that by CD103⁺ DCs from male mice (Figure 7C). Finally, sex-associated differences in CD4⁺ T cell proliferation induced by CD103⁺ DCs were assessed. As expected, CD103⁺ DCs from female mice induced greater T cell proliferation than CD103⁺ DCs from male mice did, as determined by CFSE dilution in dividing cells

(Figure 7D,E). These results suggest that CD103⁺ DCs, but not CD11b^{hi} DCs, promote female-predominant Th2 cell differentiation, followed by female-predominant Th2 cytokine production.

3.5 | Oestradiol induces enhanced Th2-type immunity in CD103⁺ DCs

Female hormones can reportedly enhance Th2-type immune responses via their effects on DCs.^{38,39} We tested the role of 17 β -oestradiol, the most common circulating form of oestrogen, in the associated Th2-type immune response in CD103⁺ DCs. Oestradiol enhanced the expression of CD86 and I-A/I-E on CD103⁺ BM-DCs stimulated with OVA, which was further enhanced by IL-33 (Figure 8A). However, in the case of CD103⁺ DCs developed from the BM cells in the absence of 17 β -oestradiol, enhanced expression of CD86 and I-A/I-E was not observed upon stimulation with OVA plus 17 β -oestradiol (Figure S2).

CD86 and I-A/I-E expression on CD103⁺ BM-DCs was not different between oestradiol-primed CD103⁺ BM-DCs and vehicle-primed CD103⁺ BM-DCs on IL-33 stimulation (Figure 8A). Oestradiol-primed CD103⁺ BM-DCs also induced ST2 expression on CD4⁺ T cells in the presence of OVA (Figure 8B). An oestrogen receptor

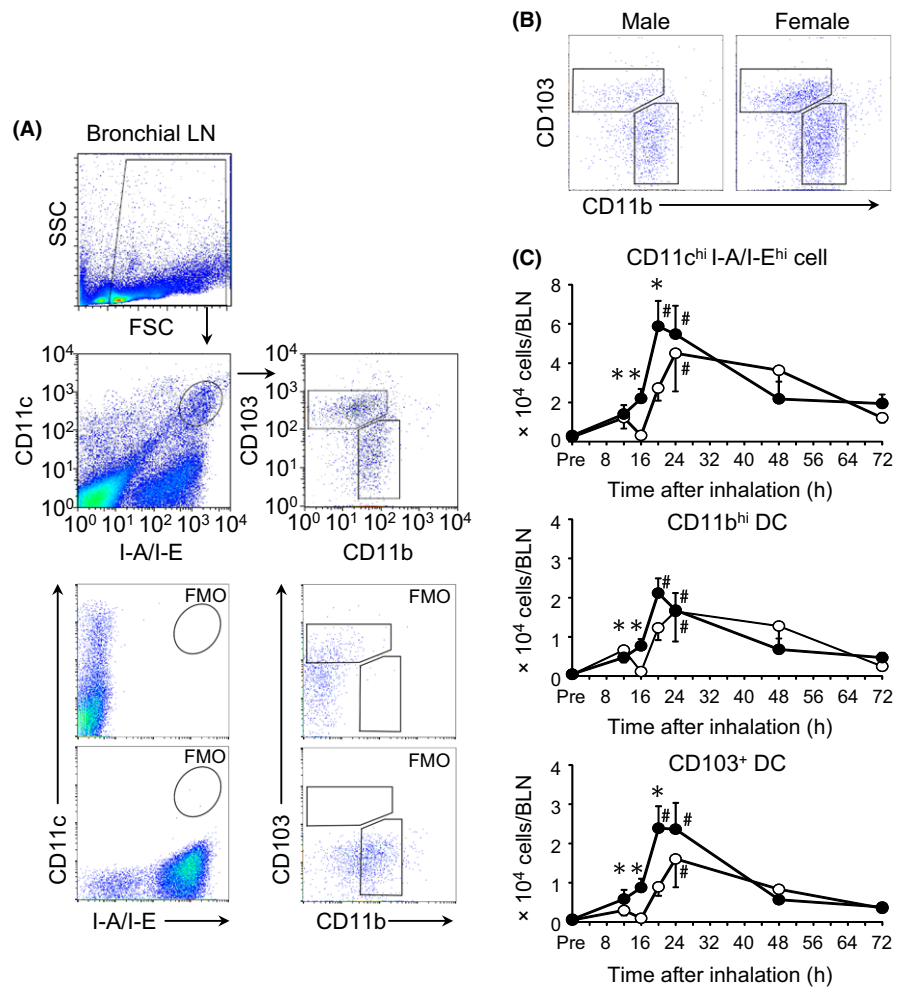


FIGURE 4 Sex-based differences in the number of DCs in the bronchial lymph node. (A) Flow cytometric gating for SSC^{low} $CD11c^{\text{hi}}$ $I\text{-}A/I\text{-}E^{\text{hi}}$ DCs from bronchial lymph nodes (BLNs) of mice. Positive populations are defined based on FMO controls. (B) Representative profile of the expression of CD11b and CD103 on DCs in the BLN of male and female mice 20 h after OVA challenge. (C) Numbers of total DCs, $CD11b^{\text{hi}}$ DCs, and $CD103^+$ DCs in the BLN were counted pre- and 12, 16, 20, 24, 48, and 72 h post-OVA challenge. Data are shown as the mean $\pm$ SEM based on two to three independent experiments ($n = 8\text{--}9$). Open circle, male mice; closed circle, female mice. * $P < .05$ compared to male mice; ** $P < .01$ compared to male mice; # $P < .05$ compared to the level before OVA inhalation

