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Fms-Like Tyrosine Kinase 3 Ligand Regulates Migratory Pattern and Antigen Uptake of Lung Dendritic Cell Subsets in a Murine Model of Allergic Airway Inflammation¹

Zhifei Shao,* Toluwalope O. Makinde,* Halvor S. McGee,* Xiang Wang,* and Devendra K. Agrawal^{2*†‡}

Fms-like tyrosine kinase 3 ligand (Flt3L) reverses the features of allergic airway inflammation and increases a Th2-suppressive regulatory lung CD11c^{high}CD11b^{low} dendritic cell (DC) subset in a mouse model. We examined the migratory pattern and Ag uptake efficiency of lung DC subsets in the therapeutic effect of Flt3L. Lung CD11c^{high}CD11b^{low} and CD11c^{low}CD11b^{high} DCs from PBS-treated, OVA-sensitized, and Flt3L-treated/OVA-sensitized BALB/c mice were sorted using MACS and FACS for phenotype analysis. Lymphatic chemokine expression in thoracic lymph nodes was determined by immunohistochemistry. Migration of two lung DC subsets to lymphatic chemokines was examined in vitro using a Transwell chemotaxis assay. Labeled Ag was intranasally delivered into mouse lung to track the migration and Ag uptake of lung DCs. The in vitro cytokine secretion of mediastinal lymph node cells was determined using ELISA. CD11c^{low}CD11b^{high} DCs have higher expression of CCR5, CCR6, and CCR7, but lower expression of CCR2 than CD11c^{high}CD11b^{low} DCs. CD11c^{low}CD11b^{high} DCs in Flt3L-treated/OVA-sensitized mice demonstrated a less mature phenotype, inefficiency in Ag uptake, and impaired migration in vitro to lymphatic chemokine than those in OVA-sensitized mice. Administration of Flt3L decreased the expression of CCR5 and CCR7 in CD11c^{low}CD11b^{high} DCs in OVA-sensitized mice. Fewer Ag-carrying cells were detected in the lungs and lymph nodes in Flt3L-treated/OVA-sensitized mice than OVA-sensitized mice with a greater decrease in CD11c^{low}CD11b^{high} DCs. Mediastinal lymph node cells from Flt3L-treated mice secreted higher levels of Th1 cytokines and IL-10 than OVA-sensitized mice in vitro. In conclusion, Flt3L-generated lung immunogenic CD11c^{low}CD11b^{high} DCs have a less mature phenotype, impaired Ag uptake, and impaired migration to draining lymph nodes. *The Journal of Immunology*, 2009, 183: 7531–7538.

Fms-like tyrosine kinase 3 (Flt3)³ ligand (Flt3L) is a synergistic hematopoietic growth factor that has emerged as a potential immunomodulator in the intervention of multiple disease conditions, such as cancer, infection, vaccination, and allergic asthma (1–6). The basis of these tentative applications is its ability to specifically expand dendritic cell (DC) population and consequently enhance the Ag presentation (7). A high level of Flt3L, however, is only detected in hematopoietic organs, such as bone marrow and peripheral lymph organs, whereas Flt3L maintains at very low levels in the peripheral circulatory system under physiological condition (8). Therefore, considering the synergistic nature of Flt3L, DC generation and DC-mediated immune response under a superphysiological level of Flt3L are primarily dependent on the cytokines or growth factors present in the micro-

environment in hematopoietic organs (9–14), for example, a Th2-dominant cytokine profile in allergic asthma (15). The fact that Flt3L treatment preferably generates some specific DC subsets (9, 11, 12) opens an intriguing question as to the phenotype and function of Flt3L-generated DCs as well as the pathway by which these DCs function in allergic conditions.

We have previously shown that Flt3L reverses the features of allergic airway inflammation, preferably increases a Th1-prone lung CD11c^{high}CD11b^{low} DC subset, and slightly decreases a Th2-prone immunogenic CD11c^{low}CD11b^{high} lung DC subset in a mouse model of acute allergic airway inflammation (15). The increasing numbers of DCs in response to inflammation are a result of enhanced hematopoiesis and a process in which DC precursors migrate toward a chemoattractant gradient. Therefore, the number of peripheral DCs appears to be dependent on the efficiency of hematopoiesis as well as the migratory capacity of DC precursors under the regulation of inflammatory mediators. One of the indicators for the migratory capacity of DC is the chemokine receptor expression that remains after the DC precursors migrate to the lungs (16, 17). Ag uptake is a key step in DC activation, upon which DCs up-regulate CCR7 expression and acquire the ability to migrate to draining lymph nodes (LN), where they prime naive CD4⁺ T cells. This process is driven by the interaction between CCR7 and its ligands, CCL19 and CCL21 (18). The differential regulation of migratory activity of lung DC subsets upon Ag uptake would be expected to influence the efficiency of the Ag presentation.

It has been reported that Flt3L treatment alters expression levels of chemokine receptors and subsequently affects the migratory capability of DCs in lungs and kidney (19, 20). Further study has

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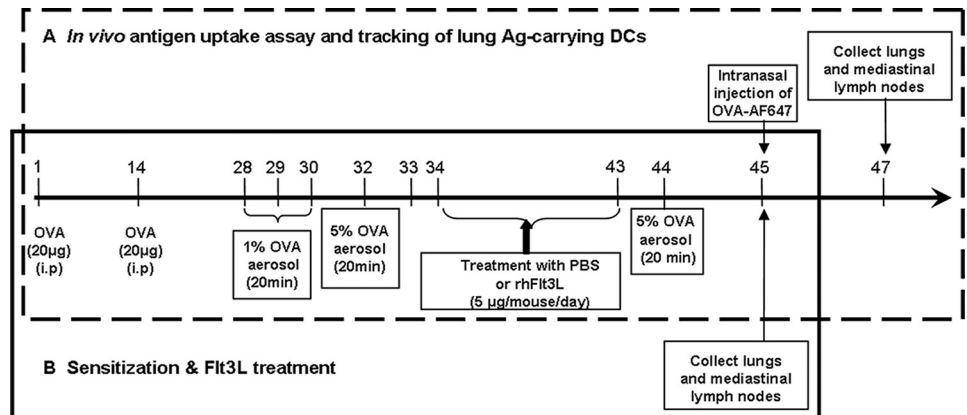
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³ Abbreviations used in this paper: Flt3, Fms-like tyrosine kinase 3; DC, dendritic cell; Flt3L, Flt3 ligand; LN, lymph node.

