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Flt-3 Ligand Increases a Lung Dendritic Cell Subset with Regulatory Properties in allergic airway inflammation

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Abstract

Background—Dendritic cell subsets display different functional role in regulating immune response and lead to various outcomes including Th1 versus Th2 or regulatory versus immunologic response. Administration of 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 dendritic cell subsets in the therapeutic effect of Flt3-Ligand.

Methods—Dendritic cells were isolated from the lungs of OVA-sensitized and challenged mice treated with rhFlt3-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 pattern by different DC subsets was examined. Also, dendritic cells were adoptively transferred in mice to examine their effect on pulmonary function.

Results—Two dendritic cell populations, CD11c^{high}CD11b^{low} and CD11c^{low}CD11b^{high}, were identified in the lungs of naïve and OVA-sensitized and challenged mice with and without treatment with Flt3-Ligand. The expression levels of CD8α, B220, CD19, F4/80, MHC II, CCR7, CD40, PDL1, PDL2, CD80, and CD86 were distinctly different between the two DC populations, which supports the notion that, CD11c^{high}CD11b^{low} and CD11c^{low}CD11b^{high} dendritic cells, potentially have regulatory and immunogenic properties, respectively. Administration of Flt3-Ligand increased the dendritic cells with regulatory potential in the lungs of antigen-sensitized mice, and CD11c^{high}CD11b^{low} dendritic cells 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 dendritic cells in a mouse model of allergic airway inflammation.

Clinical implication—Flt3-Ligand could be a potential immunomodulator in the treatment of established asthma.

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Keywords

Adoptive transfer; Airway hyperresponsiveness; Asthma; Dendritic cells; Flt3-Ligand; Mouse model; T cell Proliferation; Regulatory dendritic cells

Introduction

Asthma is an inflammatory airway disease characterized by airway eosinophilia, airway obstruction due to 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 (APCs) take up specific antigens and induce naïve T cells to differentiate into Th2 cells, which release cytokines, including IL-4, IL-5 and IL-13, governing immune response. Dendritic cells (DCs) are not only professional and the most efficient APCs, 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 immunological tolerance as well as to determine the type of T cell-mediated immune response (Th1 or Th2) following DC-T cell interaction ⁴. This differentiation is dependent on a number of factors such as the nature of antigen, the types and expression levels of co-stimulatory molecules, 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 towards tolerance or immunity. Splenic and mucosal CD8 α ⁺ DCs, but not CD8 α ⁻ DCs tolerize Th2 response ^{6, 7}, and CD8 α ⁻ DCs are more effective than CD8 α ⁺ in inducing Th2 response and eosinophilic airway inflammation ⁸. Other studies have supported the idea that CD8 α ⁺ DCs are more prone to induce Th1 response while CD8 α ⁻ DCs lead to Th2 response ^{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 (Tregs) 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 DCs subset derived from IL-10 and TGF- β 1-treated bone marrow cells with a different phenotype from CD11c⁺B220⁺Gr-1⁺DC subset can protect airway inflammation in asthmatic mice ¹⁴. The DCs demonstrate a highly flexible phenotype and often accomplish many diverse and opposing functions.

Flt3-Ligand (Flt3-L) 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 following Flt-L administration ¹⁶. We observed an increase in the number of lung DCs following the administration of Flt3-L ^{17, 18} and Flt3-L prevented and reversed allergic airway inflammation and AHR in a mouse model of asthma ^{18–20}. Additionally, the phenotype and function of DCs may be tissue-specific and they could vary depending on different isolation techniques employed ²¹. Most of the previous research obtained DCs from spleen, bone marrow, or thymus but not 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 sensitization and Flt3-L treatment.

