

Fms-like tyrosine kinase 3 ligand increases a lung DC subset with regulatory properties in allergic airway inflammation

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Background: Dendritic cell (DC) subsets display different functional roles in regulating immune responses and lead to various outcomes, including T_H1 versus T_H2 or regulatory versus immunologic responses. Administration of Fms-like tyrosine kinase 3 (Flt3) ligand prevents and reverses allergic airway inflammation and airway hyperresponsiveness in a mouse model. However, the underlying mechanisms are unclear. **Objective:** We characterized and examined the role of lung DC subsets in the therapeutic effect of Flt3 ligand.

Methods: DCs were isolated from the lungs of ovalbumin (OVA)-sensitized and OVA-challenged mice treated with recombinant human Flt3 ligand. Two populations of CD11c⁺ cells labeled with fluorochrome-conjugated antibodies were sorted. The ability of the purified cells to stimulate T-cell proliferation and cytokine secretion patterns by different DC subsets was examined. Also, DCs were adoptively transferred in mice to examine their effect on pulmonary function.

Results: Two DC populations, CD11c^{high}CD11b^{low} and CD11c^{low}CD11b^{high}, were identified in the lungs of naive and OVA-sensitized and OVA-challenged mice with and without treatment with Flt3 ligand. The expression levels of CD8 α , B220, CD19, F4/80, MHC II, CCR7, CD40, programmed death ligand 1, programmed death ligand 2, CD80, and CD86 were distinctly different between the 2 DC populations, which supports the notion that CD11c^{high}CD11b^{low} and CD11c^{low}CD11b^{high} DCs potentially have regulatory and immunogenic properties, respectively. Administration of Flt3 ligand increased the DCs with regulatory potential in the lungs of antigen-sensitized mice, and CD11c^{high}CD11b^{low} DCs acquired a maximum degree of regulatory capacity after Flt3 ligand treatment.

Conclusion: These data suggest that Flt3 ligand reverses airway hyperresponsiveness by regulating the function of lung DCs in a mouse model of allergic airway inflammation. (*J Allergy Clin Immunol* 2009;123:917-24.)

Key words: Adoptive transfer, airway hyperresponsiveness, asthma, dendritic cells, Flt3 ligand, mouse model, T-cell proliferation, regulatory dendritic cells

Abbreviations used

AHR:	Airway hyperresponsiveness
BALF:	Bronchoalveolar lavage fluid
DC:	Dendritic cell
FITC:	Fluorescein isothiocyanate
Flt3:	Fms-like tyrosine kinase 3
OVA:	Ovalbumin
PD1:	Programmed death 1
PDL1:	Programmed death ligand 1
PDL2:	Programmed death ligand 2
PE:	Phycerythrin

Asthma is an inflammatory airway disease characterized by airway eosinophilia, airway obstruction caused by an increased mucus production by goblet cells, airway remodeling, and airway hyperresponsiveness (AHR) to a variety of stimuli.^{1,2} In allergic airway inflammation antigen-presenting cells take up specific antigens and induce naive T cells to differentiate into T_H2 cells, which release cytokines, including IL-4, IL-5, and IL-13, governing immune responses. Dendritic cells (DCs) are not only professional antigen-presenting cells, they are also a heterogeneous group of cells that differ in origin, location, cell-surface phenotype, and function.³ They are responsible for bringing about immunity and are able to induce immunologic tolerance and determine the type of T cell-mediated immune response (T_H1 or T_H2) after DC/T-cell interaction.⁴ This differentiation is dependent on a number of factors, such as the nature of the antigen, the types and expression levels of costimulatory molecules, the cytokine profile in the milieu, and DC subsets that induce T-cell differentiation.⁵

An emerging concept is that DC subsets differentially skew T-cell responses toward tolerance or immunity. Splenic and mucosal CD8 α ⁺ but not CD8 α ⁻ DCs tolerize T_H2 responses,^{6,7} and CD8 α ⁻ DCs are more effective than CD8 α ⁺ DCs in inducing T_H2 responses and eosinophilic airway inflammation.⁸ Other studies have supported the idea that CD8 α ⁺ DCs are more prone to induce T_H1 responses, whereas CD8 α ⁻ DCs lead to T_H2 responses.^{9,10} The CD11c⁺B220⁺Gr-1⁺ DC subset that also expresses T-cell lineage markers, CD8 α , and CD4 has been identified and appears to be the murine counterpart of IFN- α -producing human plasmacytoid DCs.¹¹ CD8 α ⁺B220⁺ DCs could represent a subset of DCs with regulatory potential involved in the generation of regulatory T cells, and they are also less efficient in supporting CD4⁺ T-cell proliferation.¹² Moreover, they provide intrinsic protection against inflammatory responses to harmless antigen.¹³ A regulatory DC subset derived from IL-10 and TGF- β 1-treated bone marrow cells with a different phenotype from the CD11c⁺B220⁺Gr-1⁺ DC subset can protect against airway inflammation in asthmatic mice.¹⁴ DCs demonstrate a highly flexible phenotype and often accomplish many diverse and opposing functions.

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Fms-like tyrosine kinase 3 (Flt3) ligand is a synergic hematopoietic growth factor that dramatically increases the number of DCs in several tissues.¹⁵ Earlier reports have suggested the expansion of both myeloid (CD11c⁺CD11b⁺CD8 α ⁻) and lymphoid (CD11c⁺CD11b⁻CD8 α ⁺) DC subsets after Flt3 ligand administration.¹⁶ We observed an increase in the number of lung DCs after the administration of Flt3 ligand,^{17,18} and Flt3 ligand prevented and reversed allergic airway inflammation and AHR in a mouse model of asthma.¹⁸⁻²⁰ Additionally, the phenotype and function of DCs might be tissue specific and could vary depending on different isolation techniques used.²¹ Most of the previous research obtained DCs from spleens, bone marrow, or thymus but not from the lungs, where antigen uptake occurs during sensitization and challenge with allergen. Therefore we examined the phenotype and function of lung DCs in response to ovalbumin (OVA) sensitization and Flt3 ligand treatment.

METHODS

Mice

Four- to 5-week-old female BALB/c mice were purchased from Harlan Laboratories (Indianapolis, Ind) 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.

