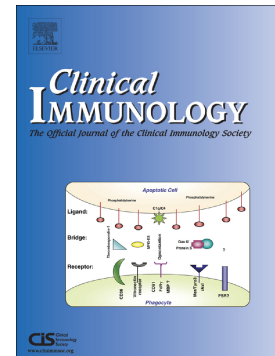


Accepted Manuscript

Overexpression of Notch ligand Delta-like-1 by dendritic cells enhances their immunoregulatory capacity and exerts antiallergic effects on Th2-mediated allergic asthma in mice

Chen-Chen Lee, Chu-Lun Lin, Sy-Jye Leu, Yueh-Lun Lee



PII: S1521-6616(17)30420-5
DOI: doi:[10.1016/j.clim.2017.10.005](https://doi.org/10.1016/j.clim.2017.10.005)
Reference: YCLIM 7952

To appear in: *Clinical Immunology*

Received date: 9 June 2017
Revised date: 22 September 2017
Accepted date: 12 October 2017

Please cite this article as: Chen-Chen Lee, Chu-Lun Lin, Sy-Jye Leu, Yueh-Lun Lee , Overexpression of Notch ligand Delta-like-1 by dendritic cells enhances their immunoregulatory capacity and exerts antiallergic effects on Th2-mediated allergic asthma in mice. The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. Yclim(2017), doi:[10.1016/j.clim.2017.10.005](https://doi.org/10.1016/j.clim.2017.10.005)

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Overexpression of Notch ligand Delta-like-1 by dendritic cells enhances their immunoregulatory capacity and exerts antiallergic effects on Th2-mediated allergic asthma in mice

Chen-Chen Lee¹, Chu-Lun Lin², Sy-Jye Leu³, and Yueh-Lun Lee³

¹Department of Microbiology and Immunology, School of Medicine, China Medical University, Taichung, Taiwan

²Graduate Institute of Medical Sciences, College of Medicine, Taipei Medical University, Taipei, Taiwan

³Department of Microbiology and Immunology, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan

Corresponding Author: Dr. Yueh-Lun Lee

Department of Microbiology and Immunology, School of Medicine, College of Medicine, Taipei Medical University, 250 Wu-Hsing Street, Taipei 11031, Taiwan

Tel: 886-2-27361661 ext. 3912

Fax: 886-2-27361661 ext. 3921

E-mail: ylee@tmu.edu.tw

Abbreviations

DCs, dendritic cells; DLL1, Delta-like-1; Th1, type 1 T helper; IL-12, interleukin-12; MHC class II, major histocompatibility complex class II; OVA, ovalbumin; IFN- γ , interferon- γ ; IgE, immunoglobulin E; AHR, airway hyperresponsiveness; APCs, antigen-presenting cells; Treg, regulatory T; BMDCs, bone marrow-derived DCs; Ad-DLL1, DLL1-expressing adenovirus; DLL1-DCs, DLL1-overexpressing DCs; RT-PCR, reverse-transcription polymerase chain reaction; MOI, multiplicity of infection; MFI, mean fluorescence intensity; TNF- α , tumor necrosis factor- α ; ELISA, enzyme-linked immunosorbent assay; MLR, mixed lymphocyte reaction; BALF, bronchoalveolar lavage fluid; cpm, counts per minute; i.p., intraperitoneal; i.n., intranasal; EU, ELISA unit; H&E, hematoxylin and eosin; PAS, periodic acid-Schiff.

Abstract

Dendritic cells (DCs) are professional antigen-presenting cells, and Notch ligand Delta-like-1 (DLL1) on DCs was implicated in type 1 T helper (Th1) differentiation. In this study, we produced genetically engineered bone marrow-derived DCs that expressed DLL1 (DLL1-DCs) by adenoviral transduction. DLL1-DCs exerted a fully mature phenotype, and had positive effects on expression levels of interleukin (IL)-12 and costimulatory molecules. Coculture of allogeneic T cells with ovalbumin (OVA)-pulsed DLL1-DCs enhanced T cell proliferative responses and promoted Th1 cell differentiation. Furthermore, adoptive transfer of OVA-stimulated DLL1-DCs into asthmatic mice alleviated the cardinal features of allergic asthma, including immunoglobulin E (IgE) production, airway hyperresponsiveness (AHR), airway inflammation, and production of Th2-type cytokines. Notably, enhanced levels of the Th1-biased IgG_{2a} response and interferon (IFN)- γ production were observed in these mice. Taken together, these data indicate that DLL1-DCs promoted Th1 cell development to alter the Th1/Th2 ratio and ameliorate Th2-mediated allergic asthma in mice.

Keywords:

dendritic cells; Delta-like-1; Th1; Th2; allergic asthma; adenovirus

ACCEPTED MANUSCRIPT

1. Introduction

Dendritic cells (DCs), as the most important professional antigen-presenting cells (APCs), serve as key regulators of the immune system, and the activation status of DCs is crucial for the activation and differentiation of T cells [1,2]. Immature DCs have a high capacity to internalize antigens but have a lower ability to activate T cells. After antigen uptake, DCs mature and migrate to regional lymph nodes, where they present antigens to naïve T cells. These antigen-activated DCs express costimulatory molecules and cytokines to provide key signals for the differentiation of CD4⁺ T cells into type 1 T helper (Th1), Th2, or regulatory T (Treg) cells [3]. As major contributors to the decision of T cells to either activate or suppress a specific immune response, DCs are a focus of various immune interventions for allergic diseases, autoimmunity, tumor treatments, and graft rejection [4-7]. Techniques for generating large amounts of clinical-grade DCs have become widely available to genetically modify the phenotype and degree of maturation of DCs. Thus, an attractive conceptual approach to enhancing the immunoregulatory potential of DCs is to genetically modify them to overexpress immunomodulatory molecules.

Notch signaling is functionally related to cell development and apoptosis [8,9]. Moreover, the important roles of Notch in adaptive immunity have expanded to include regulating T cell differentiation and function [10]. Notch is a receptor that responds to cell surface-bound ligands. Signaling is mediated by Notch ligand-receptor interactions between neighboring cells [11]. There are four mammalian Notch receptors (Notch1~4) which are bound by five ligands of the Jagged family (Jagged1 and Jagged2) and Delta-like family (Delta-like ligand 1

(DLL1), DLL3, and DLL4) [12]. Unfortunately, monoclonal antibodies that specifically recognize each Notch receptor or Notch ligand are lacking, which prevents analyses of detailed expression patterns of these molecules. In peripheral lymphoid organs, naive CD4⁺ T cells express Notch1 and Notch2 mRNA, while they do not express Notch3 or Notch4 mRNA. However, mRNA expressions of all Notch molecules increased after antigen stimulation of T cells. Using DLL1-expressing fibroblasts (L cell lines) as APCs, Amsen *et al.* showed that DLL1 constitutes an instructive signal for Th1 differentiation, whereas Jagged1 expression on APCs promotes generation of Th2 cells [13]. This discovery of the contribution of Notch signaling to Th1/Th2 differentiation opened a new pathway for regulating helper T cell differentiation. Subsequently, Elyaman's group investigated the roles of DLL1 and Jagged1 in experimental autoimmune encephalomyelitis (EAE), a Th1-mediated disease, and demonstrated that *in vivo* blockade of DLL1 during EAE reduced Th1 responses, while blockade of Jagged1 decreased Th2 cells [14]. These findings revealed that the Notch pathway plays a critical role in regulating Th1 and Th2 cell differentiation *in vivo*. Furthermore, other studies confirmed that DCs express Notch1, Notch2, DLL1, DLL4, Jagged1, and Jagged2 [15], and under different stimuli, expressions of Notch receptors and Notch ligands can be induced in DCs [16]. One report showed that the immobilized Notch ligand, DLL1, inhibited differentiation of monocytes into mature macrophages [17]. However, it permitted differentiation of monocytes into mature DCs. Overall, it appears that DLL1 is involved in DC maturation and DC-mediated T-cell differentiation.