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FIGURE 1. Protocol for sensitization and treatment. **A**, Dash frame. A total of 150 μ g OVA-AlexaFluor 647 in 30 μ l was intranasally delivered into lungs of OVA-sensitized mice on day 45. Lungs and mediastinal LN were collected for the evaluation of Ag uptake and for tracking lung DCs. **B**, Solid frame. OVA sensitization. A total dose of 50 μ g of Flt3L treatment was given to each mouse.



suggested that Flt3L-Flt3 engagement facilitates hematopoietic cell migration/homing and mobilization by enhancing or inhibiting CXCL12/CXCR4 signaling pathways, which sheds light on the molecular mechanisms of Flt3L-Flt3 interaction with respect to DC chemotaxis (21). Furthermore, Flt3L/Flt3 interaction is also involved in regulating the trafficking of normal and transformed hematopoietic cells (21). Mice pretreated with Flt3L demonstrated an altered cytokine and chemokine (MIP-2 and CCL2) expression in bronchoalveolar lavage fluid, which suggests that Flt3L may affect chemokine production and consequently intervene the recruitment of inflammatory cells (22). Lastly, Flt3L-generated mouse spleen DCs take up and process Ag less efficiently than CSF-generated mouse spleen DCs (12). These studies have provided strong evidence that Flt3L is involved in the regulation of DC migration in multiple aspects. However, whether or not Flt3L differentially alters the migratory pattern and Ag uptake of lung DC subsets and consequently determines the outcome of T cell response in OVA-induced allergic airway inflammation needs to be further elucidated.

In this study, we examined the migration-related properties of different lung DC subsets in mice in the context of OVA sensitization and Flt3L treatment. The involvement of Flt3L in DC migration helps further understand the underlying mechanisms of its therapeutic effect in allergic airway inflammation and asthma.

Materials and Methods

Mice and treatment protocols

Four- to 5-wk-old female BALB/c mice were purchased from Harlan Laboratories and maintained under specific pathogen-free conditions at Creighton University. All of the animal experiments conducted in this study were approved by the Institutional Animal Care and Use Committee of Creighton University. The sensitization and treatment protocol followed was previously described (15), as shown in Fig. 1B. Briefly, mice were divided into sensitized and nonsensitized groups. Sensitized groups received 20 μ g of grade V chicken egg OVA i.p. (Sigma-Aldrich) emulsified in 2.25 mg of Imject alum (Thermo Scientific) on days 1 and 14. Nonsensitized mice received sterile PBS i.p. The mice were challenged with either 1% OVA aerosol or PBS for 3 consecutive days starting on day 28. After establishing airway hyperresponsiveness to methacholine, as reported previously (15), sensitized mice were randomized into two groups, one of which was given daily 5 μ g of Flt3L i.p. from days 34 to 43. The other sensitized group received sterile PBS i.p. Nonsensitized mice were randomized into two groups and received either daily 5 μ g of Flt3L i.p. or the same volume of PBS i.p. from days 34 to 43. On day 44, all mice underwent OVA challenge. Lungs were collected to isolate DCs.

Intranasal delivery of AlexaFluor 647-conjugated OVA

Fluorochrome-conjugated OVA Ag was intranasally injected into OVA-sensitized and OVA-challenged mice on day 45 (Fig. 1A) using a revised noninvasive method based on previously described intranasal delivery of OVA Ag (23). Briefly, AlexaFluor 647-conjugated OVA (catalog 0-34784;

Invitrogen, Molecular Probes) was reconstituted at a concentration of 5 mg/ml in sterile PBS. Mice were completely anesthetized with 80 μ g of pentobarbital per animal (80 μ g/20 g body weight) and were held in a vertical position perpendicular to the bench. The mouse tongue was pulled out and held gently using moisture tweezers to prevent pharyngeal reflexion in response to the inhaled OVA solution. A total of 30 μ l (150 μ g) of OVA solution was delivered intranasally during the inspiration phase of a respiratory period. The mouse position and tongue were held for an additional 60 s to ensure the entry of the Ag solution into the lungs. Cough was usually observed as a consequent sign of a successful delivery. The mouse was then returned to the cage and laid on the cotton bedding at lateral position to keep body temperature constant and to prevent suffocation, which is followed by at least 2-h observation until the recovery from anesthesia. The mice were euthanized, and the lungs/mediastinal LN were collected at 48 h after the Ag delivery. Lung DCs that have taken up OVA-Ag exhibited fluorescence at the wavelength of 660 nm. The Ag-carrying cells were detected by flow cytometry after isolation procedures described in the following paragraphs.

Preparation of CD11c⁺ cells from lung and mediastinal LN

After euthanization of mice on day 45 or 47, the lungs and mediastinal LN were collected, cut into small fragments, and digested with 5 ml of collagenase D (1 mg/ml) in RPMI 1640 containing 1 mg/ml DNase (Sigma-Aldrich) at 37°C for 1 h. The digested lung and LN tissues were dissociated by repeated pipetting using a 1-ml syringe. A 40- μ m cell strainer (BD Biosciences) was used to filter the dissociated lung and LN tissues to obtain the cell suspensions. The CD11c⁺ lung cells were positively selected using an anti-mouse CD11c magnetic microbeads kit (CD11c N418, catalog 130-052-001; Miltenyi Biotec) and AutoMACS (Miltenyi Biotec), according to the protocol provided by the manufacturer (15).