Sensitization and treatment of experimental animals

The sensitization and treatment protocol followed are shown in Fig 1, A. Mice were divided into sensitized and nonsensitized groups. Sensitized groups received 20 μ g of grade V chicken egg OVA (Sigma-Aldrich, St Louis, Mo) intraperitoneally emulsified in 2.25 mg of Inject Alum on days 1 and 14. Nonsensitized mice received sterile PBS intraperitoneally. The mice were challenged with either 1% OVA aerosol or PBS for 3 consecutive days starting on day 28. AHR to methacholine was established (Fig 1, A). Sensitized mice were randomized into 2 groups, one of which was given 5 μ g of Flt3 ligand intraperitoneally from day 34 to day 43. The other group received sterile PBS intraperitoneally. Nonsensitized mice received the same volume of sterile PBS, serving as control animals. On day 44, all mice underwent OVA challenge followed by the measurement of AHR to methacholine on day 45. Bronchoalveolar lavage fluid (BALF) was collected from each animal for cytokine measurement. Lungs were collected to isolate DCs.

Noninvasive and invasive methods for measuring pulmonary function

A noninvasive single-chamber whole-body plethysmograph (Buxco Electronics, Troy, NY) without anesthesia or restraint was used to measure pulmonary functions on day 33, as described in our earlier reports.^{18,22} Enhanced pause, an index of airway obstruction,²³ was calculated. On day 45, anesthetized and tracheostomized mice were placed in a PLY4111-R/C plethysmograph single chamber (Buxco Electronics) and were mechanically ventilated (140 breaths/min; tidal volume, 0.15 mL) by using the Harvard rodent ventilator (model 683; Harvard Apparatus, South Natick, Mass). Anesthesia was administered by means of intraperitoneal injection of sodium pentobarbital (1.6 mg/20 g of body weight). Lung-specific airway resistance²⁴ was measured. In both methods mice were challenged with increasing doses of nebulized methacholine up to 100 mg/mL to measure AHR.

Isolation of lung DCs

After the mice were killed, the lungs were collected, finely chopped, 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 hour. Pellets were resuspended in 1 mL of MACS buffer (PBS supplemented with 0.5% BSA), and the cells were counted in a Beckman Coulter counter (Beckman Coulter, Fullerton, Calif). To isolate DCs, the cells were first incubated for 5 minutes with an Fc block (BD PharMingen, San Jose, Calif), followed by incubation for 30 minutes on ice with 100 μ L of CD11c microbeads (Miltenyi Biotec, Auburn, Calif) for every 100 million cells counted. The cells underwent a double-positive selection with the POSSELDs program in the AutoMACS (Miltenyi Biotec). The samples were again passed through the AutoMACS to increase the purity.

Flow cytometry of lung DCs

Antibody titrations were performed to determine the optimal antibody dilution for cell staining. The volume of the antibody was adjusted according to the numbers of cells counted in each sample. CD11c⁺ cells were collected and counted. The cells were resuspended in PBS supplemented with 4% fetal bovine serum and incubated with the following 4 antibody cocktails for 30 minutes on ice: (1) CD11c–phycoerythrin (PE), CD11b–fluorescein isothiocyanate (FITC), B220–biotin or B220–PE–Cy7, and CD8 α –peridinin chlorophyll protein complex (BD PharMingen); (2) CD11c–PE–Cy7, CD11b–PE–Cy5, programmed death ligand 1 (PDL1)–biotin, programmed death ligand 2 (PDL2)–FITC, and CD86–PE (eBioScience, San Diego, Calif); (3) CD11c–PE–Cy7, CD11b–PE–Cy5, CD80–FITC, and CD40–biotin or CCR7–Alexa700; and (4) CD11c–PE–Cy7, CD11b–PE–Cy5, F4/80–allophycocyanin or plasmacytoid dendritic cell antigen (PDCA)-1–biotin, CD19–FITC or CD8 α –FITC, CD135–PE, and MHCII–Alexa700 (eBioScience). The cells bound to biotin-conjugated primary antibody were incubated with streptavidin-conjugated allophycocyanin fluorochrome. Samples were washed by means of addition of ice-cold PBS supplemented with 4% fetal bovine serum and stored before sorting with the FACSARIA sorter (BD PharMingen). The majority of lung macrophages with high autofluorescence were distributed along the diagonal line of the dot plot and were excluded from gating of the 2 DC populations.

Adoptive transfer of lung DC subsets

CD11c^{high}CD11b^{low} Lung DCs (4×10^5 in 50 μ L of PBS) obtained from the lungs of animals from the 3 treatment groups (naive, OVA-sensitized and OVA-challenged, and OVA-sensitized, OVA-challenged, and Flt3 ligand-treated mice) were injected into the tail veins of OVA-sensitized and challenged mice on day 33 (Fig 1, B). The same number of CD11c^{low}CD11b^{high} DCs obtained from OVA-sensitized and OVA-challenged mice treated with Flt3 ligand and were also administered to sensitized mice in the same way. On day 45, mice received 5% OVA challenge followed by methacholine challenge to reassess AHR to methacholine by using the non-invasive method.

T-cell proliferation assay

DCs were resuspended in RPMI-1640 (Biowhittaker, Rockland, Me) supplemented with 10% heat-inactivated FBS and penicillin-streptomycin and incubated with OVA (100 μ g/mL) for 2 hours at 37°C. Cells were washed with the medium and incubated with mitomycin C (final concentration, 25 μ g/mL; Sigma-Aldrich). The DCs were cocultured with CD4⁺ T cells (equal ratio) isolated from the spleens of PBS-treated mice in a 96-well microtiter plate for 5 days at 37°C in a 5% CO₂ incubator. During the last 24 hours of coculture, cells were labeled with BrdU (Roche Diagnostics, Indianapolis, Ind). Incorporation of BrdU in the proliferating cells was detected by using a BrdU antibody-enzyme conjugate followed by addition of the substrate. The absorbance of the samples was measured at 450 nm, with a reference wavelength of 690 nm. Culture supernatants were collected for the measurement of secreted cytokines.

Measurement of cytokines in DC/T-cell coculture supernatants and BALF

Cytokine levels (IL-10, IL-12 [p70], and IFN- γ) were determined in the culture or BALF supernatants by using commercially available ELISAs (eBioscience), according to the manufacturer's protocol. The sensitivity of each assay was as follows: 30 pg/mL for IL-10 and 15 pg/mL for IL-12 and IFN- γ .