Materials and Methods

Mice

Four to 5 week old female Balb/c mice were purchased from Harlan Laboratories (Indianapolis, IN) 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. 1A. Mice were divided into sensitized and non-sensitized groups. Sensitized groups received 20 µg grade V chicken egg ovalbumin i.p. (OVA; Sigma-Aldrich, St. Louis, MO) emulsified in 2.25 mg of inject alum on day 1 and day 14. Non-sensitized mice received sterile PBS i.p. The mice were challenged with either 1% OVA aerosol or PBS for three consecutive days starting day 28. AHR to methacholine was established (Fig. 1A). Sensitized mice were randomized into two groups, one of which were given 10 µg Flt3-L i.p. on day 34, 36, 38, 40, 43. The other group received sterile PBS i.p. Non-sensitized mice received the same volume of sterile PBS serving as controls. 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.

Non-invasive and Invasive methods for measuring pulmonary function

A non-invasive 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}. Penh, an index of airway obstruction²³ was calculated. On day 45, anesthetized, tracheostomized mice were placed in PLY4111-R/C plethysmograph single chamber (Buxco Electronics, Troy, NY) and were mechanically ventilated (140 breaths/min and a tidal volume of 0.15 ml) using Harvard rodent ventilator (model 683, Harvard Apparatus, South Natick, MA). Anesthesia was given by intraperitoneal injection of sodium pentobarbital (1.6mg/20g body weight). Lung specific airway resistance (R_L)²⁴ 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 dendritic cells

After euthanization of mice, the lungs were collected, finely chopped and digested with 5 ml collagenase D (1 mg/ml) in RPMI-1640 containing 1 mg/ml DNase (Sigma-Aldrich, St. Louis) at 37°C for 1 hr. Pellet was resuspended in 1 ml of MACS (Magnetic-assisted cell sorter) buffer (PBS supplemented with 0.5% BSA) and the cells counted in a coulter counter. In order to isolate DCs, the cells were first incubated for 5 mins with an Fc block (BD Pharmingen, CA) followed by incubation for 30 mins on ice with 100 µl of CD11c microbeads (Miltenyi Biotec, Auburn, CA) for every 100 million cells counted. The cells underwent a double positive selection using the POSSELDs program in the AutoMACS (Miltenyi Biotec, CA). 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 the cell staining. The volume of the antibody was adjusted according to the numbers of the cell counted in each sample. CD11c⁺ cells were collected and counted. The cells were resuspended in PBS supplemented with 4% fetal bovine serum (PBS₄) and incubated with the following four antibodies cocktails for 30 mins on ice: (1) CD11cPE, CD11bFITC, B220biotin or B220PE-Cy7, CD8αPerCP (BD Pharmingen, CA); (2) CD11cPE-Cy7, CD11bPE-Cy5, PD-L1biotin,

PD-L2FITC, CD86PE (eBioScience, CA); (3) CD11cPE-Cy7, CD11bPE-Cy5, CD80FITC, CD40biotin; CCR7Alexa700; (4) CD11cPE-Cy7, CD11bPE-Cy5, F4/80APC or PDCA-1biotin, CD19FITC or CD8αFITC, CD135PE and MHCIIAlexa700 (eBioScience, CA). The cells bound to biotin-conjugated primary antibody were incubated with streptavidin-conjugated APC fluorochrome. Samples were washed by addition of ice-cold PBS₄ and stored before sorting using the FACSARIA sorter (BD, CA). Majority of lung macrophages with high autofluorescence were distributed along the diagonal line of the dot plot and were excluded from gating of the two DC populations.

Adoptive transfer of lung dendritic cell subsets

CD11c^{high}CD11b^{low} Lung DCs (4×10^5 in 50 µl PBS) obtained from the lungs of the three treatment groups (naïve, OVA-sensitized and challenged, and OVA-sensitized and challenged and Flt3-L-treated mice) were injected into the tail vein of OVA-sensitized and challenged mice on day 33 (Fig. 1B). The same number of CD11c^{low}CD11b^{high} DCs obtained from OVA-sensitized and challenged mice treated with FL 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 using an invasive method.