antagonist abolished the expression of these molecules on oestradiol-primed $CD103^+$ BM-DCs and $CD4^+$ T cells (Figure 8A,B). In addition, enhanced IL-5 production from $CD4^+$ T cells was observed after culture with oestradiol-primed $CD103^+$ BM-DCs, compared to that after culture with vehicle-treated $CD103^+$ BM-DCs, and an anti-CD86 antibody completely abolished this effect (Figure 8C).

4 | DISCUSSION

This study reports the first evidence of $CD103^+$ DCs being involved in the development of sex-based differences in Th2 cytokine production in asthma. We previously demonstrated that BLN cells from female mice with OVA-stimulated asthma, but not those treated with anti-CD3/CD28 antibodies, produced higher levels of IL-5 and IL-13 than those from male mice, suggesting the involvement of antigen-presenting cells in the sex-associated differences in Th2 cytokine production during asthma.³² With regard to migratory DCs in the BLN, Nembrini et al⁴⁰ showed, using an OVA-induced asthma mouse model, that $1\text{--}2 \times 10^5$ DCs were observed in the lung-draining lymph node 24 hours after OVA inhalation, of which $1.5\text{--}4 \times 10^4$ DCs were identified as migratory DCs based on their uptake of FITC-conjugated OVA. In their study, the numbers of migratory DCs in the lung-

draining lymph node reached a peak 24 hours after OVA inhalation and then decreased to almost half of their peak levels at the end of 48 hours.⁴⁰ Furthermore, Plantinga et al⁴¹ reported, using a house dust mite-induced asthma mouse model, that 60% of BLN DCs on day 3 post-house dust mite challenge were migratory ($CD11c^{\text{hi}}$ $MHCII^{\text{hi}}$) DCs, and 40% of BLN DCs were resident ($CD11c^{\text{hi}}$ $MHCII^{\text{dim}}$) DCs, although the sex of mice used was not described in these studies. Our data regarding the proportions of migratory DCs in the BLN 20 hours after allergen exposure (Figure 6A,B) indicate a similar tendency, in either sex, to those of migratory DCs in the BLN reported previously as mentioned above.^{40,41} Regarding the sex difference in the number of DCs, Melgert et al⁴² showed that T cell expansion in female mice appeared to be driven by a greater number of inflammatory mDCs, defined as $CD11c^{\text{hi}}$ $MHC\text{-}II^{\text{hi}}$ cells, migrating from the lungs to the lymph nodes, during asthma. Consistent with this observation, we showed that the total number of DCs, defined as $CD11c^{\text{hi}}$ $I\text{-}A/I\text{-}E^{\text{hi}}$ cells in the lung and BLN, was increased in female mice compared to those in male mice.

Chemokines, such as CCL2 and CCL20, from the airway epithelial cells activated in response to allergens, attract monocytes and DC precursors to the lung.⁴³ In contrast, TSLP, IL-25, and IL-33 in the lung confer a Th2 polarizing profile to DCs through the upregulation of B7-1, B7-2, and OX40L, which instructs naive T cells to