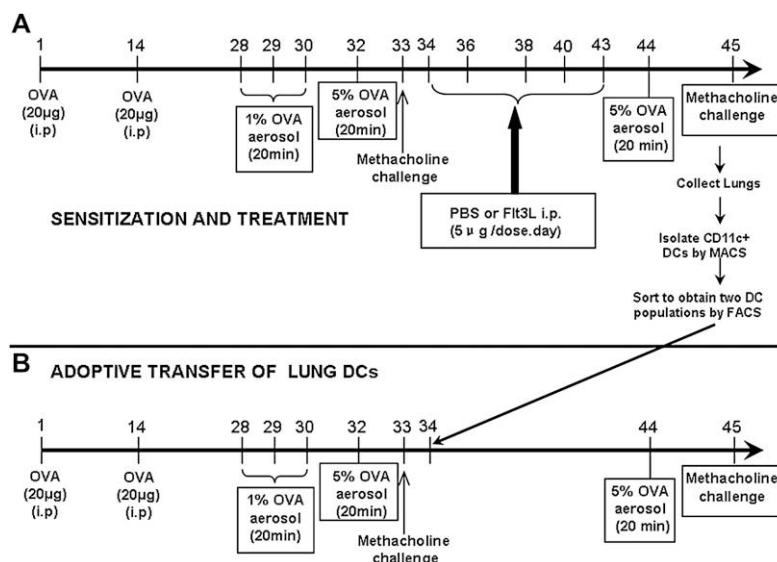


FIG 1. Protocol for sensitization, treatment, and adoptive transfer. **A**, OVA sensitization was followed by methacholine challenge. A total dose of 50 µg of Flt3 ligand (*Flt3L*) was given to each mouse before the final methacholine challenge to reassess AHR. **B**, Adoptive transfer of lung DCs. Two DC populations isolated from 3 treatment groups of mice were adoptively transferred to OVA-sensitized and OVA-challenged mice on day 34. *i.p.*, Intraperitoneal.

Lung histology

Lung lobes were removed, fixed in 4% formalin, and embedded in paraffin in an automatic tissue processor. The fixed tissues were sectioned, rehydrated, and stained with hematoxylin and eosin. Mucus secretion was identified by means of periodic acid–Schiff reaction.

Data processing and statistical analysis

Flow cytometric data were processed and analyzed with FlowJo software (Tree Star, Inc, Ashland, Ore). SPSS 10.0 (SPSS, Inc, Chicago, Ill) and GraphPad (GraphPad Software, Inc, La Jolla, Calif) software were used to conduct statistical analysis. The unpaired Student *t* test was used to determine differences between 2 groups. Multiple-group comparison was made by using 1-way ANOVA with the Bonferroni correction. A *P* value of less than .05 was considered significant. Values are expressed as means ± SEM if not indicated.

RESULTS

Flt3 ligand treatment attenuates AHR to methacholine and airway inflammation

Mice were sensitized according to the protocol (Fig 1, A). AHR to methacholine was established before Flt3 ligand treatment in OVA-sensitized and OVA-challenged mice (see Fig E1 in this article's Online Repository at www.jacionline.org). On day 33, all OVA-sensitized mice showed a significant increase in AHR to methacholine compared with that seen in nonsensitized control animals. Administration of Flt3 ligand reversed AHR on day 45, as measured by using either the noninvasive or the more rigorous invasive method. The AHR to methacholine was significantly less in Flt3 ligand-treated OVA-sensitized and OVA-challenged mice than in OVA-sensitized and OVA-challenged mice (see Fig E1).

CD11c^{high}CD11b^{low} and CD11c^{low}D11b^{high} lung DCs have distinct phenotypic properties

To identify whether different functional DC subsets are present in the lungs of nonsensitized mice, we labeled purified lung CD11c⁺

cells with fluorochrome-conjugated antibodies against CD11c, CD11b, CD8α, B220, F4/80, CD19, MHCII, CD80, CD86, CD40, PDL1, PDL2, and CCR7. Flow cytometric analysis revealed 2 distinct DC populations defined by differential expression of CD11c and CD11b in all 3 treatment groups: CD11c^{high}CD11b^{low} and CD11c^{low}CD11b^{high} (Fig 2, A). The CD11c^{high}CD11b^{low} DCs had significantly higher expression levels of CD8α, B220, CD19, PDL1, PDL2, CD80, CD86 and lower expression levels of F4/80, MHC II, CD40, and CCR7 than the CD11c^{low}CD11b^{high} DCs (Fig 2, B).

Flt3 ligand treatment increases lung CD11c^{high}CD11b^{low} DCs and decreases CD11c^{low}CD11b^{high} DCs in mice with allergic airway inflammation

Sensitized and challenged mice were given intraperitoneal injections of 5 µg of Flt3 ligand for 10 days from day 34 to day 43 (a total of 50 µg of Flt3 ligand) to assess whether OVA sensitization and Flt3 ligand treatment have any effect on the 2 lung DC populations in mice with allergic airway inflammation (Fig 1, A). Sensitized and nonsensitized control animals received the same volume of PBS intraperitoneally. OVA sensitization significantly expanded the CD11c⁺ DC population in the lungs (data not shown), which was consistent with the data in our previous reports.^{17,18} The frequency of each DC population demonstrated a dynamic change in response to OVA sensitization and Flt3 ligand treatment. CD11c^{high}CD11b^{low} DCs in the lungs were predominant (39.54% ± 1.52%) in the nonsensitized control animals and decreased drastically in the lungs of sensitized mice (3.68% ± 0.60%; Fig 2, A and C). After Flt3 ligand treatment, the number of CD11c^{high}CD11b^{low} DCs was restored, and their frequency (24.13% ± 3.17%) was significantly increased (*P* < .001) compared with that seen in the OVA-sensitized group (Fig 2, A and C).