FACS and flow cytometry analysis

Ab titrations were performed to determine the optimal Ab dilution for the cell staining. The volume of the Ab was adjusted according to the number of the cells counted in each sample. Presorted CD11c⁺ lung and mediastinal LN cells were collected and counted. The cells were resuspended in PBS supplemented with 4% FBS and incubated with the fluorochrome-conjugated Abs for 30 min on ice. The Abs used were as follows: CD11c PE-Cy7, CD11b PE-Cy5, CD86 PE, CD80 FITC, CD40 biotin, CCR5 biotin, CCR6 AlexaFluor 647, MHCII Alexa 700, and CCR7 Alexa 700 (eBioscience); goat anti-CCR2 (Abcam); and rabbit anti-goat IgG1 Ab conjugated with FITC (Abcam) as the secondary Ab. The cells bound to biotin-conjugated primary Ab were incubated with streptavidin-conjugated allophycocyanin (eBioScience). Samples were washed with ice-cold PBS supplemented with 4% FBS before fluorescence signal acquisition and cell sorting using a FACSARIA sorter (BD Biosciences). The cell gate was first defined based on forward and side scatter in microbeads-separated lung and LN CD11c⁺ cells to exclude cell debris and lymphocytes. MHCII⁺ cells were then gated within the cell gate. Lung DC subsets were further defined within MHCII⁺ cell gate based on the expression of CD11c and CD11b, as reported previously (15). Ag-carrying cells were defined as the cells that exhibit fluorescence at the wavelength of 660 nm. Cells isolated from the mice that did not receive intranasal delivery of AlexaFluor 647-conjugated OVA were non-Ag-carrying cells, and thus served as controls.

DC cytology and microscopic study

FACS-sorted lung DCs were centrifuged and resuspended in PBS. Morphology was examined and photographed using a phase-contrast microscope (Nikon Instruments). Cytospin slides were obtained using a Shandon Cytospin 4 centrifuge machine (Thermo Electron). Cytospin slides of lung cell populations after anti-CD11c magnetic microbeads separation were stained using a H&E staining kit (Newcomer Supply).

LN histology

Mediastinal LN were removed, fixed in 4% formalin, and embedded in paraffin in an automatic tissue processor. The fixed LN tissues were sectioned at 5- μ m thickness for immunohistochemistry analysis of CCL19 and CCL21. Deparaffinization, rehydration, and Ag retrieval were done before immunostaining. Goat anti-mouse CCL19 and CCL21 primary Abs (Abcam) were used at 1/200 dilution. Immunohistochemistry staining for CCL19 and CCL21 was performed using VECTOR ABC kit for goat (Vector Laboratories). Goat anti-mouse CCL19 and CCL21 primary Abs (Abcam) were used at 1/200 dilution. The LN tissue sections were counterstained with Gill 2 hematoxylin (Statlab). The tissue sections were then dehydrated using concentration gradient of ethanol and xylene. Following dehydration, the slides were mounted with mounting medium and coverslip.

Chemotaxis assay

For chemotaxis assays, 1×10^5 cells in 100 μ l of RPMI 1640 (Sigma-Aldrich) were added in the upper chamber of a Transwell (Corning Glass; catalog 3421, 6.5 mm diameter, 5.0 μ m pore size). Six hundred microliters of RPMI 1640 containing 100 ng/ml CCL19 or CCL21 (PeproTech) were added to the lower chamber. The cells were incubated for 6 h at 37°C. Trypsinization was used to detach the cells from the membrane. The cells that migrated through the membrane to the lower chamber of the Transwell were counted using a phase hemocytometer (Hausser Scientific). The chemotactic index, a measure of the specificity of migration, was calculated as follows: (number of cells migrating to chemokines)/(number of cells that migrated to medium alone).

Measurement of cytokine secretion by LN cells

The dissected mediastinal LN from OVA-sensitized and Flt3L-treated/OVA-sensitized mice were processed using the method mentioned above to obtain LN cell suspension. The cells were counted using a Beckman Coulter counter. The LN cells were resuspended at a density of 1×10^6 /ml in RPMI 1640 (Sigma-Aldrich) supplemented with 10% heat-inactivated FBS and penicillin-streptomycin and incubated in a Cellstar 12-well suspension culture plate (Greiner Bio-One) at 37°C for 72 h. Culture supernatant was collected for the measurement of secreted cytokines. Cytokine levels (IFN- γ , IL-2, IL-4, IL-10, and IL-12 (p70)) in the culture supernatant were determined using commercially available ELISAs (eBioscience), according to the manufacturer's protocol. The sensitivity of each assay was as follows: 15 pg/ml for IFN- γ , 2 pg/ml for IL-2, 4 pg/ml for IL-4, 30 pg/ml for IL-10, and 2 pg/ml for IL-12 (p70).

Data processing and statistical analysis

Flow cytometric data were processed and analyzed using Flowjo software (Tree Star). GraphPad (GraphPad Software) was used to conduct statistical analysis. Unpaired Student's *t* test was used to determine differences between two groups. Multiple group comparison was made using one-way ANOVA with the Bonferroni correction. A value of $p < 0.05$ was considered significant. Values are expressed as means $\pm$ SEM, if not indicated otherwise.

Results

Phenotype and morphology of lung DC subsets

We have previously reported (15) that two lung DC subsets, CD11c^{high}CD11b^{low} and CD11c^{low}CD11b^{high} DCs, are present in the mouse lung, as shown in Fig. 2. We also observed CD11c^{int}CD11b[−] and CD11c^{−/low}CD11b^{high} cells in a dot plot by CD11c vs CD11b, representing plasmacytoid DCs, as we previously reported (15), and granulocytes, respectively. Histology staining with H&E and phase-contrast microscopy demonstrated that CD11c^{high}CD11b^{low} and CD11c^{low}CD11b^{high} DCs had typical long dendrites and were morphologically different from CD11c-expressing plasmacytoid DCs and CD11b-expressing granulocytes such as neutrophils and eosinophils (Fig. 2).