Asthma is a chronic inflammatory disease of the lungs with multiple phenotypes. A previous study showed that asthma can be induced by both genetic and environmental factors [18]. Allergic asthma is a kind of Th2-associated asthma,

characterized by increased serum immunoglobulin E (IgE) levels, airway hyperresponsiveness (AHR), and inflammatory cell infiltration in the airway through the production of Th2-type cytokines (IL-4, IL-5, and IL-13) [19]. Although some anti-inflammatory reagents, such as corticosteroids and β -agonists, are used as first-line treatment of asthma, they do not cure the disease and have some adverse side effects [20]. Accordingly, new therapeutic methods need to be developed to treat allergic asthma. As allergic asthma is associated with Th2-dependent disease, deviation towards Th1 responses, including increases in interferon (IFN)- γ , IL-12, and IgG_{2a} to suppress the Th2 response, was considered a useful approach [21,22]. Taken together, a better understanding of how DLL1 affects the maturation, activation, and functions of DCs is a prerequisite for designing strategies for treating allergic asthma.

In this study, we attempted to alter the functional behavior of bone marrow-derived DCs (BMDCs) through Notch ligand DLL1 gene modification *in vitro* and subsequently observed the effects of such modulations on these cells. First, we constructed a DLL1-expressing adenovirus (Ad-DLL1) and transduced it into BMDCs. Phenotype changes and the immunoregulatory capacity of DLL1-overexpressing DCs (DLL1-DCs) were investigated *in vitro*. Furthermore, we evaluated the therapeutic effects of DLL1-DCs on the potential to inhibit Th2-mediated allergic asthma in a mouse model of airway inflammation. We demonstrated that overexpression of DLL1 by DCs enhanced the Th1-polarizing ability of DCs, and DLL1 may be beneficial as an adjuvant in situations where a Th1 response needs to be elicited in allergic asthma.

2. Methods

2.1 Mice

Female mouse strains BALB/c (H-2^d) and C57BL/6 (H-2^b) were obtained from the National Laboratory Animal Center and Laboratory Animal Center of National Taiwan University (Taipei, Taiwan). OT-II (H-2^b) mice expressing a transgenic T-cell receptor that is specific for the peptide sequence 323~339 of ovalbumin (OVA) were kindly provided by Prof. Chia-Rui Shen (Chang Gung University, Taoyuan, Taiwan). The mice were maintained in the Animal Center of Taipei Medical University and were used for experimentation at 5~10 weeks of age. Animal care and handling protocols were approved (no.: LAC-2013-0207) by the animal use committee of the College of Medicine, Taipei Medical University.

2.2 Construction and amplification of the Ad-DLL1

The DLL1 gene was cloned by a polymerase chain reaction (PCR) from the MSCV-IRES-GFP vector which inserted full-length murine DLL1 complementary (c)DNA (a gift from Dr. Antonio Freitas, Pasteur Institute, Paris, France) [23]. After the PCR, DLL1 cDNA was subcloned into Xba I sites of the pShuttle2 plasmid and then inserted into I-Ceu I/PI-Sce I sites of the pAdeno-X vector (TaKaRa Bio, Mountain View, CA, USA) to obtain the Ad-DLL1 vector. A control adenovirus (Ad-mock) which did not express the transgene was also prepared. Both vectors were transfected into the human embryonal kidney AD293 cell line to generate adenoviral particles. High-titer adenoviral stocks were purified using a Vivapur[®] AdenoPACK[™] 20 kit (Sartorius, Goettingen, Germany). Following purification, viral titers were measured by an end-point dilution assay, and the virus stock was stored at -80 °C.

2.3 Transduction of BMDCs with adenoviral vectors

Mouse BMDCs were prepared using tibial and femoral bone marrow from 5~8-week-old BALB/c mice. The method of culturing bone marrow cells to differentiate into DCs was described in our previous study [24]. In brief, bone marrow cells were cultured with granulocyte-macrophage colony-stimulating factor (GM-CSF) (500 U/mL; PeproTech, Rocky Hill, NJ, USA) and IL-4 (500 U/mL; eBioscience, San Diego, CA, USA) on day 0. Fresh medium containing GM-CSF and IL-4 was used to replace the old medium on days 2 and 4. On day 6 of culture, non-adherent BMDCs were collected and placed in 24-well plates at a concentration of 10^6 cells/well. Then, cells were infected with the Ad-mock or Ad-DLL1 using a multiplicity of infection (MOI) of 10, 50, 100, or 1000. After 2 h of transduction, the infecting medium was replaced with fresh culture medium for another 16~18 h. To induce DC maturation, these adenovirus-infected BMDCs (DLL1-DCs and mock DCs) and uninfected BMDCs (control DCs) were further stimulated with 50 μ g/well OVA protein (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C for 24 h. Messenger (m)RNA and protein expressions of the DLL1 transgene were respectively detected by a reverse-transcription (RT)-PCR and flow cytometry.

2.4 RT-PCR

Total RNA from DCs was extracted using TRIzol (Thermo Scientific, Rockford, IL, USA) and reverse-transcribed using a high-capacity cDNA reverse transcription kit (Thermo Scientific). Amplification reactions were performed using a Taq DNA polymerase 2x Master Mix RED kit (Ampliqon, Odense M, Denmark) in the presence of 1 μ l of total cDNA and specific primers for murine DLL1 (forward, 5'-tgacctgtgcagatggccct-3'; and reverse, 5'cgcttctagattacacctcagtcgctataacacac-3').

The PCR was performed in a Gene Amp® PCR system 2700 machine (Thermo Scientific). The thermal cycler parameters for amplification of the DLL1 gene were as follows: 1 cycle at 95 °C for 5 min followed by 30 cycles at 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min and 30 s. The level of β -actin mRNA was used as a control.