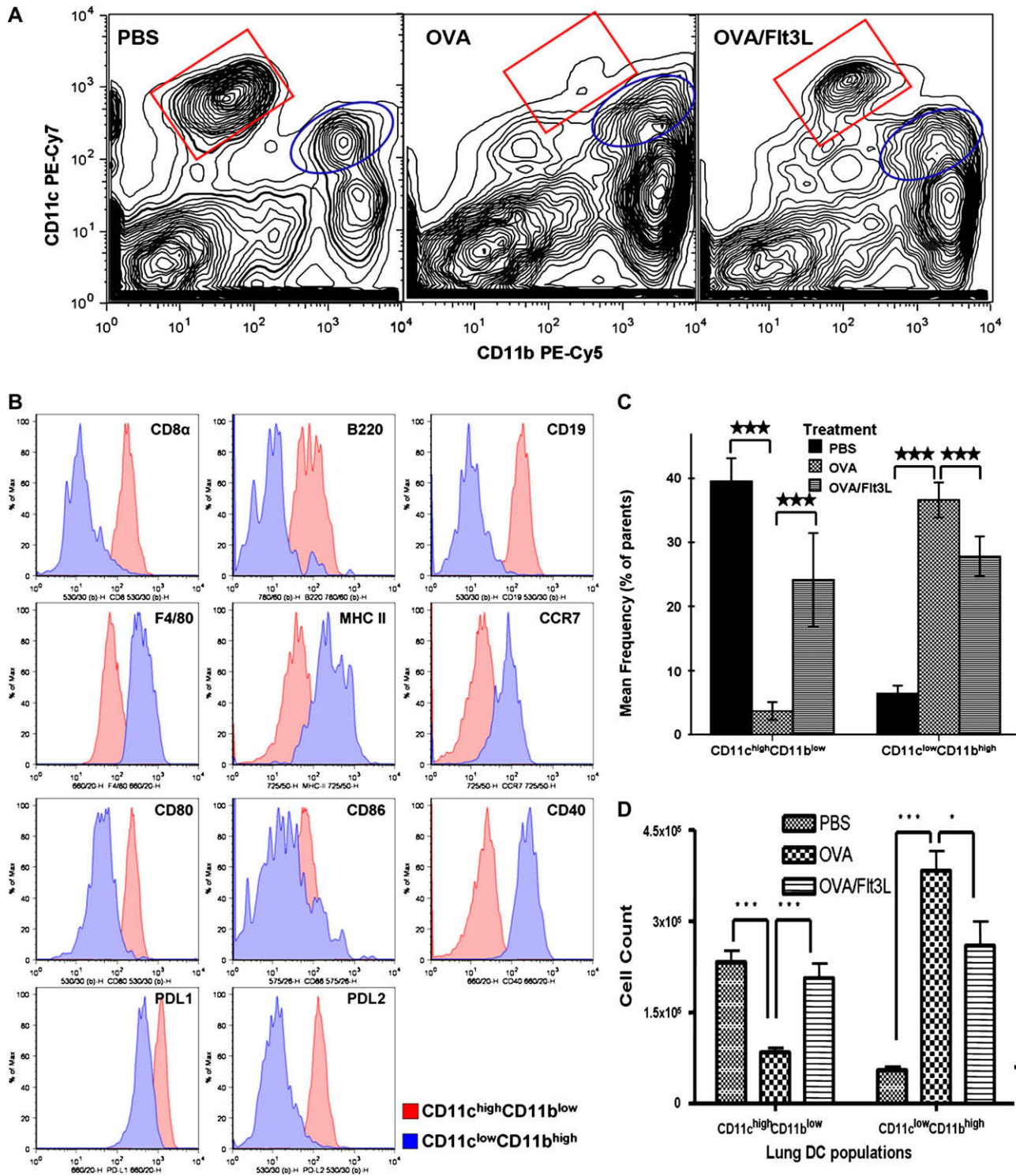


FIG 2. Dynamics and characteristics of lung DC populations. **A**, Two lung DC populations: CD11c^{high}CD11b^{low} (squares) and CD11c^{low}CD11b^{high} (circles; $n = 5$). Flt3L, Flt3 ligand. **B**, Expression of cell-surface markers on the 2 lung DC subsets in PBS-treated control mice ($n = 5$). **C** and **D**, Quantitative analyses of the 2 DC subsets. *** $P < .001$ ($n = 9-15$). Error bars indicate 95% confidence limits.

CD11c^{low}CD11b^{high} DCs were more prominent ($36.62\% \pm 1.19\%$) in the lungs of sensitized mice than in nonsensitized control animals ($6.40\% \pm 0.54\%$; Fig 2, A and C). After Flt3 ligand treatment, the frequency of CD11c^{low}CD11b^{high} DCs in lungs ($27.77\% \pm 1.36\%$) was significantly reduced ($P <$

.001) compared with the number of DCs in the lungs of OVA-sensitized mice ($36.62\% \pm 1.19\%$; Fig 2, A and C). The relative changes in proportion were also reflected by similar changes in absolute number of the 2 lung DC subsets (Fig 2, D).

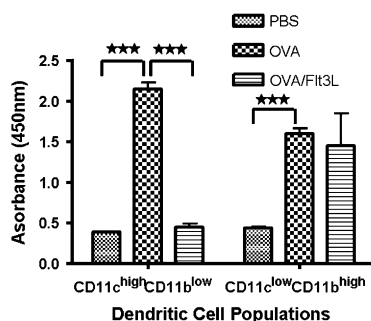


FIG 3. T-cell proliferation by 2 DC populations. CD11c^{high}CD11b^{low} DCs isolated from OVA-sensitized and Flt3 ligand (*Flt3L*)-treated mice showed inability to activate CD4⁺ T-cell proliferation. ****P* < .001, *n* = 5.

CD11c^{high}CD11b^{low} DCs in OVA-sensitized mice lose the ability to induce T-cell proliferation after Flt3 ligand treatment

The 2 distinct populations of DCs were sorted and cocultured individually with naive CD4⁺ T cells for 5 days to measure T-cell proliferation capacity. Both CD11c^{high}CD11b^{low} and CD11c^{low}CD11b^{high} DCs obtained from OVA-sensitized mice showed high capacity in inducing proliferation of naive CD4⁺ T cells (Fig 3). This capacity was diminished in the case of CD11c^{high}CD11b^{low} DCs after Flt3 ligand treatment and is comparable with that seen in nonsensitized control animals. However, CD11c^{low}CD11b^{high} DCs retain the capacity to stimulate naive CD4⁺ T cells *in vitro* after Flt3 ligand treatment (Fig 3). These data suggest that Flt3 ligand treatment can induce the suppressive capability of lung CD11c^{high}CD11b^{low} DCs, at least in part, by causing their inability to induce the proliferation of CD4⁺ T cells.