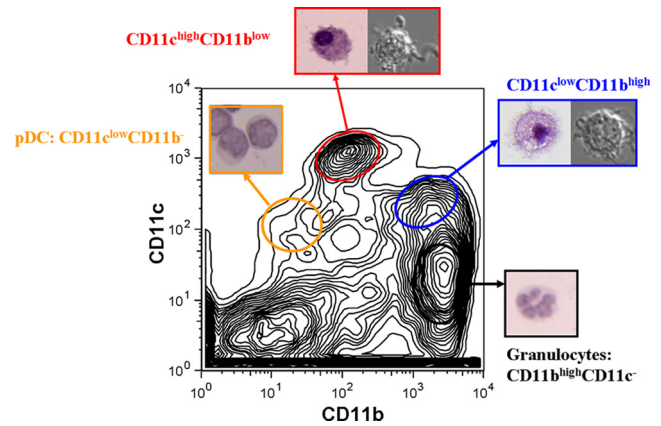


FIGURE 2. Phenotype and morphology of lung DC subsets. Lung DC subsets and other cell populations in a CD11c vs CD11b dot plot. Four cell populations were gated and sorted using BD FACSARIA cell sorter. Cytopsin slides were stained with H&E. CD11c^{high}CD11b^{low} and CD11c^{low}CD11b^{high} DCs had typical long dendrites and were morphologically different from CD11c-expressing plasmacytoid DCs and CD11b-expressing granulocytes, such as neutrophils and eosinophils. (Images and figure are the representatives of at least three independent experiments.)

Flt3L modifies the expression of chemokine receptors and costimulatory molecules in lung CD11c^{low}CD11b^{high} DCs in OVA-sensitized mice

CD11c^{low}CD11b^{high} lung DCs had higher expression of CCR5, CCR6, and CCR7, and low CCR2 expression in comparison with CD11c^{high}CD11b^{low} DCs regardless of the treatment. OVA sensitization and challenge remarkably up-regulated the expression of the chemokine receptors responsible for recruiting DC precursors, including CCR2, CCR5, and CCR6 in both CD11c^{high}CD11b^{low} and CD11c^{low}CD11b^{high} lung DCs (Fig. 3A), although there was a significant decrease in the numbers of CD11c^{high}CD11b^{low} DCs in OVA-sensitized and OVA-challenged mice (15). Interestingly, although Flt3L treatment significantly increased CD11c^{high}CD11b^{low} lung DCs, it did not up-regulate the expression of CCR2, CCR5, and CCR6 in this lung DC subset (Fig. 3A). Nevertheless, the expression of CCR5 in the CD11c^{low}CD11b^{high} lung DCs was down-regulated by Flt3L treatment in OVA-sensitized mice (Fig. 3A), which is well correlated with a decreased number of CD11c^{low}CD11b^{high} lung DCs. In addition, CD11c^{low}CD11b^{high} lung DCs had a less mature phenotype characterized by down-regulated expression of CD40, CD86, and CCR7 in Flt3L-treated/OVA-sensitized mice as compared with those in OVA-sensitized mice (Fig. 4).

Flt3L modifies the expression of lymphatic chemokine secretion in mediastinal draining LN

As shown in Fig. 3B, the expression levels of CCL21 and CCL19 were up-regulated by OVA sensitization in the T cell-rich zone of the cortex, and CCL21 was up-regulated also in afferent lymphatic vessels and high endothelial venules in the mediastinal LN. In Flt3L-treated/OVA-sensitized mice, the expression of CCL19 and CCL21 appears to be down-regulated compared with their expression in OVA-sensitized mice, as measured by immunohistochemistry (Fig. 3B).

CD11c^{low}CD11b^{high} lung DCs in Flt3L-treated/OVA-sensitized mice demonstrate impaired migratory capacity to lymphatic chemokines in vitro

We next examined the migratory capacity of lung DCs in response to lymphatic chemokines in OVA-sensitized and Flt3L-treated/