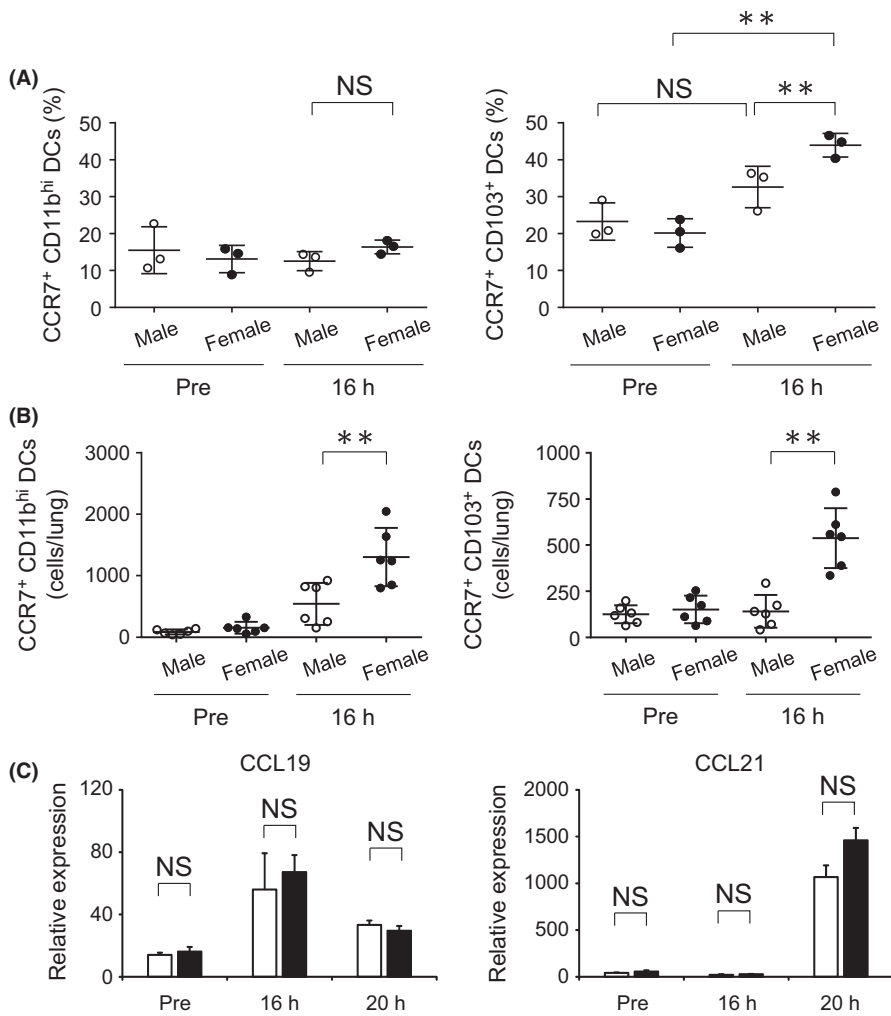


FIGURE 5 Sex-based differences in the migration capacity of pulmonary DCs to the BLN. (A) The percentages of CCR7⁺ population in the pulmonary CD11b^{hi} DCs and CD103⁺ DCs, and (B) the total numbers of CCR7⁺ CD11b^{hi} DCs and CCR7⁺ CD103⁺ DCs in the lungs pre- and 16 h post-OVA challenge. (C) CCL19 and CCL21 mRNA expression in the BLN pre- and 16 and 20 h post-OVA challenge was quantitated by RT-PCR. Data are shown as mean ± SEM based on three to six mice. Experiments were repeated twice with similar results. Open bar and circles, male mice; closed bar and circles, female mice. ***P* < .01; NS, not significant

differentiate into Th2 cells.^{44–47} Development of CD11b⁺ DCs from precursors in the lung depends on cytokines M-CSF and GM-CSF levels, but does not require an interaction between Flt3 and Flt3L, whereas CD103⁺ DC development from common DC precursors relies on the stepwise activity of Flt3 signalling.⁴⁸ In contrast, Plantinga et al⁴¹ reported that Flt3L^{−/−} mice lacked both CD11b⁺ and CD103⁺ DCs, and exhibited significantly reduced numbers of eosinophil, neutrophil, and lymphocytic infiltration in the BAL fluids compared to that in the asthmatic WT mice, after exposure to house dust mites. In the present study, we showed that Flt3L and IL-33 expression in the lung tissues was enhanced in female mice compared to male mice (Figure 3), whereas TSLP expression levels were similar between the sexes, and IL-25 was not detected in either sex (data not shown). These observations suggest that close proximity of lung DCs to IL-33- or Flt3L-secreting cells might be responsible, at least in part, for the sex-associated differences in DC development and in conferring a Th2 polarizing profile to DCs in the asthmatic lung.

Developmental lineage affects the differential expression of CCR7, a chemokine receptor required for migration to lung-draining mediastinal lymph nodes; Ly-6C^{hi} CD11b⁺ DCs, and CD14^{hi} CD11b⁺ DCs, which are Flt3-independently derived from monocytes in the lungs,²⁴ express lower levels of *Ccr7*, whereas pulmonary CD103⁺

DCs express higher levels of *Ccr7* in the lung 24 hours after LPS instillation, indicating that migratory capacities of CD103⁺ DCs are superior to those of CD11b⁺ DCs.²⁵ Furthermore, Besnard et al³⁷ demonstrated that IL-33 induces CCR7 expression on DCs and DC activation in the lung. Recently, Chen et al⁴⁹ suggested the possibility that airway exposure to IL-33 might increase the number of cells expressing ST2, a receptor for IL-33, in the lung. Here, the enhanced population of ST2-expressing CD103⁺ DCs in female mice was accompanied by increased expression of IL-33 mRNA and protein in the lungs of these animals (Figures 3B,C and 6E). Consistent with this observation, the increased CCR7⁺ cell population in CD103⁺ DCs and the increased number of CCR7⁺ CD103⁺ DCs in the lung were observed in female mice (Figure 5A,B). These results suggest that the development of the CD103⁺ DC lineage, in association with CCR7 expression and further activation by enhanced IL-33 in females, might be involved in the sex-related differences in CCR7 expression on CD103⁺ DCs, which might lead to the corresponding differences in the number of CD103⁺ DCs in the BLN (Figure 4B,C).