Cytokine secretion pattern favors T_H2 suppression

In DC/T-cell coculture supernatants, higher levels of IL-12 and IFN- γ were detected in the supernatants of CD11c^{high}CD11b^{low} DCs than in the supernatants of CD11c^{low}CD11b^{high} DCs. There was no significant difference in the IL-12 and IFN- γ levels among the PBS-, OVA-, and Flt3 ligand-treated groups (Fig 4, A-C). Higher levels of IL-10 were observed in the supernatants of CD11c^{high}CD11b^{low} DCs isolated from OVA/Flt3 ligand-treated mice than those from PBS-treated or OVA-sensitized mice. These observations, together with the increased frequency of lung CD11c^{high}CD11b^{low} DCs in Flt3 ligand-treated sensitized mice, suggested that Flt3 ligand treatment might facilitate T_H2 suppression by enhancing T_H1 responses involving IL-10. This was supported by the BALF cytokine levels: the levels of IL-12, IL-10, and IFN- γ in the BALF were significantly higher in Flt3 ligand-treated and OVA-sensitized mice than in mice that were only OVA sensitized (Fig 4, D-F).

CD11c^{high}CD11b^{low} DC subset in OVA-sensitized mice acquires the capability of suppressing AHR on Flt3 ligand treatment

Adoptive transfer of the 2 lung DC subsets was performed (Fig 1, B) to confirm the regulatory capability of lung CD11c^{high}CD11b^{low} DCs and to examine the effect of Flt3 ligand. OVA-sensitized and OVA-challenged mice that received adoptive transfer of CD11c^{high}CD11b^{low} DCs isolated from nonsensitized

mice demonstrated a reduction in AHR compared with those receiving CD11c^{high}CD11b^{low} DCs obtained from sensitized control animals and those receiving CD11c^{low}CD11b^{high} DCs obtained from sensitized and Flt3 ligand-treated mice (Fig 5, A). The maximum attenuation in AHR, however, was achieved in the mice receiving CD11c^{high}CD11b^{low} DCs isolated from sensitized and Flt3 ligand-treated mice (Fig 5, A). Moreover, consistent with adoptive transfer data, lung histologic study showed reduced airway inflammation in the lungs of the mice receiving adoptively transferred CD11c^{high}CD11b^{low} DCs obtained from OVA-sensitized and Flt3 ligand-treated mice compared with recipient mice of other experimental groups (Fig 5, B). Typically, in the lungs of OVA-sensitized and OVA-challenged mice, the inflammatory cells, including eosinophils, neutrophils, and basophils, infiltrate in the periphery of the airways, and mucus hypersecretion by goblet cells is seen. These histologic features of allergic airway inflammation were attenuated in mice receiving CD11c^{high}CD11b^{low} DCs obtained from OVA-sensitized and Flt3 ligand-treated mice (Fig 5, B).

Effect of administration of Flt3 ligand on costimulatory molecules

We next determined whether Flt3 ligand has any effect on the expression levels of costimulatory molecules. The expression of the costimulatory molecules, including CD40, CD80, CD86, PDL1, and PDL2, was upregulated after OVA sensitization. Flt3 ligand treatment, however, differentially affected the expression levels of costimulatory molecules in the 2 DC populations (Fig 6). Administration of Flt3 ligand upregulated CD86 and PDL2 levels in CD11c^{high}CD11b^{low} DCs but downregulated CD40 and CD86 in CD11c^{low}CD11b^{high} DCs in mice with allergic airway inflammation (Fig 6).

DISCUSSION

The purpose of the present study was to determine whether different functional DC subsets are present in the lungs of mice and whether Flt3 ligand affects the generation of a regulatory DC subset in the lungs of OVA-sensitized mice and thus plays a role in regulating allergic immune response.

Here we identified 2 lung DC populations with distinct expression of CD11c, CD11b, CD8 α , B220, CD19, and MHCII molecules. A number of studies have reported that mucosal, splenic, and bone marrow-derived CD8 α ⁺ DCs have T_H2-suppressive capacity,⁶⁻¹⁰ and Flt3 ligand has been shown to preferably generate CD8 α ⁺ conventional DCs in the spleen.²⁵ We observed that CD11c^{high}CD11b^{low} DCs are functionally similar to the mucosal⁷ and splenic⁶ CD8 α ⁺ DCs and are more prone to induce a skewed T_H1 response because of increased release of IL-12 and IFN- γ , which can serve as T_H2 suppressors.²⁶ In addition, higher expression levels of PDL1 and PDL2 of CD11c^{high}CD11b^{low} DCs in Flt3 ligand-treated OVA-sensitized mice suggest that they are able to induce a strong T-cell suppression. In contrast, a higher expression of MHCII, CD40, and CCR7 in CD11c^{low}CD11b^{high} DCs is associated with a better capability to present antigen and to migrate to draining lymph nodes, which is important to elicit a T_H2 response. The *in vivo* functional effects after adoptive transfer of DCs and the *in vitro* T-cell proliferation assay further confirmed that the 2 DC populations, CD11c^{high}CD11b^{low} and CD11c^{low}CD11b^{high} DCs, possess