2.5 Flow cytometric analysis

After OVA stimulation, harvested DCs were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD11c or phycoerythrin (PE)-conjugated anti-I-A/I-E (MHC class II), anti-CD80, anti-CD86 (eBioscience), and anti-Delta-like1 antibodies (Biolegend, San Diego, CA, USA) to assess the DLL1 level and maturation status of DCs, which were performed for 30 min in the dark at 4 °C. Cells were washed and resuspended in FACS buffer (1% fetal bovine serum (FBS) and 0.1% NaN₃ in a 1× phosphate-buffered saline (PBS) solution). Staining with isotype control antibodies was performed in all experiments. Flow cytometry was performed using a FACSCalibur (Becton Dickinson, Mountain View, CA, USA), and the mean fluorescence intensity (MFI) was analyzed with Cellquest pro software (Becton Dickinson).

2.6 Cytokine and chemokine detection

DCs infected with adenoviral vectors were further pulsed with OVA for 24 h. Culture supernatants were collected, and levels of IL-12, IL-10, IL-1 β , IL-6, and tumor necrosis factor (TNF)- α were measured according to the instructions of the enzyme-linked immunosorbent assay (ELISA) kits (eBioscience). In a mixed lymphocyte reaction (MLR) and OVA-specific T cell response, the level of IFN- γ in the supernatants was detected by an ELISA kit (R&D Systems, Minneapolis, MN,

USA). In asthma models, splenocytes (10^7 cells/well) from each group of mice were stimulated with 50 $\mu\text{g/mL}$ OVA in 24-well plates, and culture supernatants were collected after 72 h of incubation. Levels of IL-4, IL-5, IL-10, IL-13, IFN- γ , TNF- α , and eotaxin in bronchoalveolar lavage fluid (BALF) and the spleen were analyzed using ELISA kits (eotaxin and IL-5 kits were from R&D Systems; IL-4 and IL-13 kits were from eBioscience).

2.7 $CD4^+$ T cell isolation

Splenocytes from C57BL/6 mice were resuspended in MACS buffer (0.5% bovine serum albumin (BSA) and 2 mM EDTA in a $1\times$ PBS solution) at 10^8 cells/mL and stained with anti-CD4 magnetic beads (Miltenyi Biotec, Auburn, CA, USA), which was performed for 15 min at 4 $^{\circ}\text{C}$. Cells were washed and resuspended in MACS buffer to positively select $CD4^+$ T cells for further experiments.

2.8 Measurement of the $CD4^+$ T cell response in vitro

For the MLR, BMDCs (10^6 cells/well) of day 6 culture from BALB/c mice were infected with the Ad-mock (at an MOI of 100) or the Ad-DLL1 (at an MOI of 100) and then treated with OVA protein (50 $\mu\text{g/mL}$). After 48 h of culture, DCs were irradiated and cocultured with allogeneic $CD4^+$ T cells (2×10^5 /well) from C57BL/6 mice at different proportions of irradiated DCs (4×10^4 , 2×10^4 , 10^4 , 0.5×10^4 , and 0.25×10^4 /well). For the antigen-specific T-cell response, treated and irradiated BMDCs from C57BL/6 mice were cocultured with syngeneic $CD4^+$ T cells from OT-II transgenic mice, and ratios of DCs: T-cells were the same as those used for the MLR. Cells were cultured for 48 h and then pulsed with [^3H]-thymidine for an 18-h incubation. Values of [^3H]-thymidine incorporation in T cells were determined with a dry scintillation counter and are expressed as counts per minute (cpm). In addition,

secretion levels of IFN- γ in supernatants were determined by an ELISA.

2.9 Adoptive transfer of DLL1-DCs to allergen-sensitized mice

All groups of mice ($n = 6/\text{group}$) were sensitized by an intraperitoneal (i.p.) injection of 10 μg OVA plus 4 mg alum (Thermo Scientific) on days 1, 10, and 20. On days 28 and 29, mice were intranasally (i.n.) challenged with 100 μg OVA. Finally, mice were further challenged with 5% OVA in 0.9% NaCl by inhalation for 30 min daily for 3 consecutive days (days 30, 31, and 32). On day 33, the AHR of mice was measured. To evaluate the immunomodulatory effect of adenovirus-transduced DCs in asthmatic mice, three groups of mice were intravenously injected with 5×10^5 OVA-pulsed mock DCs (at an MOI of 100), OVA-pulsed DLL1-DCs (at an MOI of 100), or OVA-pulsed Ad-uninfected DCs (control DCs) on day 20. OVA-sensitized and -challenged mice without DC transfer served as an asthmatic positive control (PC group).

2.10 OVA-specific serum antibody assay

Serum samples were collected from mice on day 33. OVA-specific IgE and IgG_{2a} antibody titers in serum were detected with ELISA kits (Becton Dickinson Biosciences, San Jose, CA, USA) as previously described [25]. Serum was diluted 1:10 for the IgE determination and 1:100 for IgG_{2a} quantification. Levels of antibodies were compared with IgE and IgG_{2a} standards of predetermined concentrations (immunoglobulin concentrations: IgE = 1.5 $\mu\text{g}/\text{ml}$ and IgG_{2a} = 15.5 $\mu\text{g}/\text{ml}$). The OVA-specific IgE or IgG_{2a} standards were derived by pooling sera from OVA-sensitized mice. The concentration of standard serum was arbitrarily assigned as 1 ELISA unit (EU). Sample results are expressed as a ratio of the value to the standard.

2.11 Measurement of the AHR

The AHR was measured 24 h after the last OVA challenge. The detailed method was previously described [26]. Briefly, tracheostomized mice that were anesthetized were exposed to increasing concentrations (1~32 mg/mL) of aerosolized methacholine (Sigma-Aldrich), and the resulting AHR was measured by the lung resistance (R_L). The R_L was continuously computed (Labview, National Instruments, Dallas, TX, USA) by fitting the pressure, flow, and volume to an equation of motion.

2.12 Analysis of BALF and lung tissue histology

Mice were sacrificed after measuring the AHR, and the lungs were immediately lavaged via a tracheal cannula with Hank's balanced salt solution (HBSS) which was free of ionized calcium and magnesium. The lavage fluid was centrifuged, and supernatants were collected for the chemokine and cytokine assays. Cells from the BALF were washed and resuspended in 1 ml RPMI-1640 medium supplemented with 2% FBS. The cytocentrifuged preparation of BALF cells was fixed and stained with Liu's staining solution (Chi I Pao, Taipei, Taiwan). The total cell number was counted for at least 200 cells of preparations via a hemocytometer. Different cell types were enumerated as macrophages, eosinophils, neutrophils, and lymphocytes, based on standard morphological criteria. The lungs were fixed in 10% buffered formaldehyde after the lavage, routinely processed, embedded in paraffin wax, and sectioned at 5 μ m for hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) staining.

2.13 Data analysis

Results are expressed as the mean $\pm$ standard error of the mean (SEM). Statistical analyses were performed using a one-way analysis of variance (ANOVA) followed by

Turkey's multiple-comparison test. p values of <0.05 were considered statistically significant.