T cell proliferation assay

DCs were resuspended in RPMI-1640 (Biowhittaker, ME) supplemented with 10% heat-inactivated FBS and penicillin-streptomycin and incubated with OVA (100 µg/ml) for 2 hrs at 37°C. Cells were washed with the medium and incubated with mitomycin C (final concentration 25 µg/ml) (Sigma-Aldrich, St. Louis, MO). The DCs were co-cultured with CD4⁺ T cells (equal ratio) isolated from the spleen 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 hrs co-culture, cells were labeled with BrDU (Roche diagnostics, IN). Incorporation of BrDU in the proliferating cells was detected by a BrDU antibody-enzyme conjugate followed by addition of the substrate. The absorbance of the samples was measured at 450 nm with reference wavelength of 690 nm. Culture supernatant was collected for the measurement of secreted cytokines.

Measurement of cytokines in DC-T cells coculture supernatant and BAL fluid

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

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 (H&E). Mucus secretion was identified by periodic acid-Schiff (PAS) reaction.

Data processing and statistical analysis

Flow cytometric data were processed and analyzed using Flowjo software (Tree Star, Inc. OR). SPSS 10.0 (SPSS Inc. Chicago IL) and GraphPad (GraphPad Software Inc, CA) were 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.

Results

Flt3-L treatment attenuates AHR to methacholine and airway inflammation

Mice were sensitized according to the protocol (Fig 1A). AHR to methacholine was established prior to Flt3-L treatment in OVA-sensitized and challenged mice (Supplement Fig. 1). On day 33 all OVA-sensitized mice showed a significant increase in AHR to methacholine compared to non-sensitized controls. Administration of Flt3-L reversed AHR on day 45 as measured by either non-invasive or the more rigorous invasive method. The AHR to methacholine was significantly lower in Flt3-L-treated OVA-sensitized and challenged mice than OVA-sensitized and challenged mice (Supplement Fig. 1).

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

In order to identify whether different functional DC subsets are present in the lungs of non-sensitized mice, we labeled purified lung CD11c⁺ cells with fluorochrome-conjugated antibodies against CD11c, CD11b, CD8 α , B220, F4/80, CD19, MHC class II molecule, CD80, CD86, CD40, PDL1, PDL2, and CCR7. Flow cytometric analysis revealed two distinct DC populations defined by differential expression of CD11c and CD11b; CD11c^{high}CD11b^{low} (Fig. 2A, square) and CD11c^{low}CD11b^{high} (Fig. 2A, circle) in all three treatment groups. The CD11c^{high}CD11b^{low} DCs had significantly higher expression levels of CD8 α , B220, CD19, PDL1, PDL2, CD80, CD86 and lower expression levels of F4/80, MHC II, CD40, and CCR7 than CD11c^{low}CD11b^{high} DCs (Fig. 2B).

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

In order to assess whether OVA sensitization and Flt3-L treatment have any effect on the two lung DCs populations in mice with allergic airway inflammation, sensitized and challenged mice were given i.p. injections of 10 μ g Flt3-L for 5 days on day 34, 36, 38, 40 and 43 (a total of 50 μ g Flt3-L) (Fig. 1A). Sensitized and non-sensitized controls received the same volume of PBS i.p. 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-L treatment. CD11c^{high}CD11b^{low} DCs in the lung were predominant (39.54 $\pm$ 1.52 %) in the non-sensitized controls and dropped drastically in the lungs of sensitized mice (3.68 $\pm$ 0.60 %) (Fig. 2A, C). Following Flt3-L treatment, the number of CD11c^{high}CD11b^{low} DCs was restored and its frequency (24.13 $\pm$ 3.17 %) was significantly increased (p <0.001) compared to the OVA-sensitized group (Fig. 2A, C). CD11c^{low}CD11b^{high} DCs were more prominent (36.62 $\pm$ 1.19 %) in the lungs of sensitized mice than non-sensitized controls (6.40 $\pm$ 0.54 %) (Fig. 2A, C). Following Flt3-L treatment, the frequency of CD11c^{low}CD11b^{high} DCs in the lung (27.77 $\pm$ 1.36 %) was significantly reduced (p <0.001) compared to the number of DCs in the lung of OVA-sensitized mice (36.62 $\pm$ 1.19 %) (Fig. 2A, C). The relative changes in proportion were also reflected by similar changes in absolute number of the two lung DC subsets (Fig 2D).