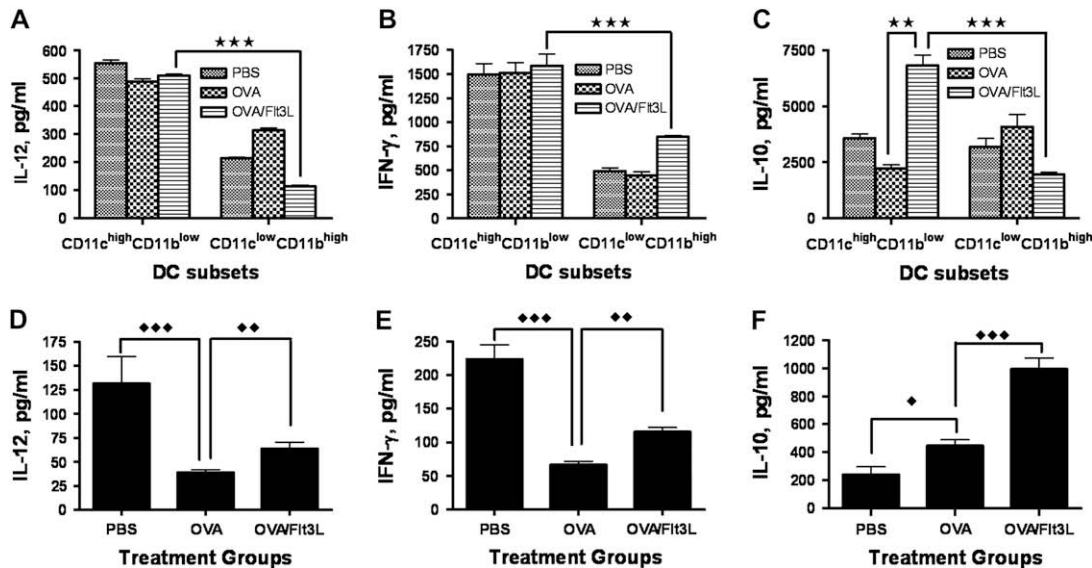


FIG 4. Cytokine secretion patterns in DC/T-cell coculture supernatants and BALF. Levels of IL-12 (A), IFN- γ (B), and IL-10 (C) in DC/T-cell coculture supernatants. D-F, Levels of IL-12 (Fig 4, D), IFN- γ (Fig 4, E), and IL-10 (Fig 4, F) in BALF. *** $P < .001$, ** $P < .01$, * $P < .05$ ($n = 3-6$).

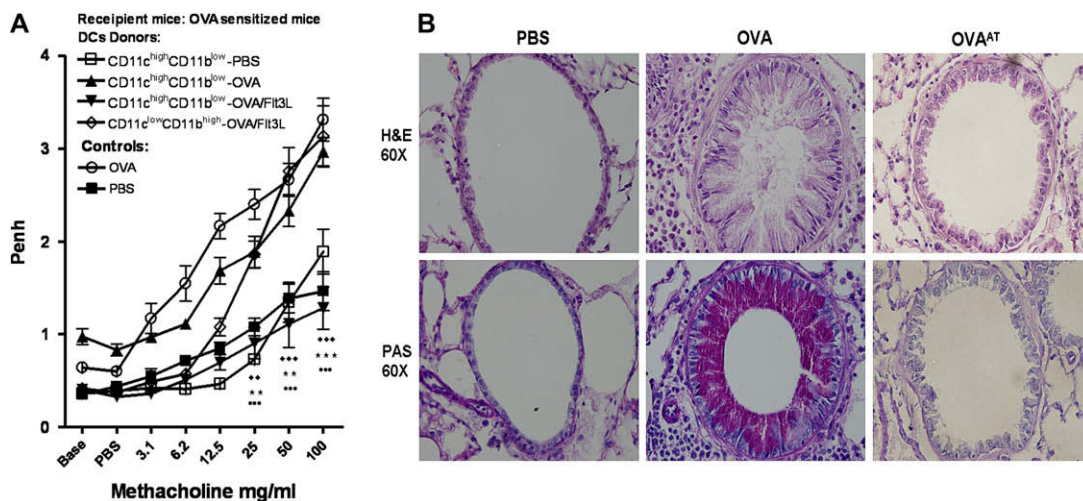


FIG 5. Adoptive transfer of lung DCs. A, Pulmonary function of sensitized mice after adoptive transfer of DCs from different donors. ●●● $P < .001$, CD11c^{high}CD11b^{low}-OVA+Flt3 ligand versus OVA; ♦♦♦ $P < .001$ and ♦♦ $P < .01$, CD11c^{high}CD11b^{low}-OVA+Flt3 ligand versus CD11c^{low}CD11b^{high}-OVA+Flt3 ligand; *** $P < .001$ and ** $P < .01$, CD11c^{high}CD11b^{low}-OVA+Flt3 ligand versus CD11c^{high}CD11b^{low}-OVA (n = 3-5). The OVA-sensitized and OVA-challenged mice that received adoptively transferred CD11c^{high}CD11b^{low} cells showed an attenuated AHR on day 45. Penh, Enhanced pause. B, Sensitized mice receiving adoptive transfer of CD11c^{high}CD11b^{low} DCs isolated from OVA-sensitized mice treated with Flt3 ligand (OVA^{AT}-OVA) showed decreased airway inflammation and decreased mucus cell hyperplasia ($\times 60$ magnification). H&E, Hematoxylin and eosin; PAS, periodic acid-Schiff.

regulatory and immunogenic properties, respectively, in allergic airway inflammation. Lung plasmacytoid DCs with suppressive properties have a different phenotype from CD11c^{high}CD11b^{low} lung DCs and thus are easily excluded from our sort (see Fig E2 in this article's Online Repository at www.jacionline.org).

We further demonstrated that Flt3 ligand not only specifically gives rise to the T_H2-suppressive CD11c^{high}CD11b^{low} DC subset in OVA-sensitized mice but also that Flt3 ligand modifies their phenotype and function toward a more intense T_H2 suppression

involving IL-10. This is supported by the findings that adoptive transfer of CD11c^{high}CD11b^{low} DCs to OVA-sensitized and OVA-challenged mice attenuated established AHR, with the maximal effect seen in mice that received these DCs isolated from Flt3 ligand-treated mice. The findings correlate well with the inability of CD11c^{high}CD11b^{low} DCs to induce T-cell proliferation on Flt3 ligand treatment. The regulatory role of CD11c^{high}CD11b^{low} DCs is further supported by significantly enhanced IL-10 production in the BALF and in the supernatants of CD11c^{high}CD11b^{low}