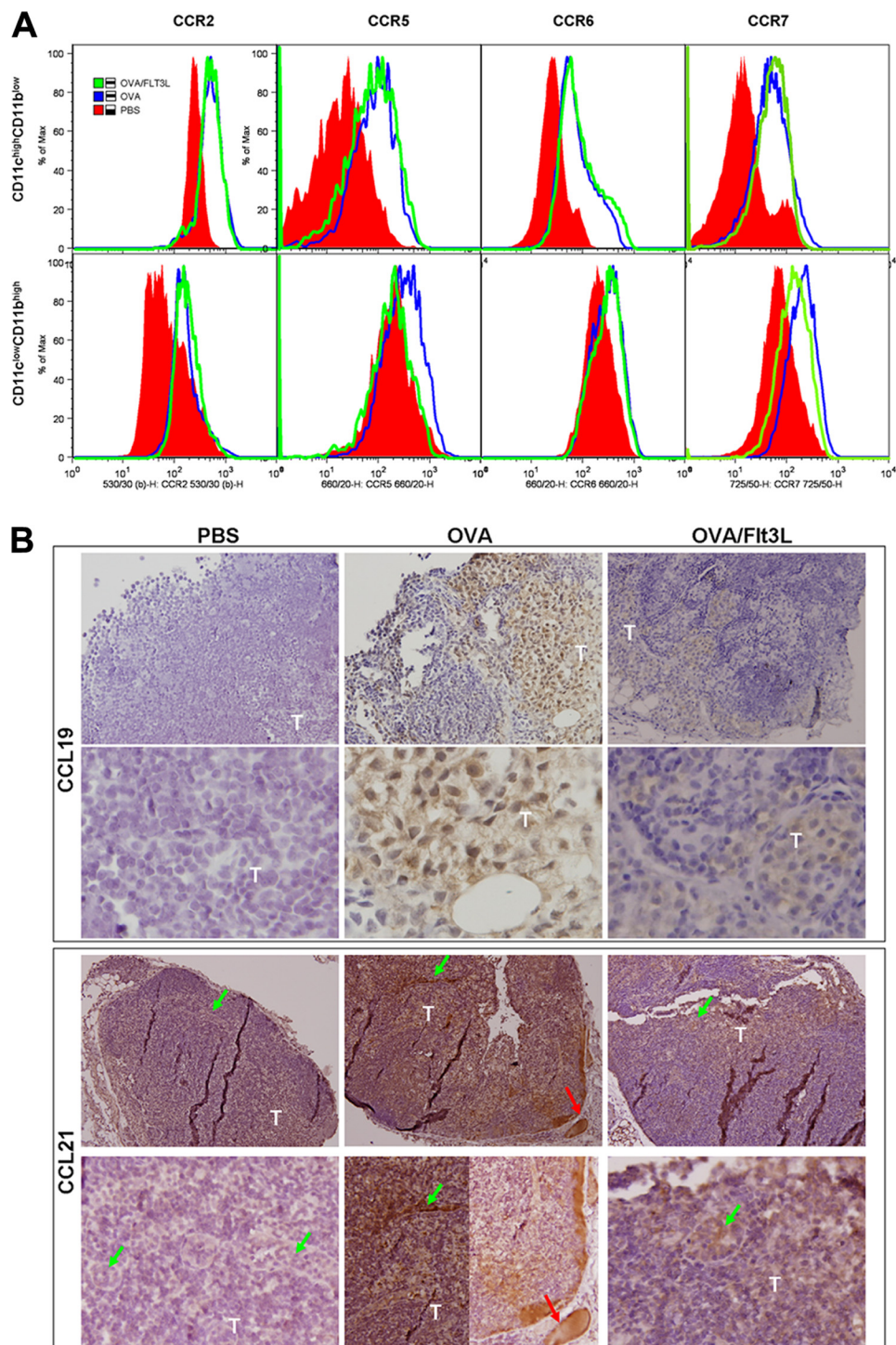


FIGURE 3. Expression of chemokine receptors and lymphatic chemokines. **A**, Two lung DC subsets, CD11c^{high}CD11b^{low} (upper panel) and CD11c^{low}CD11b^{high} (lower panel) DCs, have different expression levels of chemokine receptors, CCR2, CCR5, CCR6, and CCR7. Flt3L down-regulated the expression of CCR5 and CCR7 in CD11c^{low}CD11b^{high} (lower panel) DCs (histograms are the representative of at least three independent experiments). **B**, Expression of CCL19 (upper panel, $\times 20$) and CCL21 (middle panel, $\times 20$, and lower panel, $\times 20$, with digital zoom-in) was up-regulated by OVA sensitization and down-regulated by Flt3L. Green arrow, afferent lymphatic vessel; red arrow, high endothelial venules; T, T cell-rich zone. (Images are the representative of at least three independent experiments involving three animals.)

OVA-sensitized mice. OVA sensitization significantly enhanced the migratory ability of both CD11c^{high}CD11b^{low} and CD11c^{low}CD11b^{high} lung DCs in response to lymphatic chemokines CCL19 and CCL21 (Fig. 5). Moreover, Flt3L treatment did not alter the migration capacity of CD11c^{high}CD11b^{low} DCs, but significantly decreased the migration activity of CD11c^{low}CD11b^{high} lung DCs in response to lymphatic chemokines in the cells isolated from OVA-sensitized mice (Fig. 5). This is well correlated with an Flt3L-induced down-regulation of CCR7 expression in this DC subset (Fig. 3A). In addition, CCL19 induced a significantly higher degree of transmigration activity than CCL21 for both CD11c^{high}CD11b^{low} and CD11c^{low}CD11b^{high} lung DCs (Fig. 5).

Lung CD11c^{low}CD11b^{high} DCs in Flt3L-treated/OVA-sensitized mice demonstrate inefficient Ag uptake

The efficiency of Ag uptake by the two lung DC subsets was examined in Flt3L-treated/OVA-sensitized and OVA-sensitized mice. Intranasal delivery of fluorochrome-conjugated OVA Ag was used to track the migratory activity of lung Ag-carrying DCs. As shown in Fig. 6A, 48 h after intranasal injection of AlexaFluor 647-conjugated OVA, higher number of Ag-carrying lung cells (71.4%) was detected in the lungs of OVA-sensitized mice than that in Flt3L-treated/OVA-sensitized mice (46.4%). The dot plot of Ag-carrying cells by CD11c vs CD11b revealed that CD11c^{high}CD11b^{low} lung DCs maintained normal

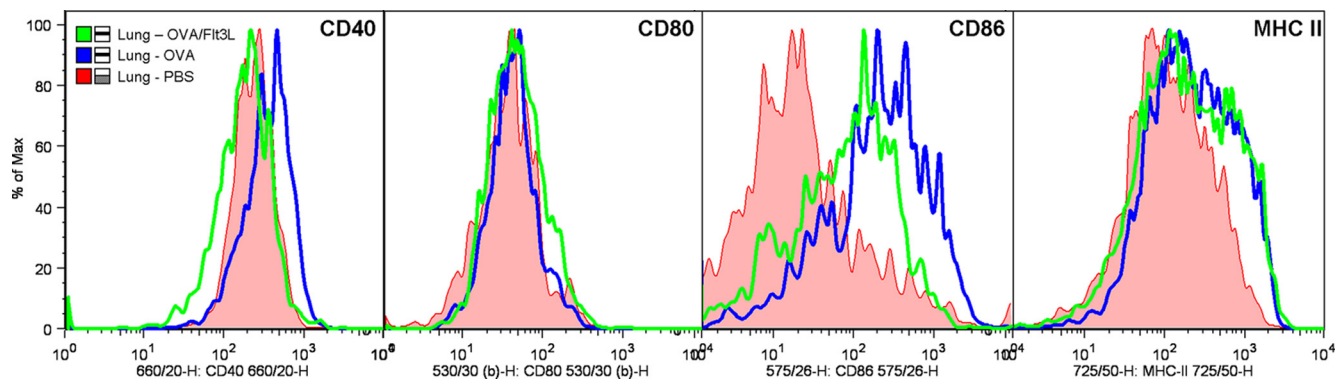


FIGURE 4. Phenotype of $CD11c^{low}CD11b^{high}$ DC subset in Flt3L-treated/OVA-sensitized mice. $CD11c^{low}CD11b^{high}$ DCs isolated from Flt3L-treated/OVA-sensitized mice displayed a less mature phenotype than those from OVA-sensitized mice, as indicated by CD40 and CD86 expression. (The figures are the representative of at least three independent flow cytometric experiments.)