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

The two distinct populations of DC were sorted and co-cultured individually with naïve 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 naïve CD4⁺ T cell (Fig. 3). This capacity was diminished in the case of CD11c^{high}CD11b^{low} DCs after Flt3-L treatment and is comparable to that in non-sensitized controls. However, CD11c^{low}CD11b^{high} DCs retain the capacity to stimulate naïve CD4⁺ T

cells *in vitro* after Flt3-L treatment (Fig. 3). These data suggest that Flt3-L 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 Th2 suppression

In DC-T cell coculture supernatant, higher levels of IL-12 and IFN- γ were detected in the supernatant of CD11c^{high}CD11b^{low} than CD11c^{low}CD11b^{high} DCs. There was no significant difference in the IL-12 and IFN- γ levels between PBS, OVA and Flt3-L-treated groups (Fig. 4 A–C). Higher level of IL-10 was observed in the supernatant of CD11c^{high}CD11b^{low} DCs isolated from OVA-Flt3-L-treated mice than PBS or OVA-sensitized mice. These observations, together with the increased frequency of lung CD11c^{high}CD11b^{low} DCs in Flt3-L treated sensitized mice, suggested that Flt3-L treatment may facilitate a Th2-suppression by enhancing Th1 response 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-L-treated OVA-sensitized than OVA-sensitized mice (Fig. 4D–F).

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

Adoptive transfer of the two lung DC subsets was performed (Fig 1B) to confirm the regulatory capability of lung CD11c^{high}CD11b^{low} DCs and to examine the effect of Flt3-L. OVA-sensitized and challenged mice that received adoptive transfer of CD11c^{high}CD11b^{low} DCs isolated from non-sensitized mice demonstrated a reduction in AHR as compared to those receiving CD11c^{high}CD11b^{low} DCs obtained from sensitized controls and those receiving CD11c^{low}CD11b^{high} DCs obtained from sensitized and Flt3-L treated mice (Fig. 5A). The maximum attenuation in AHR, however, was achieved in the mice receiving CD11c^{high}CD11b^{low} DCs isolated from sensitized and Flt3-L treated mice (Fig. 5A). Moreover, consistent with adoptive transfer data, lung histological 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-L treated mice compared to recipient mice of other experimental groups (Fig. 5B). Typically, in the lungs of OVA-sensitized and 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 histological features of allergic airway inflammation were attenuated in mice receiving CD11c^{high}CD11b^{low} DCs obtained from OVA-sensitized and Flt3-L treated mice. (Fig. 5B).

Effect of administration of Flt3-L on costimulatory molecules

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

Discussion

The purpose of the present study was to determine whether different functional DC subsets are present in the lungs of mouse and whether Flt3-L 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 two 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 Th2 suppressive capacity^{6–10} and Flt3-L has been shown to preferably generates CD8 α ⁺ conventional DC in the spleen²⁵. We observed that CD11c^{high}CD11b^{low} DCs are functionally similar to the mucosa⁷ and spleen⁶ CD8 α ⁺ DCs and are more prone to induce a skewed Th1 response due to increased release of IL-12 and IFN- γ , which can serve as Th2 suppressors²⁶. In addition, higher expression levels of PDL1 and PDL2 of CD11c^{high}CD11b^{low} DCs in Flt3-L-treated OVA-sensitized mice suggest that they are able to induce a strong T cells suppression. In contrast, a higher expression of MHC II, 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 Th2 response. The *in vivo* functional effects following adoptive transfer of DCs and the *in vitro* T cell proliferation assay further confirmed that the two DC populations, CD11c^{high}CD11b^{low} and CD11c^{low}CD11b^{high} DCs, possess 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 (Suppl Fig 2.).