ACCEPTED MANUSCRIPT

3. Results

3.1 Overexpression of DLL1 in Ad-DLL1-infected DCs

First, we prepared a recombinant adenovirus carrying the DLL1 sequence (Ad-DLL1), and BMDCs were infected with viral particles using various MOIs of the Ad-DLL1. An Ad-mock containing no transgene in the expression cassette was used as a control vector. Adenovirus-infected BMDCs (DLL1-DCs and mock DCs) and uninfected BMDCs (control DCs) were further stimulated with the OVA protein to induce cell maturation. After 24 h of incubation, RNA extractions from these cells were collected, and the mRNA of DLL1 was detected by an RT-PCR. As shown in Figure 1A, DLL1 mRNA exhibited low constitutive expression in immature DCs, control DCs, and mock DCs, while levels of DLL1 mRNA were obviously enhanced in DLL1-DCs. Furthermore, expressions of DLL1 surface molecules on DCs were analyzed by flow cytometry. Results revealed that DLL1-DCs overexpressed DLL1 in a dose-dependent manner (Figure 1B). In contrast, low expression of DLL1 was observed in immature DCs, control DCs, and mock DCs.

3.2 Ad-DLL1 infection promoted DC maturation and activation

It is well known that fully mature DCs secrete high levels of IL-12 and proinflammatory cytokines (TNF- α , IL-1 β , and IL-6), and also express high levels of MHC class II costimulatory surface molecules (CD80 and CD86) to prime activation of naïve T cells [27]. IL-12, which stimulates the proliferation and development of Th1 cells, was significantly induced in DCs infected with the Ad-DLL1 in a dose-dependent manner compared to the secretion from control DCs (Figure 2A). IL-10 is an anti-inflammatory cytokine and can be produced by many types of

immune cells including DCs, Treg cells, and Th2 cells [28]. In contrast to the induction of high amounts of IL-12, IL-10 was slightly upregulated by DCs following Ad-DLL1 delivery at an MOI of 1000 (Figure 2B). Additionally, because proinflammatory cytokines (IL-1 β , IL-6, and TNF- α) are involved in enhancing airway inflammation in asthmatic patients [29], we further investigated whether DLL1 infection modulated production of these cytokines by DCs. Results demonstrated that DCs infected with a high adenovirus titer of 1000 MOI, of either the Ad-mock or Ad-DLL1, displayed high levels of proinflammatory cytokines (Figure 2C-E). In contrast, at MOIs of 10 and 100, Ad-DLL1 infection did not enhance IL-1 β or IL-6 production by DCs, although these DCs still exhibited increased TNF- α production. Furthermore, to investigate phenotype changes of DCs by Ad-DLL1 infection, we measured expressions of surface markers on DCs after OVA stimulation. Immature DCs showed low levels of MHC class II CD80 and CD86 markers, and these markers were upregulated in OVA-stimulated control DCs and mock DCs (Figure 2F, G). Moreover, all of these maturation markers had markedly increased in DLL1-DCs. These data indicate that Ad-DLL1-infected DCs were trending towards a fully mature phenotype in the presence of OVA stimulation. Collectively, infection with the Ad-DLL1 at an MOI of 100 promoted DC maturation and induced high amounts of IL-12 but did not markedly increase proinflammatory cytokine production; thus we predicted that this dose might be optimal for performing further experiments.

3.3 DLL1-DCs enhanced T-cell proliferation and induced IFN- γ production by T cells

DCs are superior APCs and exhibit the ability to direct T-cell proliferation and polarization. Therefore, an allogenic MLR assay was performed to clarify whether DLL1-DCs possess immunomodulatory effects on T cells. Different groups of

irradiated DCs were cocultured with allogenic CD4⁺ T cells at various ratios (1:10, 1:20, 1:40, and 1:80). As shown in Figure 3A, DLL1-DCs significantly promoted T-cell proliferation compared to control DCs and mock DCs (Figure 3A). We also assessed levels of the Th1-type cytokine, IFN- γ , in culture supernatants and found that IFN- γ production by T cells at a DC/T-cell ratio of 1:10 was more dramatically induced by DLL1-DCs than by control DCs and mock DCs (Figure 3B). Furthermore, syngeneic OVA-specific OT-II CD4⁺ T cells were cocultured with differently treated DCs, and the T-cell response was measured. Consistent with the above results, the OT-II CD4⁺ T cell population driven by OVA-pulsed DLL1-DCs showed a higher T-cell proliferative response than those of other groups of DCs (Figure 3C). In addition, DLL1-DCs exhibited significant enhancement of IFN- γ production in T cells at a DC/T-cell ratio of 1:10 (Figure 3D). Taken together, the results demonstrated that overexpression of DLL1 by DCs enhanced their ability to activate naïve T cells and direct Th1 cell development.

3.4 DLL1-DC treatment downregulated anti-OVA IgE production in mice with OVA-induced asthma

Based on our *in vitro* findings that DLL1-DCs can promote Th1 development, we hypothesized that these cells have the ability to control Th2-mediated asthma by altering the Th1/Th2 balance in an OVA-induced asthmatic animal model. Therefore, we next focused on the antiallergic effects of DLL1 in allergic asthma. The experimental protocol is schematically represented in Figure 4A. OVA-stimulated control DCs, mock DCs, and DLL1-DCs were respectively transferred to three groups of OVA-sensitized mice. Positive control mice (the PC group) received PBS injection instead of DCs. Serum samples were collected on day 33, and titers of OVA-specific antibodies were assayed. The Th2-type IL-4 cytokine was suggested to promote IgE

production in mice, while the Th1-type IFN- γ cytokine enhanced IgG_{2a} production [30]. Compared to the PC group, mice injected with DLL1-DCs expressed a significantly lower level of IgE (Figure 4B). In addition, DLL1-DC-treated mice exhibited a higher level of IgG_{2a} in serum compared to the other groups (Figure 4C). Overall, these results indicated that DLL1-DCs inhibited the Th2 response, while inducing a Th1 response in mice with OVA-induced asthma.

3.5 DLL1-DCs reduced the severity of AHR and allergen-induced airway inflammation

We investigated whether administration of DLL1-DCs could attenuate AHR and airway inflammation. As shown in Figure 5A, PC mice challenged with methacholine (1~32 mg/ml) had high, dose-dependent R_L values. Adoptive transfer of control DCs or mock DCs had no suppressive effect on the development of AHR. On the contrary, in DLL1-DC-treated mice, there was an obvious decrease in R_L values at the maximal dose of methacholine (32 mg/mL) relative to the other groups (Figure 5A). Furthermore, inflammatory cell infiltrates in the BALF and lungs were analyzed. As expected in this model, PC mice exposed to OVA challenge had markedly increased numbers of total cells and eosinophils in the BALF (Figure 5B). In addition, a histological analysis revealed a considerable increase in inflammatory cell infiltration (by H&E staining) and mucus production (by PAS staining) in the lungs (Figure 5C, D). The control DC and mock DC groups also exhibited similar severities of airway inflammation as did the PC group. In contrast, DLL1-DC treatment decreased inflammatory cell recruitment and also reduced mucus secretion.