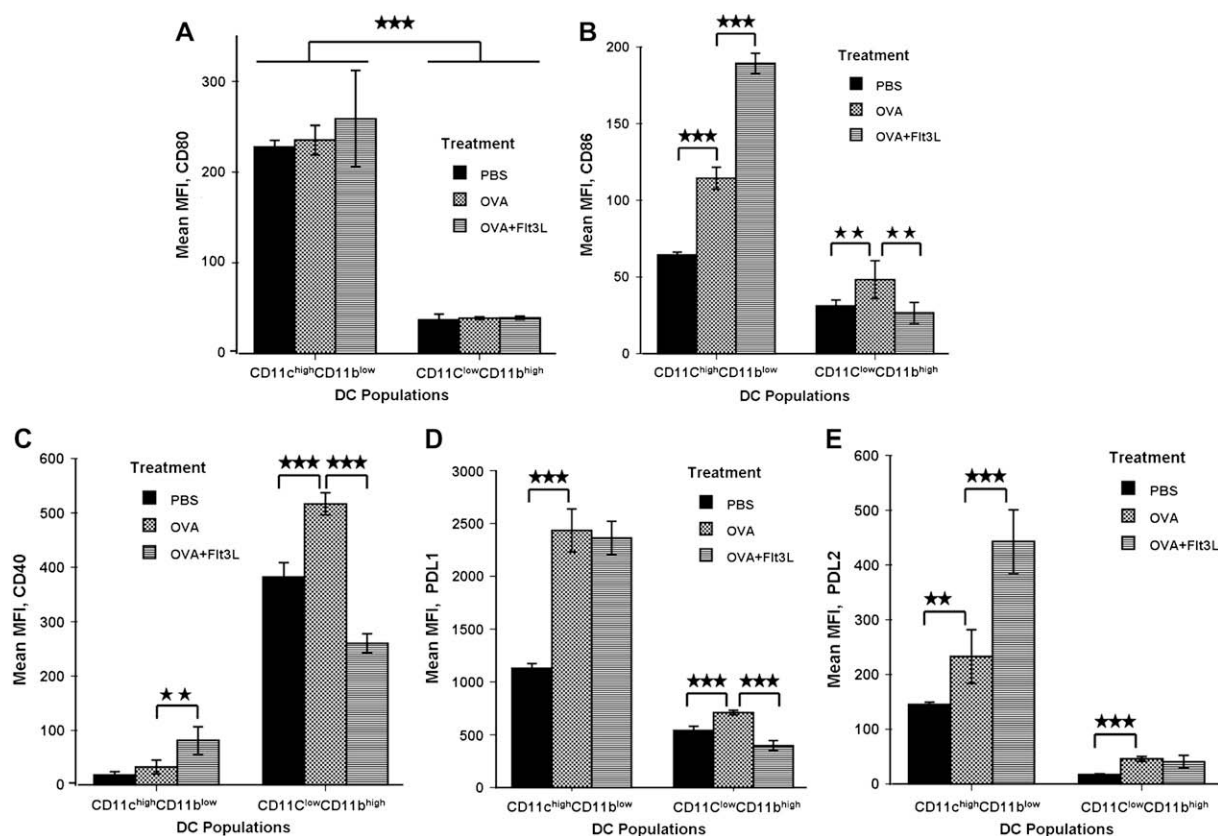


FIG 6. Expression levels of costimulatory molecules. The expression levels of CD80, CD86, CD40, PDL1, and PDL2 in CD11c^{high}CD11b^{low} and CD11c^{low}CD11b^{high} DC populations are differentially regulated by Flt3 ligand: **A**, CD80; **B**, CD86; **C**, CD40; **D**, PDL1; and **E**, PDL2. ****P* < .001 and ***P* < .01 (*n* = 6–12). Error bars indicate 95% confidence limits. MFI, Mean fluorescence intensity.

DC/T-cell coculture in the Flt3 ligand-treated group. Thus the generation of regulatory CD11c^{high}CD11b^{low} DCs in the lung and the release of IL-10 could be the underlying mechanisms for the therapeutic effect of Flt3 ligand in allergic airway inflammation.

In the nonsensitized mice the CD11c^{high}CD11b^{low} DC subset was not able to induce T-cell proliferation, possibly because of an immature phenotype and inefficient antigen uptake. CD11c^{high}CD11b^{low} DCs isolated from OVA-sensitized and OVA-challenged mice were able to induce T-cell proliferation, which is also consistent with the results from the adoptive transfer of DCs in which AHR was not attenuated. This suggests that CD11c^{high}CD11b^{low} DCs require IL-10 to exert their regulatory effect in resolving allergic airway inflammation and AHR.

The reason for increased numbers of the CD11c^{high}CD11b^{low} DC subset is because Flt3 ligand administration mobilizes more Flt3-expressing DC precursors from bone marrow into the blood.²⁷ Those cells serve as immediate precursors of DCs, leading to a dynamic change in the frequency of different DC subsets in the lungs.^{27–31} Although Flt3 ligand is required and important for maintaining the homeostasis of the DC pool in peripheral lymph organs,²⁷ little is known about whether Flt3 ligand-expanded spleen DCs can migrate to the lung in response to OVA challenge. The current study supports our previously published results that Flt3 ligand prevents^{19,32} and reverses^{18,20} AHR to methacholine in OVA-sensitized and OVA-challenged mice. In addition, we have previously shown that Flt3 ligand treatment induces an

increase in CD11⁺CD11b⁺ DCs¹⁸ accompanied by a decrease in dendrites and cytoplasmic veils,¹⁷ indicating that Flt3 ligand treatment gives rise to a less mature DC phenotype. Another study also reported that DCs expanded by murine Flt3 ligand were able to induce suppressive effects on T cells.³³ Flt3 ligand knockout mice have no defect on T-cell priming and secondary response,³⁴ suggesting that Flt3 ligand-dependent lung DCs might have a different function than conventional DCs. Triccas et al³⁵ and Ulrich et al³⁶ have reported an enhanced T_H1 response on the administration of Flt3 ligand, which is consistent with our results from the *in vitro* DC/T-cell coculture and BALF cytokine levels.

The engagement of DC costimulatory molecules to their receptors on naive T cells allows immunity or suppression to occur. The alteration of the expression of the costimulatory molecules PDL2, CD86, and CD40 on Flt3 ligand treatment provides a clue for the mechanisms of Flt3 ligand on DCs. We demonstrated that the expression levels of PDL1 and PDL2 were significantly higher in regulatory CD11c^{high}CD11b^{low} DCs than immunogenic CD11c^{low}CD11b^{high} DCs. In addition, PDL2 and CD86 were significantly upregulated after Flt3 ligand treatment only in regulatory CD11c^{high}CD11b^{low} DCs, and CD40 was downregulated only in immunogenic CD11c^{low}CD11b^{high} DCs. B7 family members, B7-H1 (PDL1) and B7-DC (PDL2), with their binding receptor, programmed death 1 (PD1), have been identified and shown to play bidirectional regulatory roles in T-cell proliferation.^{37,38} The relatively greater role of IFN- γ in stimulating PDL1 expression and IL-4 in stimulating PDL2

expression suggests that PDL1 and PDL2 might have distinct functions in regulating T_H1 and T_H2 responses.³⁷ There are also a number of studies showing that the PDL2-PD1 pathway inhibited T-cell proliferation and cytokine production.³⁹⁻⁴² The correlation of Flt3 ligand treatment and increased PDL2 expression open an intriguing putative pathway that the inhibitory effect of PDL2-PD1 interaction on T-cell proliferation might be enhanced by Flt3 ligand treatment.