We further demonstrated that Flt3-L not only specifically gives rise to the Th2-suppressive CD11c^{high}CD11b^{low} DCs subset in OVA-sensitized mice, Flt3-L also modifies their phenotype and function towards a more intense Th2 suppression involving IL-10. This is supported by the findings that adoptive transfer of CD11c^{high}CD11b^{low} DCs to OVA-sensitized and challenged mice attenuated established AHR with the maximal effect in mice that received these DCs isolated from Flt3-L-treated mice. The findings correlate well with the inability of CD11c^{high}CD11b^{low} DCs to induce T cell proliferation upon Flt3-L 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 supernatant of CD11c^{high}CD11b^{low} DC-T cell coculture in Flt3-L-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-L in allergic airway inflammation.

In the non-sensitized mice, CD11c^{high}CD11b^{low} DC subset was not able to induce T-cell proliferation, possibly due to an immature phenotype and inefficient antigen uptake. CD11c^{high}CD11b^{low} DCs isolated from OVA-sensitized and challenged mice were able to induce T cell proliferation, which is also consistent with the results from the adoptive transfer of DCs where AHR was not attenuated. This suggests that CD11c^{high}CD11b^{low} CD8 α ⁺ DCs require IL-10 to exert their regulatory effect in resolving allergic airway inflammation and AHR.

The reason for increased number of CD11c^{high}CD11b^{low} DC subset is because Flt3-L administration mobilizes more Flt3-expressing DC precursors from bone marrow into the blood²⁷. 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-L is required and important for maintaining the homeostasis of dendritic cell pool in peripheral lymph organs²⁷, little is known whether or not Flt3L-expanded spleen DCs can migrate to the lung in response to OVA challenge. The current study supports our previously published results that Flt3-L prevents^{19, 32} and reverses^{18, 20} AHR to methacholine in OVA-sensitized and challenged mice. In addition, we have previously shown that Flt3-L treatment induces an increase in CD11⁺CD11b⁺ DCs¹⁸ accompanied by a decrease in dendrites and cytoplasmic veils¹⁷, indicating that Flt3-L treatment gives rise to a less mature DC phenotype. Other study also reported that DCs expanded by murine Flt3-L were able to induce suppressive effects on T cells³³. Flt3-L knockout mice have no defect on T cell priming and secondary response³⁴, suggesting that Flt3-L-dependent lung DCs may have a different function than conventional

DCs. Triccas and colleagues³⁵ and Ulrich et al³⁶ have reported an enhanced Th1 response upon the administration of Flt3-L, which is consistent with our results from the *in vitro* DC-T cell co-culture and BALF cytokine levels.

The engagement of DC costimulatory molecules to their receptors on naïve T cell allows immunity or suppression to occur. The alteration of the expression of costimulatory molecules, PD-L2, CD86 and CD40 upon Flt3-L treatment provides a clue for the mechanisms of Flt3-L on DCs. We demonstrated that the expression levels of PD-L1 and PD-L2 were significantly higher in regulatory CD11c^{high}CD11b^{low} DCs than immunogenic CD11c^{low}CD11b^{high} DCs. In addition, PD-L2 and CD86 were significantly upregulated after Flt3-L 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 (PD-L1) and B7-DC (PD-L2) with its binding receptor PD1, have been identified and shown to play bi-directional regulatory roles in T cell proliferation^{37, 38}. The relatively greater role of IFN- γ in stimulating PD-L1 expression and IL-4 in stimulating PD-L2 expression suggests that PD-L1 and PD-L2 may have distinct functions in regulating Th1 and Th2 responses³⁷. There are also a number of studies showing that the PD-L2-PD1 pathway inhibited T cell proliferation and cytokine production^{39–42}. The correlation of Flt3-L treatment and increased PD-L2 expression open an intriguing putative pathway that the inhibitory effect of PD-L2-PD1 interaction on T cell proliferation might be enhanced by Flt3-L treatment.