CD103⁺ DCs comprise a primary DC subset in the lung and mediastinal lymph nodes that prime Th2 responses upon allergen encounter, whereas CD11b^{hi} DCs prime Th1 responses.²⁸ Furthermore, CD103⁺ DCs in the BLN are specialized for the cross-presentation of

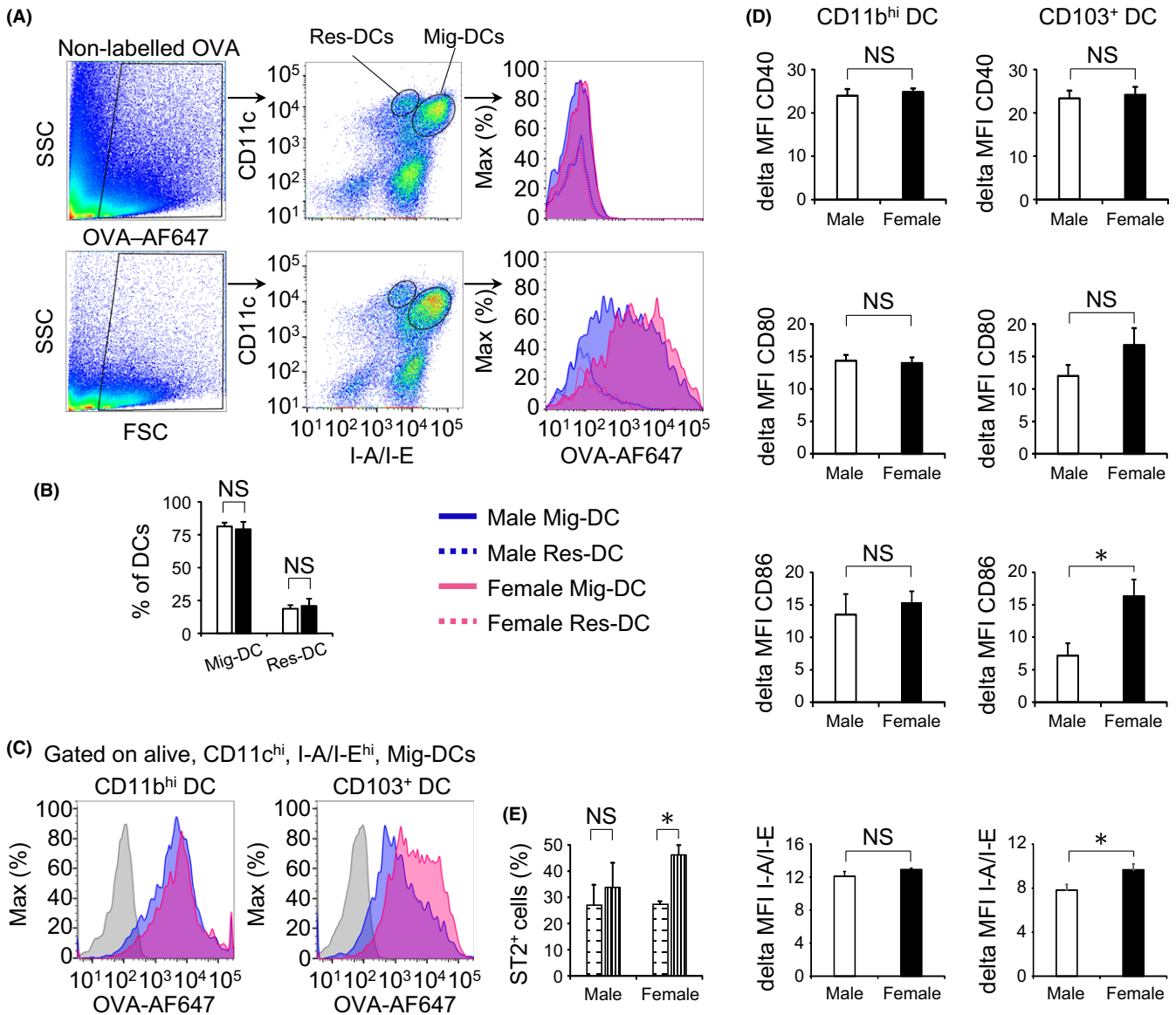


FIGURE 6 Sex-based differences in Th2 priming ability of DCs in the BLN. (A) Sex-based differences in OVA uptake by CD11c^{hi} I-A/I-E^{dim} BLN-resident DCs and CD11c^{hi} I-A/I-E^{hi} migratory DCs (from the lung to the BLN). Solid blue line, migratory DCs from male mice; solid red line, migratory DCs from female mice; dotted blue line, resident DCs from male mice; dotted red line, resident DCs from female mice. (B) The percentages of resident DCs and migratory DCs in BLN DCs of male and female mice. (C) Sex-based differences in OVA uptake by CD11b^{hi} and CD103⁺ DCs in the BLN. Representative results of three independent experiments are shown. Grey line, DCs that took up non-conjugated OVA; blue line, migratory DCs of each subset from male mice; red line, migratory DCs of each subset from female mice. (D) Sex-based differences in mean fluorescent intensity of CD40, CD80, CD86, and I-A/I-E on migratory CD11b^{hi} DCs and CD103⁺ DCs in the BLN. (E) Differences in ST2 expression on migratory BLN CD11b^{hi} DCs and CD103⁺ DCs from male and female mice. Horizontal line bars, CD11b^{hi} DCs; vertical line bars, CD103⁺ DCs. Open bar, male mice; closed bar, female mice. Data are shown as the mean $\pm$ SEM based on four to six mice. Experiments were repeated twice with similar results. *P < .05; NS, not significant