3.6 DLL1-DCs suppressed the production of Th2 cytokines and promoted the secretion of a Th1 cytokine

We next assayed the release of mediators in the BALF to evaluate the effect of DLL1-DCs on T-cell responses in the lungs. Levels of eotaxin, a chemokine for recruiting eosinophils, and Th2-type cytokines (IL-4, IL-5, and IL-13) were increased in PC mice, whereas administration of control DCs and mock DCs had no inhibitory effects (Figure 6A-D). In contrast, DLL1-DC treatment resulted in significant downregulation of the production of these mediators. Moreover, the production of IFN- γ , an essential cytokine for the development of the Th1 response, showed an increasing trend in DLL1-DC-treated mice (Figure 6E). Levels of IL-10 in the BALF were also detected, and data showed no difference among these groups (Figure 6F).

To further identify whether DLL1-DC treatment affected the production of cytokines by OVA-stimulated splenocytes of mice, we isolated splenocytes from each group of mice and cultured them with OVA for 3 days. Levels of IL-4, IFN- γ , TNF- α , and IL-10 in culture supernatants were examined. Results revealed that mice treated with DLL1-DCs had a marked reduction in IL-4 production by splenocytes, while the level of IFN- γ production was obviously increased in DLL1-DC-treated mice (Figure 6G, H). There was no effect of DLL1-DC treatment on TNF- α or IL-10 production in the spleen (Figure 6I, J). Collectively, these data indicated that DLL1-DCs might relieve the allergic syndrome through shifting the Th1/Th2 response toward a more-balanced state.

4. Discussion

DCs are highly efficient APCs capable of priming activation of T cells. Based on their immunostimulatory capacity, DCs have been used as vaccines for treating cancers and infectious diseases. In this regard, a promising approach is the use of viral vectors encoding immunoregulatory molecules to enhance specific functions of DCs. Among virus-based vectors, Ads have been widely used in gene therapy and vaccine applications during the past two decades. Advantages of Ads include high titers of recombinant virus production *in vitro*, efficient infection of target cells, and an inability to cause severe disease in natural hosts [31,32]. In this context, our present study clarified the *in vitro* effects of DLL1-overexpressing Ad transduction on the phenotype and function of mouse BMDCs, and these data may be important considerations when applying these DCs in mouse models and clinical trials.

Costimulatory and MHC molecules on DCs were identified to play important roles in affecting T-cell activation and differentiation [33]. In addition, T-cell differentiation can be affected by IL-12 and IL-4, and these cytokines are the main respective inducers of Th1 and Th2 subsets [34]. In cultured BMDCs, we found that Ad-mock transduction resulted in slight upregulation of MHC II expression after OVA stimulation. However, the relative degree of activation was less than that with transduction of an Ad vector encoding DLL1 for DC activation. DLL1-DCs provide higher levels of costimulatory and MHC molecules optimal for priming naïve T-cell activation. Meanwhile, these more-mature DCs can secrete higher levels of IL-12 to efficiently force the development of Th1 cells. Consistent with this evidence, one report demonstrated that activation of Notch signaling using OP9 cells expressing

DLL1 upregulated the expressions of MHC II and CXCR4 in lipopolysaccharide-stimulated DCs [35]. Furthermore, another study showed that deletion of RBP-J, a transcription factor required for Notch signaling, in DCs resulted in lower expressions of MHC II, CD80, and CD86, and inefficient T-cell activation [36]. Taken together, these results indicate that Notch triggering may promote DC maturation and activation. Thus, it is possible to upregulate their immunoregulatory ability through DLL1 and Notch interactions, and blocking experiments with an anti-DLL1 antibody need to be performed to confirm this hypothesis in a future study. On the other hand, there is some dispute about the induction of DC maturation by Ads. Some reports documented that Ad-transduced DCs display a significantly activated phenotype manifested by increased expressions of surface activation markers (MHC II, CD40, CD80, and CD86), and cytokine production (IL-12, IL-6, IL-1 β , and TNF- α), whereas others showed little or no enhancement of these markers [37-40]. These differences in results probably reflect phenotype changes possibly affected by culture conditions, vector doses, and different species.

The role of the Notch signaling in Th cell differentiation has received much attention, and several studies addressed the therapeutic potential of manipulating the Notch pathway to suppress aberrant immune activation. In particular, DLL1 Notch ligand expression by APCs is specifically required for Th1 cell development. In results of the MLR, we showed that Ad-DLL1-infected DCs significantly promoted the proliferation and production of IFN- γ by T cells. These data imply that overexpression of DLL1 by DCs enhances their ability to drive Th1 cell differentiation, which is consistent with the finding that the DLL1-Fc protein as a soluble Notch agonist on T cells promotes Th1 development [41]. In another study, using DLL1 retrovirus-transduced IL-12^{-/-} DCs, Sun *et al.* revealed that

DLL1-promoted Th1 cell responses are IL-12-independent [42]. They further demonstrated that DLL1 favoring Th1 cell development is a result of its ability to inhibit IL-4 production and Th2 cell development.

In our animal model, we demonstrated that the severity of AHR and the development of airway eosinophilia were significantly reduced in asthmatic mice after DLL1-DC treatment. At the same time, we found that DLL1-DC treatment ameliorated asthma through inhibiting Th2 responses by reducing Th2-type cytokine secretion. In Th2-mediated allergic asthma, Th2 cytokines (IL-4, IL-5, and IL-13) mainly cause asthma syndromes. IL-4 acts on B cells by driving immunoglobulin isotype switching towards the production of IgE, which sensitizes mast cells to allergen exposure [43]. In particular, IL-5 induces the maturation and activation of eosinophils, which produce cytotoxic proteins for tissue destruction in the airways. Additionally, IL-13 increases epithelial permeability and mucus production, which thus promotes the development of AHR [44]. Overall, these Th2-type cytokines promote critical effects of the asthmatic response. Thus, according to the above information, inhibiting Th2 immunity may attenuate the clinical syndromes of allergic asthma.

Before giving DLL1-DCs to mice, we pretreated DLL1-DCs with OVA stimulation to elevate the allergen presentation by DLL1-DCs, which may have induced increased MHC II and costimulatory molecule expressions and high levels of IL-12 production, and promoted the development of OVA-specific Th1 cells. Actually, a single injection of OVA-pulsed DLL1-DCs reduced the production of OVA-specific IgE but increased levels of OVA-specific IgG_{2a} in asthmatic mice. Moreover, the Th1-type cytokine, IFN- γ , was enhanced in DLL1-DC-treated mice. Thus, we suggest

that DLL1-DCs promoted the secretion of IFN- γ by Th1 cells, and further induced the production of IgG_{2a} by IFN- γ -stimulating B cells. Th1 responses are proposed to protect against asthma through the production of IFN- γ . Some studies demonstrated that allergen-immunized mice deficient in the IFN- γ receptor exhibit an increased duration of eosinophilia and Th2 responses [22,45], and adoptive transfer of allergic-specific Th1 cells reduces Th2-cytokine-dependent AHR and airway eosinophilia in an asthma model via mechanisms that depend on IFN- γ [46]. The mechanisms of IFN- γ 's action are shown to suppress mRNA synthesis of Th2 cytokines and Th2 cell proliferation *in vitro* [45]. In addition, IFN- γ exhibits its suppressive effect by inhibiting lung APC functions and migration, thus attenuating T cell priming in the airway [47]. Mitchell *et al.* proved that IFN- γ also acts on the airway epithelium to inhibit mucus hyperproduction and airway eosinophilia in allergic airway diseases [48]. However, there are contradictory reports on the antiallergic effect of IFN- γ . For example, one study revealed that mixed populations of Th1 and Th2 cells caused severe airway inflammation [49]. Another report showed that local expression of IFN- γ in the lungs increased eosinophilic infiltration and promoted Th2-type cytokine production [50]. Collectively, the role of IFN- γ during the development of allergic asthma is controversial, and these conflicting results show that IFN- γ can both attenuate and augment allergic airway diseases, depending on the concentration and timing of application or expression. Thus, the precise mechanisms by which Th1 cells affect Th2 responses and the specific conditions that determine the net effect of IFN- γ on allergic responses remain to be determined.