Ag uptake ability in Flt3L-treated/OVA-sensitized mice, but $CD11c^{low}CD11b^{high}$ DCs demonstrated an impaired Ag uptake, as indicated by a decreased number of Ag-carrying cells in this subset in Flt3L-treated/OVA-sensitized mice (Fig. 6A) as compared with OVA-sensitized mice. Interestingly, such difference was not observed between the lung DCs in PBS-treated mice and Flt3L-treated/PBS-treated mice. Regardless of Flt3L treat-

ment, $CD11c^{high}CD11b^{low}$ DCs were more capable at OVA Ag uptake than $CD11c^{low}CD11b^{high}$ lung DCs possibly due to their intrinsic properties (Fig. 6A).

Ag-carrying $CD11c^{low}CD11b^{high}$ DCs in Flt3L-treated/OVA-sensitized mice show impaired migration to mediastinal draining LN in vivo

Both Ag-carrying $CD11c^{high}$ and $CD11c^{low}$ DC subsets were detected in mediastinal draining LN with less Ag-carrying cells detected in Flt3L-treated/OVA-sensitized mice primarily due to less Ag-carrying DCs in the lungs (Fig. 6B). Flt3L did not alter the migratory efficiency of $CD11c^{high}CD11b^{low}$ lung DCs because they were still the majority of the Ag-carrying cells in LN (Fig. 6B). The $CD11c^{low}CD11b^{high}$ Ag-carrying DCs, however, demonstrated impaired migration to the LN in Flt3L-treated/OVA-sensitized mice at 48 h after Ag exposure (Fig. 6B) as shown by a cell frequency of 5.66% in Flt3L-treated mice in contrast to a cell frequency of 27.7% in OVA-sensitized mice. This might be a consequence of down-regulated CCR7 expression by Flt3L treatment (Fig. 3A). However, in PBS-treated and Flt3L-treated/PBS-treated mice, no such change was observed. Flt3L treatment did not alter the migratory pattern of both lung DC subsets in PBS-treated mice (Fig. 6B).

The LN cells in Flt3L-treated/OVA-sensitized mice demonstrate a Th1-prone immune response

The entire cell fraction freshly isolated from mediastinal draining LN was cultured in RPMI 1640 supplemented with 10% FBS for 72 h to mimic overall in vivo cytokine production in LN. As shown in Fig. 7, LN cells isolated from OVA-sensitized/Flt3L-treated mice secreted significantly higher levels of IL-2, IL-10, IL-12(p70), and IFN- γ . No difference in IL-4 levels was detected between the groups. The shift in cytokine production from Th2 to Th1 was associated with the change in numbers of migratory DCs in mediastinal LN.

Discussion

The Flt3L reverses airway inflammation and airway hyperresponsiveness by increasing a Th1-prone regulatory lung DC subset and decreasing a Th2-prone lung DC subset (15). In this study, we defined the role of DC migration and Ag uptake by lung DC subsets in the context of OVA sensitization and the Flt3L-induced therapeutic effect in allergic airway inflammation.

The two lung DC subsets we reported previously demonstrate typical phenotypic and morphological characteristics of DCs, such as long dendrites, coexpression of costimulatory molecules, MHCII, and chemokine receptors. DC maturation is accompanied by a gradual

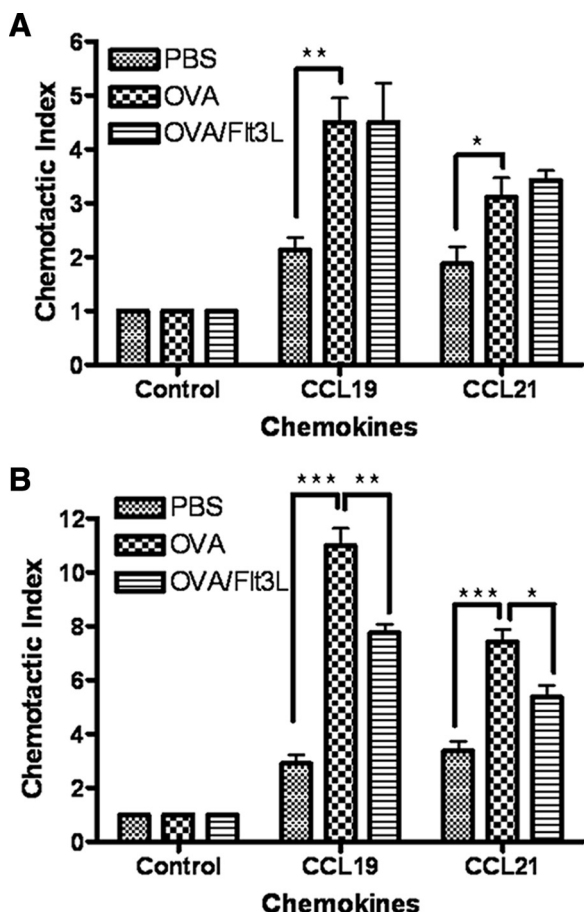


FIGURE 5. In vitro migration assay of lung DCs. The migratory capacity of lung DC subsets in response to lymphatic chemokines, CCL19 and CCL21, was determined in vitro using Transwell. $CD11c^{high}CD11b^{low}$ DC subset (A) is less capable in migrating to lymphatic chemokines than $CD11c^{low}CD11b^{high}$ DC subset (B). $CD11c^{low}CD11b^{high}$ DCs demonstrated an impaired migration to lymphatic chemokines in Flt3L-treated/OVA-sensitized mice (***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$, $n = 4$).