inhaled antigens to CD8⁺ T cells, in contrast to CD11b⁺ DCs, which are characterized by the differential expression of MHC-I and MHC-II processing pathways.^{50,51} In accordance with the cross-presentation activity of CD103⁺ DCs, these cells mediated sex-associated differences in the Th2 cytokine production, but not IFN- γ production, from not only CD4⁺ T cells (Figure 7B), but also CD8⁺ T cells (Figure 7C). In the enhanced Th2 cytokine production, the expression of CD40, CD80, CD86, or OX40L is required for directional T cell

differentiation and activation of Th1, Th2, and Th17 cells.⁵² Moreover, OX40L, Jagged2, ICOSL, and TSLPR expression on DCs has been associated with Th2 induction by DCs during Th2 cell differentiation.⁵³⁻⁵⁵ In the current study, mRNA expression of OX40L, Jagged2, ICOSL, and TSLPR in CD103⁺ DCs is not different between male and female mice (Figure S3). Therefore, the enhanced expression of CD86 on CD103⁺ DCs may be directly responsible for the corresponding enhanced Th2 cell differentiation (Figures 6D and 8C).

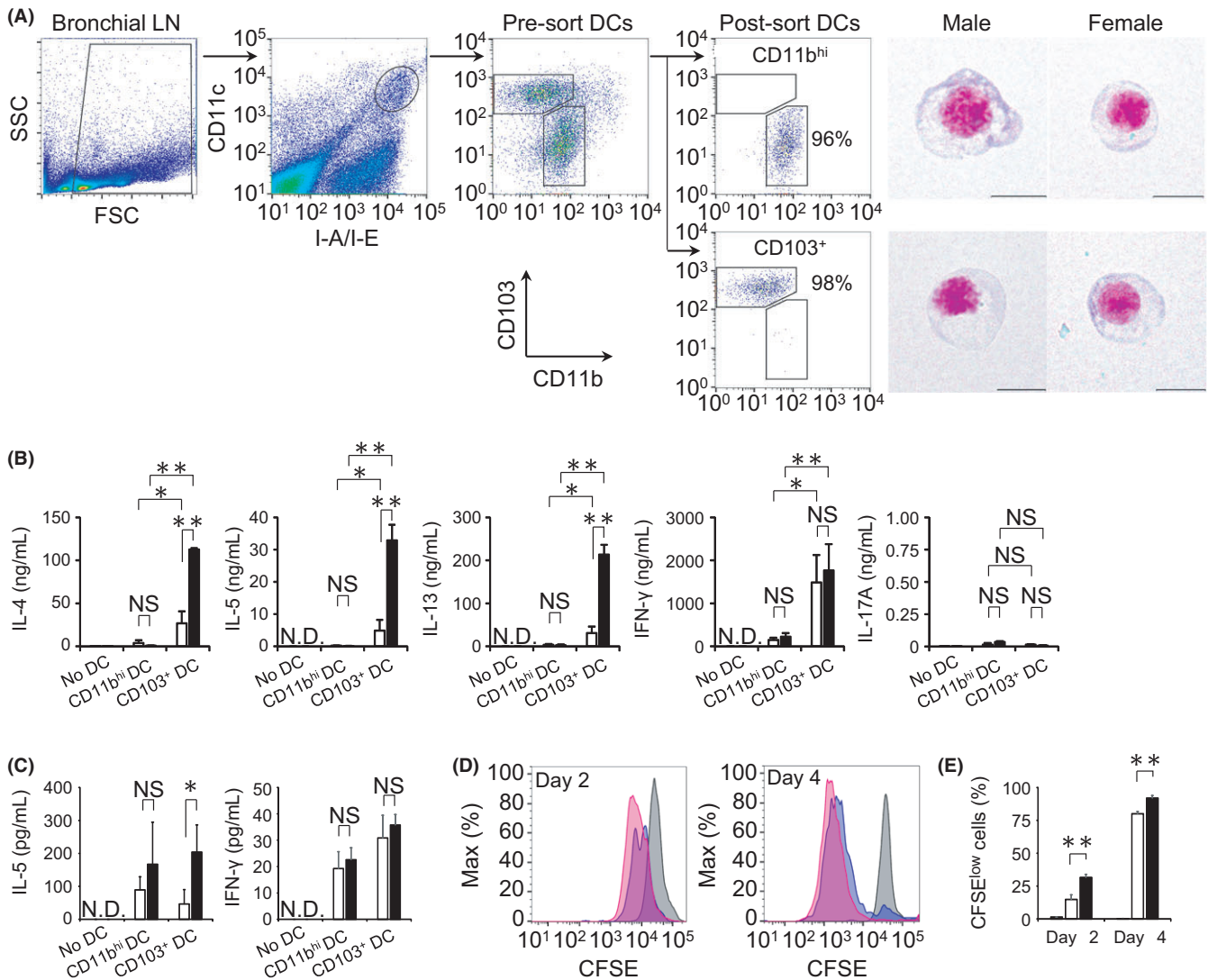


FIGURE 7 Sex-based differences in T cell differentiation promoted by BLN DCs. (A) Purity of sorted BLN CD11b^{hi} DCs and CD103⁺ DCs. Representative images of each population are shown. Scale bar = 10 μ m. (B) Cytokine levels in cultures of CD4⁺ T cells from the spleen of OT-II transgenic (Tg) mice with CD11b^{hi} DCs or CD103⁺ DCs from the BLN of male or female mice. (C) Cytokine production in CD8⁺ T cells from OT-I transgenic (Tg) mice cultured with each DC subset from the BLN of male or female mice. Cytokine levels in the culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA). (D, E) 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE)-labelled CD4⁺ T cells were cultured with CD103⁺ DCs from male or female mice. (D) The dilution of the CFSE signal on