A shift in the balance of cytokines from Th2 responses to Th1 responses may worsen existing disease. TNF- α , which induces the recruitment of inflammatory cells and stimulates smooth muscle contraction in the airway, is produced by Th1 cells,

mast cells, and macrophages [51]. Thus, it is possible that upregulation of TNF- α by DLL1-DC transfer could potentiate the inflammatory response in asthma. In our study, although DLL1-DCs exhibited an increased level of TNF- α *in vitro*, adoptive transfer of these DCs did not affect the level of TNF- α *in vivo*. Therefore, we suggest that DLL1-DC treatment did not modulate Th2-mediated immune responses via TNF- α overexpression. In addition, IL-10 is one of the cytokines utilized by thymus-derived CD4⁺FOXP3⁺ Treg cells and CD4⁺FOXP3⁻ type 1 regulatory T (Tr1) cells to suppress Th2-driven allergic diseases [52]. In this regard, we identified whether the counteraction of DLL1-DCs on efferent Th2 cells was by enhancing IL-10 production, and found that expression of IL-10 in DLL1-DCs and DLL1-DC-treated mice was not upregulated. This indicates that the suppressive mechanism of DLL1-DCs on Th2 responses does not occur through IL-10 induction. However, we could not rule out the possible involvement of TGF- β and Treg populations in our system.

5. Conclusions

In vitro, Ad-DLL1 infection enhanced DC maturation and activation, and these modified DCs induced the production of IFN- γ by T cells. In a mouse model of OVA-induced asthma, a single injection of these DCs suppressed IgE production, the AHR, inflammatory cell infiltration, and Th2 cytokines in the BALF and spleen, but induced OVA-specific Th1 responses. Our results suggest that DLL1-overexpressing DC-induced attenuation of the allergic response in mice is associated with enhanced Th1 responses which inhibit Th2 responses. This study provides useful information to facilitate the use of Ad-DLL1-infected DCs for immunomodulatory treatment of allergic asthma and cancer.

Funding

This study was supported by a research grant (MOST 103-2320-B-038-032-MY3) from the Ministry of Science and Technology, Taiwan.

Declaration of funding

The authors declare that they have no financial conflicts of interest.

Acknowledgements

The retroviral vector encoding the mouse DLL1 gene was donated by Dr. Antonio A. Freitas.

References

- [1] P. Blanco, A.K. Palucka, V. Pascual, J. Banchereau, Dendritic cells and cytokines in human inflammatory and autoimmune diseases, *Cytokine Growth Factor Rev.* 19 (2008) 41-52.
- [2] P. Bousso, T-cell activation by dendritic cells in the lymph node: lessons from the movies, *Nat. Rev. Immunol.* 8 (2008) 675-684.
- [3] M.F. Lipscomb, B.J. Masten, Dendritic cells: immune regulators in health and disease, *Physiol. Rev.* 82 (2002) 97-130.
- [4] K. Palucka, J. Banchereau, Cancer immunotherapy via dendritic cells, *Nat. Rev. Cancer* 12 (2012) 265-277.
- [5] C.C. Gross, H. Wiendl, Dendritic cell vaccination in autoimmune disease, *Curr. Opin. Rheumatol.* 25 (2013) 268-274.
- [6] M.A. Gill, The role of dendritic cells in asthma, *J. Allergy Clin. Immunol.* 129 (2012) 889-901.
- [7] K.S. Devi, N. Anandasabapathy, The origin of DCs and capacity for immunologic tolerance in central and peripheral tissues, *Semin. Immunopathol.* 39 (2017) 137-152.
- [8] M.N. Cruickshank, D. Ulgiati, The role of notch signaling in the development of a normal B-cell repertoire, *Immunol. Cell Biol.* 88 (2010) 117-124.
- [9] F. Radtke, H.R. MacDonald, F. Tacchini-Cottier, Regulation of innate and adaptive immunity by Notch, *Nat. Rev. Immunol.* 13 (2013) 427-437.
- [10] D. Amsen, C. Helbig, R.A. Backer, Notch in T cell differentiation: all things considered. *Trends Immunol.* 36 (2015) 802-814.
- [11] F. Radtke, N. Fasnacht H.R. Macdonald, Notch signaling in the immune system. *Immunity* 32 (2010) 14-27.
- [12] Y. Shang, S. Smith, X. Hu, Role of Notch signaling in regulating innate immunity and inflammation in health and disease, *Protein Cell* 7 (2016) 159-174.
- [13] D. Amsen, J.M. Blander, G.R. Lee, K. Tanigaki, T. Honjo, R.A. Flavell, Instruction of distinct CD4 T helper cell fates by different notch ligands on antigen-presenting cells. *Cell* 117 (2004) 515-526.
- [14] W. Elyaman, E.M. Bradshaw, Y. Wang, M. Oukka, P. Kivisakk, S. Chiba, et al., Jagged1 and delta1 differentially regulate the outcome of experimental autoimmune encephalomyelitis. *J. Immunol.* 179 (2007) 5990-5998.
- [15] I. Maillard, T. Fang, W.S. Pear, Regulation of lymphoid development,