In conclusion, we report the presence of potentially regulatory and immunogenic DC subsets in the lung of a mouse model of allergic airway inflammation. Flt3-L treatment is not only able to increase regulatory Th1-prone DC subset in OVA-sensitized mice but can also enhance their regulatory capability. The regulatory effects of Flt3-L-generated DCs might be achieved by changes in the expression of costimulatory molecules and an enhanced IL-10 secretion.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AHR	Airway hyperresponsiveness
APC	Antigen presenting cell
BALF	Bronchoalveolar lavage fluid
DC	Dendritic cell
Flt3-L	Fms like tyrosine kinase 3 Ligand
OVA	ovalbumin

pDC	Plasmacytoid dendritic cells
PD1	Programmed-death 1
PDL1	Programmed-death ligand 1
PDL2	Programmed-death ligand 2
Tregs	Regulatory T cells

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Fig. 1. Protocol for sensitization, treatment and adoptive transfer

A. OVA sensitization was followed by methacholine challenge. A total dose of 50 mg Flt3-L treatment was given to each mouse prior to the final methacholine challenge to reassess the AHR. **B.** Adoptive transfer of the lung DCs: Two DC populations isolated from three treatment groups of mice were adoptively transferred to OVA sensitized and challenged mice on day 34.

Fig. 2. Dynamics and characteristics of lung dendritic cell populations

A. Two lung DC populations: CD11c^{high}CD11b^{low} (square) and CD11c^{low}CD11b^{high} (circle) DCs (n = 5). **B.** Expression of cell surface markers on the two lung DC subsets in PBS-treated control mice (n = 5). **C–D.** Quantitative analyses of the two DC subsets (*** P < 0.001, n = 9–15; error bars indicate 95% confidence limits).

Fig. 3. T cell proliferation by two DCs populations

CD11c^{high}CD11b^{low} isolated from OVA sensitized and Flt3-L treated mice showed inability to active CD4⁺ T cell proliferation (*** p<0.001, n=5).

Fig. 4. Cytokine secretion patterns in DC-T coculture supernatant and BALF
Levels of IL-12 (A), IFN- γ (B), and IL-10 (C) in DC-T cells co-culture supernatant; levels of IL-12 (D), IFN- γ (E), and IL-10 (F) in the BALF. (★★★ $P < 0.001$, ★★ $P < 0.01$, ★ $P < 0.05$, $n = 3 \sim 6$).

Fig. 5. Adoptive transfer of lung DCs

*A. Pulmonary function of sensitized mice after adoptive transfer of DCs from different donors. (●●● p<0.001, CD11c^{high}CD11b^{low}-OVA+Flt3-L vs OVA; ◆◆◆ p<0.001, ◆◆ p<0.01, CD11c^{high}CD11b^{low}-OVA+Flt3-L vs CD11c^{low}CD11b^{high}-OVA+ Flt3-L; ★★★ P<0.001, ★★ P<0.01, CD11c^{high}CD11b^{low}-OVA+Flt3-L vs CD11c^{high}CD11b^{low}-OVA, n=3~5). The OVA sensitized and challenged mice that received adoptively transferred CD11c^{high}CD11b^{low} cells showed an attenuated AHR on day 45. *B.* sensitized mice receiving adoptive transfer of CD11c^{high}CD11b^{low} DCs isolated from OVA sensitized mice treated with Flt3-L (OVA^{AT}-OVA), showed decreased airway inflammation and decreased mucus cell hyperplasia (60x magnification).*

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-L. A. CD80, B. CD86, C. CD40, D. PDL1, E. PDL2 (*** P < 0.001, ** P < 0.01, n = 6-12; error bars indicate 95% confidence limits).