day 2 or 4 post-culture was assayed by flow cytometry. Representative results of two independent experiments are shown. Grey line, CFSE-labelled CD4⁺ T cells cultured without DCs; blue line, CD4⁺ T cells cultured with CD103⁺ DCs from male mice; red line, CD4⁺ T cells cultured with CD103⁺ DCs from female mice. (E) Percentage of CFSE^{low} cells in CD4⁺ T cells cultured with CD103⁺ DCs from the BLN of male and female mice after 2 or 4 days of culture. Grey bar, CFSE-labelled CD4⁺ T cells cultured without DCs; open bar, CFSE-labelled CD4⁺ T cells cultured with CD103⁺ DCs from male mice; closed bar, CFSE-labelled CD4⁺ T cells cultured with CD103⁺ DCs from female mice. Data are shown as the mean $\pm$ SD based on triplicate cultures. Experiments were repeated twice with similar results. * P < .05; ** P < .01; NS, not significant; N.D., not detected

Oestrogen likely induces enhanced immune responses in females through interaction with its receptors.^{38,56} Yang et al³⁹ showed that 17 β -oestradiol upregulates costimulatory molecules and MHC-II on CD11c-positive murine splenic DCs, which promotes T cell priming. In the present study, CD86 and I-A/I-E expression on CD103⁺ DCs was not enhanced through culture with 17 β -oestradiol alone, but rather through incubation with IL-33, as previously suggested by Rank et al⁵⁷ Furthermore, sustained

exposure to 17 β -oestradiol, including that during the period of CD103⁺ DC development, might enhance CD103⁺ DC activation and maturation in females upon allergen exposure, which could lead to female-enhanced Th2 cell differentiation and activation (Figure 8 and Figure S2). Meanwhile, 17 β -oestradiol enhanced the expression of CD86 and I-A/I-E on DCs after stimulation with OVA, but not with IL-33 (Figure 8A). Our results suggest that 17 β -oestradiol has a limited role in the IL-33-ST2 axis-mediated