- differentiation, and function by the Notch pathway, *Annu. Rev. Immunol.* 23 (2005) 945-974.
- [16] B. Perez-Cabezas, M. Naranjo-Gomez, P. Bastos-Amador, G. Requena-Fernandez, R. Pujol-Borrell, F.E. Borrás, Ligation of Notch receptors in human conventional and plasmacytoid dendritic cells differentially regulates cytokine and chemokine secretion and modulates Th cell polarization, *J. Immunol.* 186 (2011) 7006-7015.
- [17] K. Ohishi, B. Varnum-Finney, R.E. Serda, C. Anasetti, I.D. Bernstein, The Notch ligand, Delta-1, inhibits the differentiation of monocytes into macrophages but permits their differentiation into dendritic cells, *Blood* 98 (2001) 1402-1407.
- [18] A.B. Mukherjee, Z. Zhang, Allergic asthma: influence of genetic and environmental factors, *J. Biol. Chem.* 286 (2011) 32883-32889.
- [19] S.I. Mayr, R.I. Zuberi, F.T. Liu, Role of immunoglobulin E and mast cells in murine models of asthma, *Braz. J. Med. Biol. Res.* 36 (2003) 821-827.
- [20] A.A. Alangari, Corticosteroids in the treatment of acute asthma, *Ann. Thorac. Med.* 9 (2014) 187-192.
- [21] J. Li, F. Zhang, J. Li, The immunoregulatory effects of traditional Chinese medicine on treatment of asthma or asthmatic inflammation, *Am. J. Chin. Med.* 43 (2015) 1059-1081.
- [22] F.D. Finkelman, S.P. Hogan, G.K. Hershey, M.E. Rothenberg, M. Wills-Karp, Importance of cytokines in murine allergic airway disease and human asthma, *J. Immunol.* 184 (2010) 1663-1674.
- [23] A. de La Coste, E. Six, N. Fazilleau, L. Mascarell, N. Legrand, M.P. Mailhe, et al., In vivo and in absence of a thymus, the enforced expression of the Notch ligands delta-1 or delta-4 promotes T cell development with specific unique effects. *J. Immunol.* 174 (2005) 2730-2737.
- [24] Y.H. Shieh, H.M. Huang, C.C. Wang, C.C. Lee, C.K. Fan, Y.L. Lee. Zerumbone enhances the Th1 response and ameliorates ovalbumin-induced Th2 responses and airway inflammation in mice. *Int. Immunopharmacol.* 24 (2015) 383-391.
- [25] C.C. Lee, C.C. Wang, H.M. Huang, C.L. Lin, S.J. Leu, Y.L. Lee. Ferulic acid induces Th1 responses by modulating the function of dendritic cells and ameliorates Th2-mediated allergic airway inflammation in mice. *Evid. Based Complement. Alternat. Med.* 2015 (2015) 678487.
- [26] C.L. Lin, G. Hsiao, C.C. Wang, Y.L. Lee, Imperatorin exerts antiallergic effects in Th2-mediated allergic asthma via induction of IL-10-producing regulatory T cells by modulating the function of dendritic cells. *Pharmacol.*

- Res. 110 (2016) 111-121.
- [27] M.B. Lutz, G. Schuler, Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity?, *Trends Immunol.* 23(2002) 445-449.
 - [28] C.L. Maynard, C.T. Weave, Diversity in the contribution of interleukin-10 to T-cell mediated immune regulation. *Immunol. Rev.* 226 (2008) 219-233.
 - [29] A. Saggini, G. Maccauro, D. Tripodi, M.A. De Lutiis, F. Conti, P. Felaco, et al., Allergic inflammation: role of cytokines with special emphasis on IL-4, *Int. J. Immunopathol. Pharmacol.* 24 (2011) 305-311.
 - [30] E. Mohr, A.F. Cunningham, K.M. Toellner, S. Bobat, R.E. Coughlan, R.A. Bird, et al., IFN- γ produced by CD8 T cells induces T-bet-dependent and -independent class switching in B cells in responses to alum-precipitated protein vaccine, *Proc. Natl. Acad. Sci. U S A* 107 (2010) 17292-17297.
 - [31] M.B. Appaiahgari, S. Vрати, Adenoviruses as gene/vaccine delivery vectors: promises and pitfalls. *Expert. Opin. Biol. Ther.* 15 (2015) 337-351.
 - [32] Y.S. Ahi, D.S. Bangari, S.K. Mittal, Adenoviral vector immunity: its implications and circumvention strategies, *Curr. Gene Ther.* 11 (2011) 307-320.
 - [33] S. Ville, N. Poirier, G. Blancho, B. Vanhove, Co-stimulatory blockade of the CD28/CD80-86/CTLA-4 balance in transplantation: impact on memory T cells?, *Front. Immunol.* 6 (2015) 411.
 - [34] J. Zhu, H. Yamane W.E. Paul, Differentiation of effector CD4 T cell populations, *Ann. Rev. Immunol.* 28 (2010) 445-489.
 - [35] Y.C. Wang, X.B. Hu, F. He, F. Feng, L. Wang, W. Li, et al., Lipopolysaccharide-induced maturation of bone marrow-derived dendritic cells is regulated by notch signaling through the up-regulation of CXCR4, *J. Biol. Chem.* 284 (2009) 15993-16003.
 - [36] F. Feng, Y.C. Wang, X.B. Hu, X.W. Liu, G. Ji, Y.R. Chen, et al., The transcription factor RBP-J-mediated signaling is essential for dendritic cells to evoke efficient anti-tumor immune responses in mice, *Mol. Cancer* 9 (2010) 90.
 - [37] Z.C. Hartman, D.M. Appledorn, A. Amalfitano, Adenovirus vector induced innate immune responses: impact upon efficacy and toxicity in gene therapy and vaccine applications, *Virus Res.* 132 (2008) 1-14.
 - [38] R.J. Korst, A. Mahtabifard, R. Yamada, R.G. Crystal, Effect of adenovirus gene transfer vectors on the immunologic functions of mouse dendritic cells, *Mol. Ther.* 5 (2002) 307-315.
 - [39] E.A. Hirschowitz, J.D. Weaver, G.E. Hidalgo, D.E. Doherty, Murine dendritic

- cells infected with adenovirus vectors show signs of activation, *Gene Ther.* 7 (2000) 1112-1120.
- [40] C.J. Kirk, J.J. Mule, Gene-modified dendritic cells for use in tumor vaccines, *Hum. Gene Ther.* 11 (2000) 797-806.
- [41] Y. Maekawa, S. Tsukumo, S. Chiba, H. Hirai, Y. Hayashi, H. Okada, et al., Delta1-Notch3 interactions bias the functional differentiation of activated CD4⁺ T cells, *Immunity* 19 (2003) 549-559.
- [42] J. Sun, C.J. Krawczyk, E.J. Pearce, Suppression of Th2 cell development by Notch ligands Delta1 and Delta4, *J. Immunol.* 180 (2008) 1655-1661.
- [43] P.C. Fulkerson, M.E. Rothenberg, Targeting eosinophils in allergy, inflammation and beyond, *Nat. Rev. Drug Discov.* 12 (2013) 117-129.
- [44] J.L. Ingram, M. Kraft, IL-13 in asthma and allergic disease: asthma phenotypes and targeted therapies, *J. Allergy Clin. Immunol.* 130 (2012) 829-842; quiz 843-824.
- [45] L. Cohn, R.J. Homer, N. Niu, K. Bottomly, T helper 1 cells and interferon γ regulate allergic airway inflammation and mucus production, *J. Exp. Med.* 190 (1999) 1309-1318.
- [46] T.J. Huang, P.A. MacAry, P. Eynott, A. Moussavi, K.C. Daniel, P.W. Askenase, et al., Allergen-specific Th1 cells counteract effector Th2 cell-dependent bronchial hyperresponsiveness and eosinophilic inflammation partly via IFN- γ , *J. Immunol.* 166 (2001) 207-217.
- [47] K. Nakagome, K. Okunishi, M. Imamura, H. Harada, T. Matsumoto, R. Tanaka et al., IFN- γ attenuates antigen-induced overall immune response in the airway as a Th1-type immune regulatory cytokine, *J. Immunol.* 183 (2009) 209-220.
- [48] C. Mitchell, K. Provost, N. Niu, R. Homer, L. Cohn, IFN- γ acts on the airway epithelium to inhibit local and systemic pathology in allergic airway disease, *J. Immunol.* 187 (2011) 3815-3820.
- [49] D.A. Randolph, R. Stephens, C.J. Carruthers, D.D. Chaplin, Cooperation between Th1 and Th2 cells in a murine model of eosinophilic airway inflammation, *J. Clin. Invest.* 104 (1999) 1021-1029.
- [50] M. Koch, M. Witzentrath, C. Reuter, M. Herma, H. Schütte, N. Suttrop, et al., Role of local pulmonary IFN- γ expression in murine allergic airway inflammation, *Am. J. Respir. Cell Mol. Biol.* 35(2006) 211-219.
- [51] L. Gallelli, M.T. Busceti, A. Vatrella, R. Maselli, G. Pelaia, Update on anticytokine treatment for asthma, *Biomed. Res. Int.* 2013 (2013)104315.
- [52] M. Noval Rivas, T.A. Chatila, Regulatory T cells in allergic diseases, *J. Allergy Clin. Immunol.* 138 (2016) 639-652.