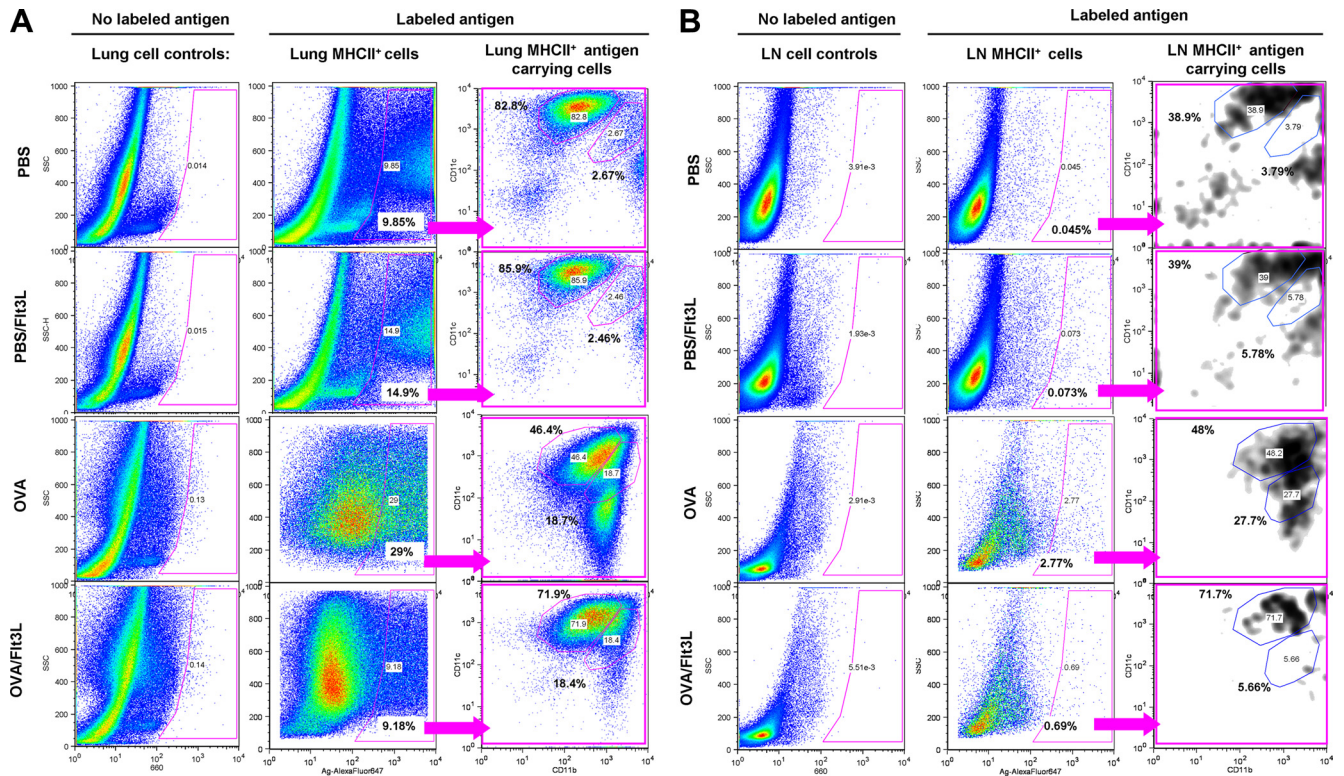


FIGURE 6. Phenotype and migration of Ag-carrying DCs. MHCII⁺ lung cells were gated first and plotted by side scatter vs Ag-AlexaFluor 647. Lung and LN Ag-carrying cells were gated based on the controls: lung or LN cells isolated from the mice that did not receive intranasal injection of AlexaFluor 647-conjugated OVA Ag (wavelength 660). **A**, Lung Ag-carrying MHCII⁺ cells in PBS-treated, Flt3L-treated/PBS-treated, OVA-sensitized, and Flt3L-treated/OVA-sensitized mice. **B**, LN Ag-carrying MHCII⁺ cells in PBS-treated, Flt3L-treated/PBS-treated, OVA-sensitized, and Flt3L-treated/OVA-sensitized mice. (The figures are the representative of three independent experiments involving three animals in each treatment group.)

up-regulation of costimulatory molecules and adhesion molecules, which can be observed in dot plot. However, the expression of CD11c, CD11b, CD8 α , CCR7, MHCII, CD40, CD80, and CD86 is distinctly different between the two lung DC subsets (15), and does not demonstrate a gradual increase from one DC subset to the other. Therefore, the two lung DC subsets do not appear to be immature and mature states of the same DC population.

The increased number of CD11c^{high}CD11b^{low} lung DC subset (15) in Flt3L-treated/OVA-sensitized mice seems to be solely due to the hematopoietic effect of Flt3L administration, but not the regulation of chemokine receptor expression. Although OVA sensitization and a Th2-dominated milieu remarkably up-regulate the expression of chemokine receptors CCR2, CCR5, and CCR6 in CD11c^{high}CD11b^{low} lung DCs, we did not observe an increased number of this DC subset in OVA-sensitized mice. In addition, the increased number of CD11c^{high}CD11b^{low} lung DCs was not associated with the expression levels of chemokine receptors in Flt3L-treated/OVA-sensitized mice. These findings suggest that the expression of chemokine receptors is selectively regulated by Ag

sensitization in this mouse model, whereas the inflammatory cytokine-induced hematopoiesis contributes more to the dynamics of lung DC recruitment than the expression of chemokine receptors.