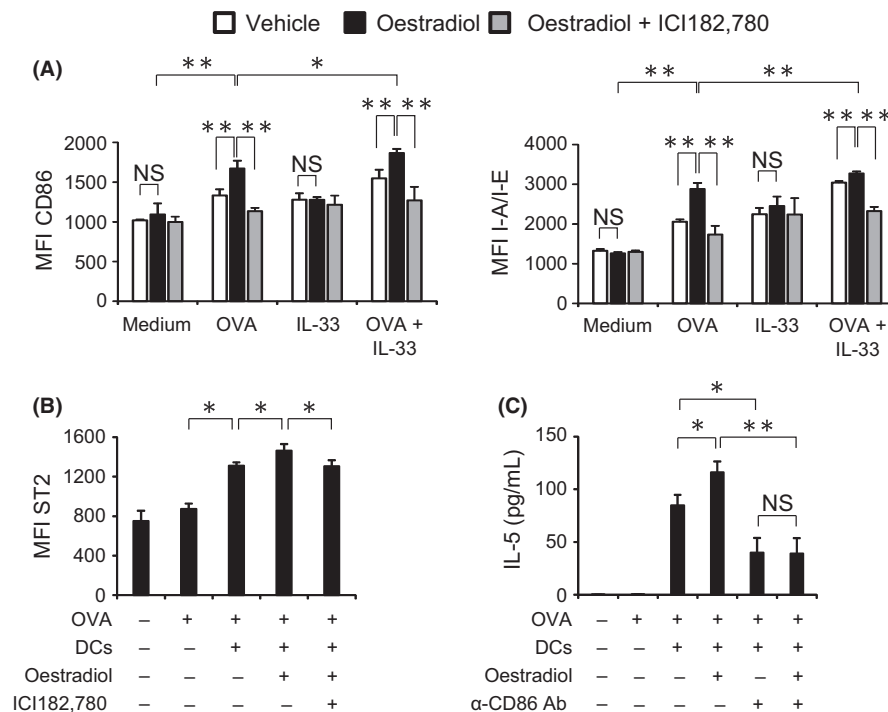


FIGURE 8 17β-oestradiol enhances Th2 cell priming by bone marrow-derived CD103⁺ DCs. CD103⁺ bone marrow (BM)-DCs were induced from BM cells of male mice in the presence of 17β-oestradiol, 17β-oestradiol plus ICI182,780, or vehicle. (A) Each set of CD103⁺ BM-DCs was cultured in the presence of OVA, IL-33, or OVA plus IL-33 for 24 h. CD86 and I-A/I-E expression levels on CD103⁺ BM-DCs were assayed by flow cytometry. (B) BLN CD4⁺ T cells were cultured with 17β-oestradiol-primed, 17β-oestradiol plus ICI182,780-primed, or vehicle-primed CD103⁺ BM-DCs in the presence or absence of OVA. ST2 expression on CD4⁺ T cell from BLNs was determined by flow cytometry. (C) BLN CD4⁺ T cells were cultured with 17β-oestradiol-primed or vehicle-primed CD103⁺ BM-DCs in the presence or absence of OVA and recombinant anti-mouse CD86 antibody. IL-5 levels in the culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA). Data are shown as the mean ± SD based on triplicate cultures. Experiments were repeated twice with similar results. **P* < .05; ***P* < .01; NS, not significant

activation of CD103⁺ DCs in female mice; however, this requires further investigation.

The present study has important implications for understanding the pathophysiological role of DCs in sex-associated asthma phenotypes. Recently, the possibility that CD103⁺ DCs might even have tolerogenic ability towards inhaled allergens through the induction of regulatory T cells in the absence of DC maturation stimulus has been reported.^{58,59} Further research is needed to completely investigate the precise role of CD103⁺ DCs in the pathogenesis of asthma. Furthermore, it still remains unclear whether BDCA3⁺ DCs, the human homolog of mice CD103⁺ DCs, play an important role in enhanced Th2 cytokine production in female patients with asthma. Therefore, the results of this study have limitations regarding their application to humans. Additional clinical studies are already ongoing in our laboratory to confirm the results, which could lead to the development of more effective strategies to treat female patients with asthma.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

AUTHOR CONTRIBUTIONS

IO and TM conceived and designed the experiments; CM, KK, TM, and KO-D performed the experiments; TM, IO, TT, CM, KK, TK, and JI analysed the data; IO, JI, and MT contributed reagents/materials/analysis tools; TM, CM, IO, TT, and MT contributed to the writing of the manuscript.

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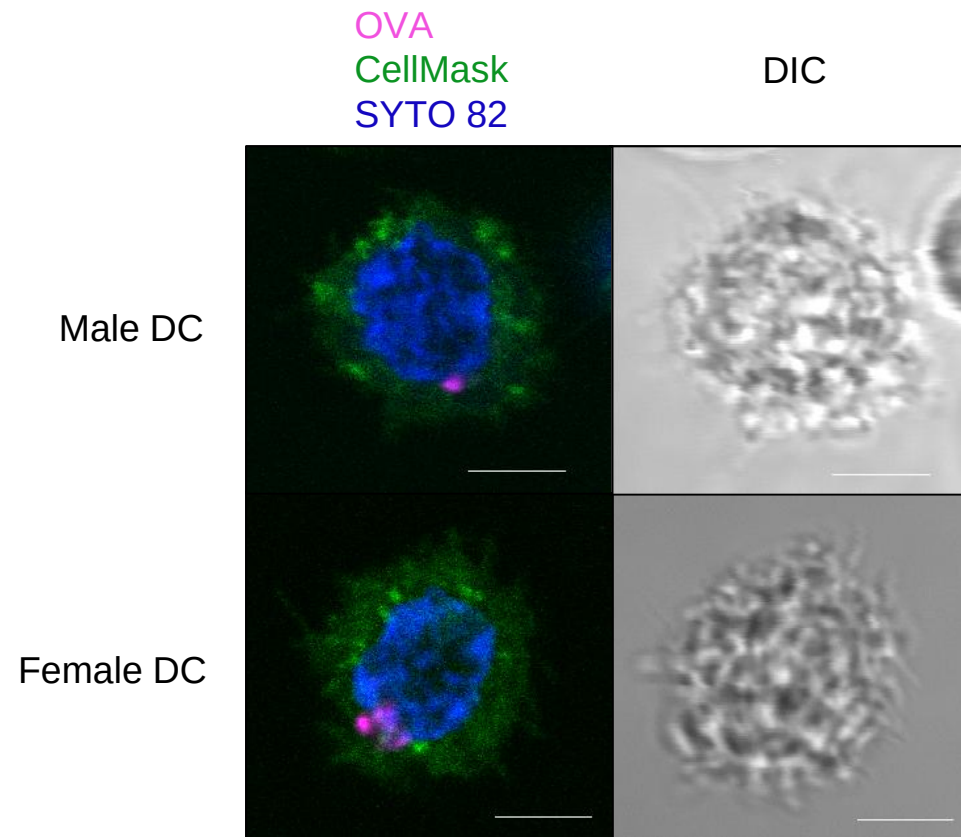
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SUPPORTING INFORMATION