In conclusion, we report the presence of potentially regulatory and immunogenic DC subsets in the lungs of mice in a model of allergic airway inflammation. Flt3 ligand treatment is not only able to increase the regulatory T_H1 -prone DC subset in OVA-sensitized mice but also can enhance its regulatory capability. The regulatory effects of Flt3 ligand-generated DCs might be achieved by changes in the expression of costimulatory molecules and enhanced IL-10 secretion.

Clinical implications: Flt3 ligand could be a potential immunomodulator in the treatment of established asthma.

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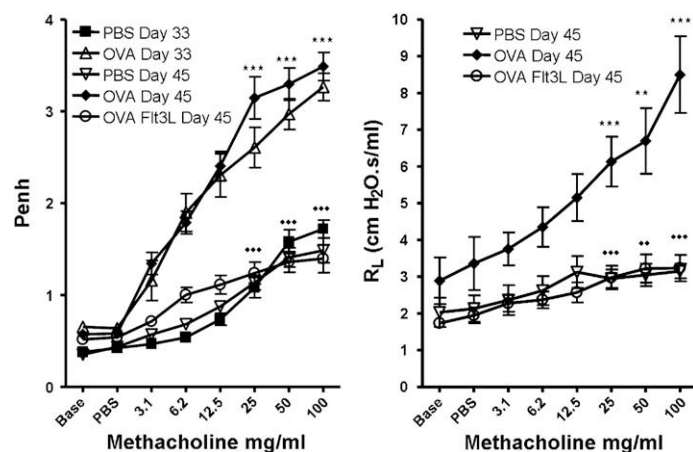


FIG E1. Flt 3 ligand treatment attenuates AHR to methacholine. *Left*, Enhanced pause (*Penh*) on day 33 ($n = 12$). *** $P < .001$, OVA day 45 versus PBS day 45. ♦♦♦ $P < .001$, OVA–Flt3 ligand day 45 versus OVA day 45. *Right*, Lung-specific resistance (R_L) on day 45 ($n = 5\sim8$). *** $P < .0001$ and ** $P < .01$, OVA day 45 versus PBS day 45. ♦♦♦ $P < .0001$ and ♦♦ $P < .01$, OVA–Flt3 ligand day 45 versus OVA day 45.

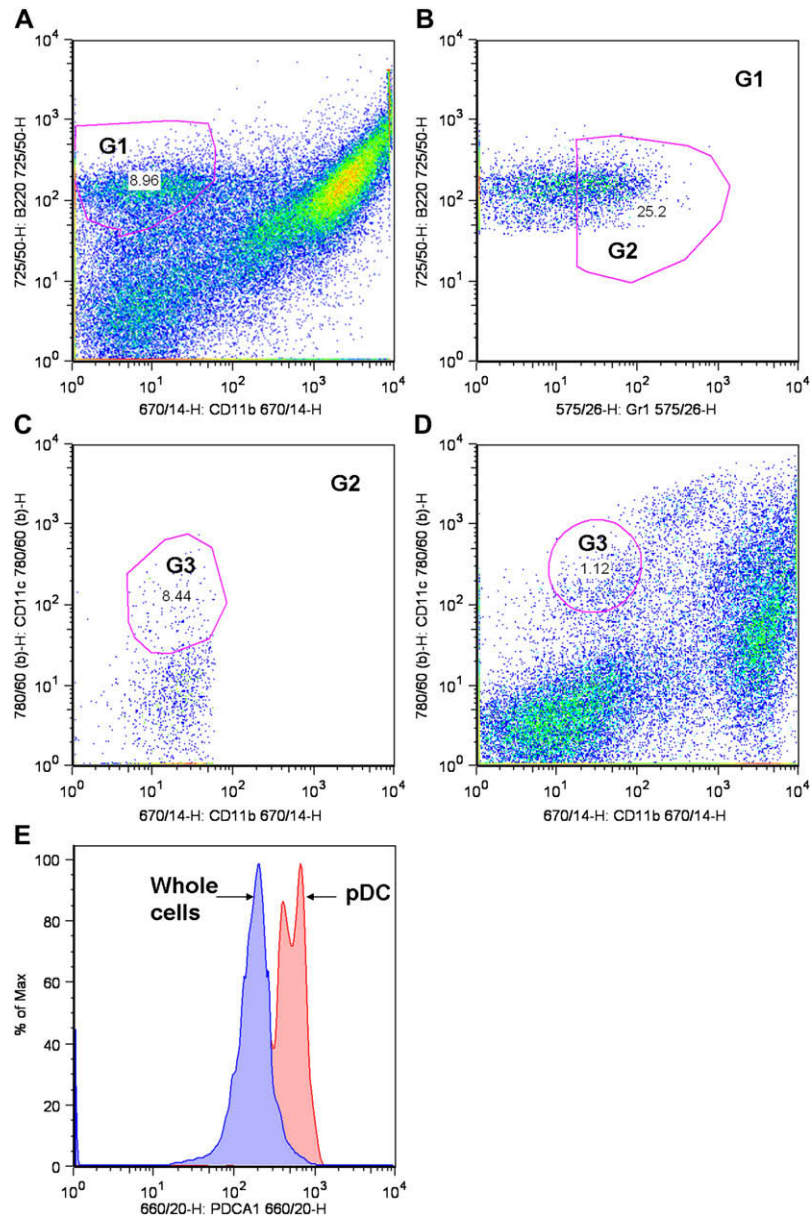


FIG E2. Identification of lung plasmacytoid DCs (*pDC*) by using specific cell-surface markers. **A**, CD11c bead-purified lung cells in B220 versus CD11b dot plots. *G1*, B220⁺CD11b⁻ cells. **B**, *G1* cells in B220 versus Gr-1 dot plot. *G2*, B220⁺CD11b⁻Gr-1⁺ cells. **C**, *G2* cells Cd11c versus Cd11b dot plot. *G3*, Gate for pDCs: B220⁺Cd11b⁻Gr-1⁺ cells. **D**, CD11c bead-purified lung cells in CD11c versus CD11b dot plot. *G3*, Gate for pDCs not included in the gates for sorting CD11c^{high}CD11b^{low} and CD11c^{low}CD11b^{high} lung DCs. **E**, mPDCA-1 expression in pDCs and CD11c bead-purified lung cells. pDCs have higher levels of mPDCA-1 expression than lung cells.