A decreased number of CD11c^{low}CD11b^{high} DCs in the lungs of Flt3L-treated/OVA-sensitized mice is well correlated to the decreased expression levels of CCR5, a crucial chemokine receptor in recruiting myeloid lung DCs in allergic asthma. The down-regulation of CCR7, CD40, and CD86 also appears to be an Flt3L-dependent effect. The less mature phenotype of CD11c^{low}CD11b^{high} DCs in Flt3L-treated/OVA-sensitized mice was consistent with the functional properties of these cells, including inefficient Ag uptake and impaired migratory potential in response to lymphatic chemokines, CCL19 and CCL21, in Flt3L-treated/OVA-sensitized mice. The inefficiency Ag uptake has been reported in spleen DCs in Flt3L-treated mice (12), and our group has reported that the Flt3L-generated lung and spleen DCs exhibit a semimature phenotype indicated by fewer dendrites and cytoplasmic veils (24). These findings somewhat contradict the traditional belief that Flt3L facilitates the DC maturation. However, it should be noted that Flt3L as a

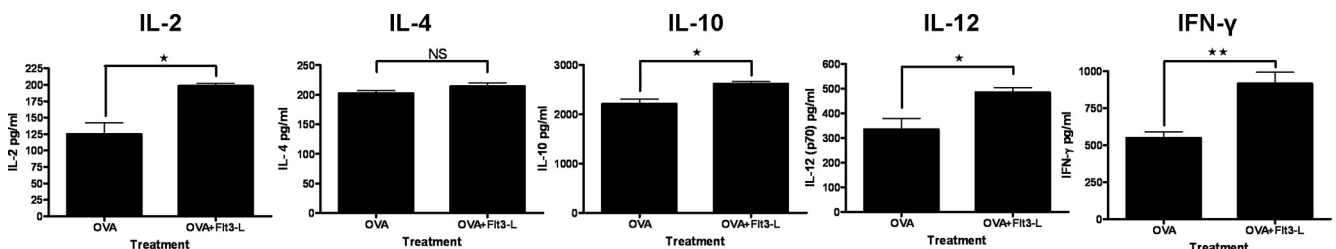


FIGURE 7. Cytokine production by mediastinal LN cells. The whole LN cell suspension was cultured for 72 h, and the supernatant was collected for the measurement of IL-2, IL-4, IL-10, IL-12 (p70), and IFN- γ production (**, $p < 0.01$; *, $p < 0.05$; $n = 3-6$).

hematopoietic growth factor primarily regulates the hematopoiesis and may cause DCs to have an underdeveloped phenotype. It is Ag loading that triggers DC maturation. The synergistic effect of Flt3L in combination with Th2 cytokines might contribute to the subtle regulation of DC phenotype in the process of hematopoiesis, which also explains why the similar differential effects on the lung DC subsets in PBS-treated mice were not observed.

The CD11c^{high}CD11b^{low} lung DC subset has better Ag uptake ability, but less efficient migration to lymphatic cytokines, whereas the CD11c^{low}CD11b^{high} DC subset demonstrates opposite properties characterized by less Ag uptake and a fast migratory pattern. The in vitro Ag presentation assay showed that both DC subsets were able to induce T cell proliferation in OVA-sensitized mice (15). Therefore, both DC populations can function as APCs, although the pattern of migration and the amount of Ag uptake might differ. CD11c^{high}CD11b^{low} lung DCs demonstrated better ability to take up Ag, but they do not seem to perform fast migration, as shown by low-level expression of chemokine receptors CCR7 and in vitro/vivo migration assays. On the contrary, the CD11c^{low}CD11b^{high} DC subset appears to serve as fast response inflammatory DCs in allergic asthma because the majority of the lungs DCs in allergic airway inflammation have this phenotype. These cells are efficient in presenting Ag and migration to LN despite a lower amount of Ag loading. Although a greater percentage of CD11c^{high}CD11b^{low} Ag-carrying DCs was present in the lungs of OVA-sensitized mice, only a small percentage of them is able to migrate to the LN in comparison with the CD11c^{low}CD11b^{high} DC subset, most of which seems to function well in migration to LN. Lastly, the differential expression of the chemokine receptor CCR2 also helps define the functional role of CD11c^{high}CD11b^{low} DCs because CCR2 has been thought to play a more important role in DC recruitment in Th1 response (25, 26). Most importantly, Flt3L is shown to play a regulatory role in the multiple aspects of CD11c^{high}CD11b^{low} and CD11c^{low}CD11b^{high} DC subsets, increasing Th1-prone DC subset (15), decreasing the migration and Ag uptake of Th2-prone DC subsets, and finally leading to Th2 suppression. These data are also supported by a Th1-prone cytokine secretion pattern by both LN cells and the cells in DC-T cell coculture system (15).

Flt3 receptor is expressed by hematopoietic precursors, such as common lymphoid and myeloid hematopoietic precursors, and steady-state DCs in peripheral lymph organs, such as the spleen and LN (27). It has been reported that Flt3L-Flt3 interactions are essential to the regulation of homeostatic DC development in the spleen (28). However, alteration of CCR7 expression in lung DCs in Flt3L-treated/OVA-sensitized mice most likely occurs at the stage of the common lymphoid/myeloid precursors because no evidence has reported that DCs in peripheral lymphoid organs migrate to the lung during allergic airway inflammation. The suppressive effect of Flt3L in allergic asthma is not only the result of an impaired migration to lymphatic chemoattractants in Flt3L-generated lung DCs, but it is also linked to a decrease in the secretion of CCL19/CCL21 by LN cells. This suppressive effect is controlled and influenced by the systemic cytokine milieu, which is dominated by the types of immune response induced such as Th1, Th2, or tolerance, and possibly IL-10 in the case of Flt3L-induced therapeutic effect in allergic asthma. Therefore, the modification of CCL19/CCL21 expression seems to be an indirect result of Flt3L administration.

The therapeutic effect of Flt3L is not only limited to the OVA-induced mouse model of allergic airway inflammation, but it has been observed in a clinically relevant cockroach-induced mouse model of asthma (29). Our study helps define the role of Th1 activation in counteracting Th2 response in allergic asthma. If such functional, distinct lung DC subsets are present in human asthmatic patients and

have similar responses to superphysiological levels of Flt3L, Flt3L could be an optional immunomodulator for asthma.

The result of this study demonstrates that Flt3L generated a Th1-prone lung DC subset in asthmatic mice solely through its synergistic hematopoiesis effect, but not through regulating the expression of chemokine receptors. The CD11c^{low}CD11b^{high} DC subset in the lungs displays a less mature phenotype in Flt3L-treated/OVA-sensitized mice, which is responsible for an impaired migration and Ag uptake.

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Disclosures

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