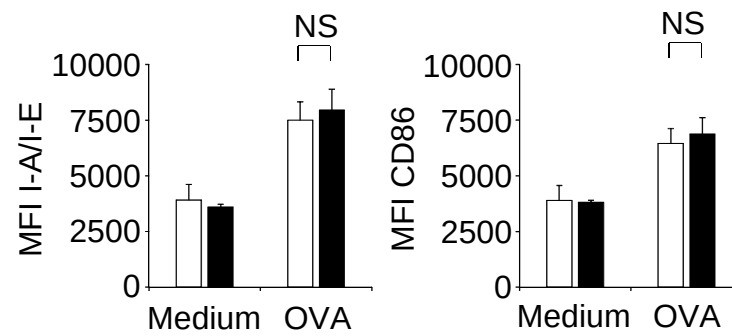
Additional Supporting Information may be found online in the supporting information tab for this article.

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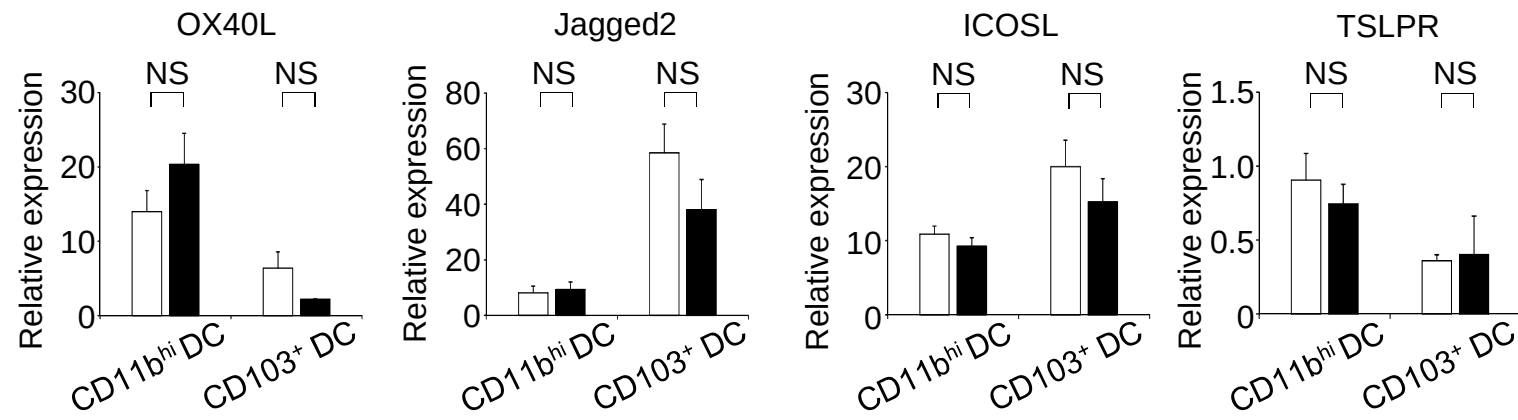
Supplementary Figure
Figure S1. Masuda et al.



Supplementary Figure
Figure S2. Masuda et al.



Supplementary Figure
Figure S3. Masuda et al.



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Sex-based differences in CD103⁺ dendritic cells promote female-predominant Th2 cytokine production during allergic asthma

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Supporting Information

Figure S1. Trafficking of OVA within BLN DCs. Male and female mice were sensitized by intraperitoneal injections with non-labeled OVA and then challenged intratracheally by Alexa Fluor 647-conjugated OVA. DCs were prepared from BLN of the male and female mice. Cells were stained with CellMask plasma membrane stain and SYTO 82 fluorescent nucleic acid stain. Cells were analyzed using a confocal laser scanning microscope. Scale bar = 5 μ m. DIC, differential interference contrast. Representative results of two independent experiments are shown.

Figure S2. Sex-based differences in CD86 and I-A/I-E expression on CD103⁺ BM-DCs. CD103⁺ BM-DCs were induced from the BM cells in the absence of 17 β -estradiol. CD103⁺ BM-DCs were cultured in the presence of 17 β -estradiol (closed bar) or vehicle (open bar) with OVA for 24 h. CD86 and I-A/I-E expression levels on CD103⁺ BM-DCs were assayed by flow cytometry. Data are shown as the mean $\pm$ SD based on triplicate cultures. Experiments were repeated twice with similar results. NS, not significant.

Figure S3. Sex-based differences in mRNA expression in BLN DCs. BLN DCs were isolated from mice 20 h after OVA challenge. Expression levels of OX40L, Jagged2, ICOSL, and TSLPR mRNA in DCs were determined by real-time RT-PCR. Data are shown as the mean $\pm$ SEM of four to six mice. Experiments were repeated twice with similar results. NS, not significant.