Figure legends

Figure 1 DLL1 expression following transduction of bone marrow-derived dendritic cells (BMDCs) with a DLL1-expressing adenovirus (Ad-DLL1). Levels of (A) mRNA and (B) protein expressions of DLL1 in Ad-infected DCs were respectively measured by RT-PCR and FACS analyses. DCs were generated from the bone marrow of BALB/c mice and infected with the Ad-DLL1 at various multiplicities of infection (MOIs) of 10, 50, and 100. DCs transduced with the Ad-mock encoding no transgene served as a control (at an MOI of 100). At 24 h after further OVA stimulation, cells were collected and assessed.

Figure 2 Transduction of DCs with Ad-DLL1 induced activation of the DC phenotype. (A-E) IL-12, IL-10, and proinflammatory cytokines (IL-1 β , IL-6, and TNF- α) production from Ad-infected DCs. (F, G) Expression of MHC class II surface molecules, CD80 and CD86, on Ad-infected DCs. After 6 days of culture, DCs were infected with various MOIs of the Ad-DLL1 or Ad-mock and then treated with OVA (50 μ g/ml). After 24 h of incubation with OVA, supernatants were analyzed for cytokine production by an ELISA, and cells were harvested to detect surface activation markers by flow cytometry. Representative flow cytometric profiles of expressions of these surface molecules on gated CD11c⁺ cells are shown. The mean fluorescence intensity (MFI) was calculated, and results are expressed as the mean $\pm$ SEM from three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control DCs. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. mock DCs.

Figure 3 DLL1-DCs enhanced T cell proliferation and IFN- γ production by activated

T cells *in vitro*. (A, B) The ability of DLL1-DCs to stimulate an allogeneic T cell response. DCs from BALB/c mice transduced with Ad-DLL1 or Ad-mock at an MOI of 100. These DCs were stimulated with OVA (50 μ g/ml) for another 24 h and then irradiated. In a mixed lymphocyte reaction, allogeneic CD4⁺ T cells from C57BL/6 mice were cocultured with different proportions of γ -irradiated DCs at DC: T-cell ratios of 1:10, 1:20, 1:40, and 1:80 in 96-well plates for 54 h. Supernatants were collected for cytokine analysis, and cells were pulsed with [³H]-thymidine for another 18 h. The proliferative capacity was expressed as counts per minute (cpm). Levels of IFN- γ in supernatants were analyzed by an ELISA. (C-D) For the OVA-specific CD4⁺ T-cell response, different treated DCs from C57BL/6 mice were cocultured with OT-II CD4⁺ T cells for 54 h. Proliferative responses were assessed, and IFN- γ levels of supernatants were analyzed. Results are expressed as the mean $\pm$ SEM from three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control DCs. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. mock DCs.

Figure 4 Levels of anti-OVA specific antibodies in DLL1-DC-treated asthmatic mice. (A) Schematic diagram of the animal sensitization and challenge protocol. Mice were randomly assigned to four groups. On days 1, 10, and 20, all groups of mice were sensitized by an intraperitoneal (i.p.) injection of OVA. On day 20, three groups of mice were respectively intravenously (i.v.) injected with 5×10^5 OVA-pretreated control DCs, mock DCs (at an MOI of 100), and DLL1-DCs (at an MOI of 100). Positive control (PC) mice were injected with PBS instead of cells. These mice were further challenged with OVA for 5 consecutive days (days 28~32), and the AHR was measured 1 day after the last challenge. BALF was collected after measuring the AHR. (B, C) DLL1-DC treatment reduced IgE production but enhanced IgG_{2a} secretion in mice with OVA-induced asthma. Results are expressed as the mean $\pm$ SEM of six

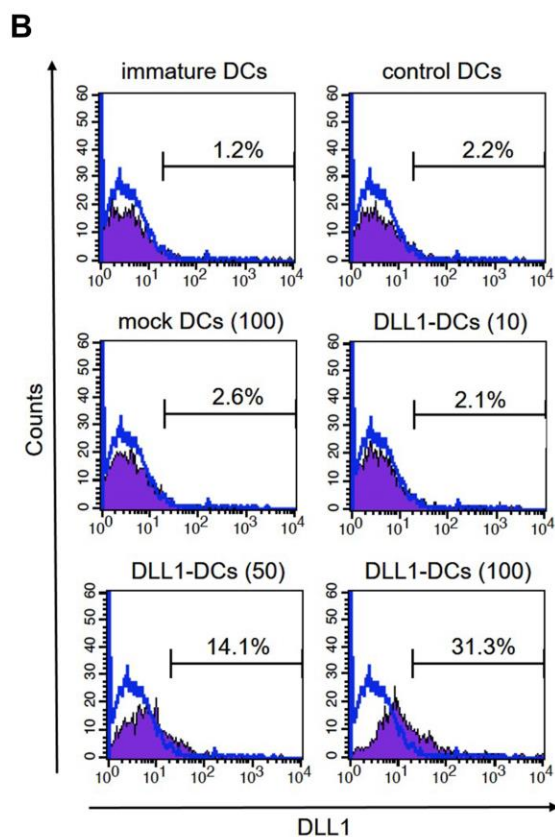
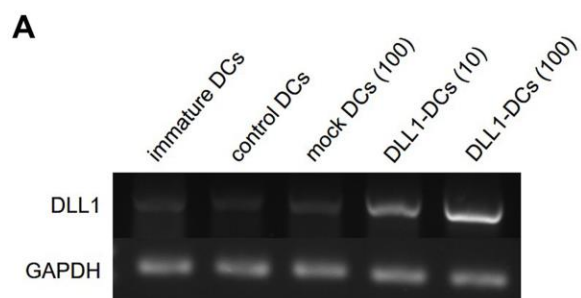
mice in each group. * $p < 0.05$, *** $p < 0.001$ vs. the positive control (PC) group. # $p < 0.05$ vs. the control-DC group. + $p < 0.05$ vs. the mock-DC group.

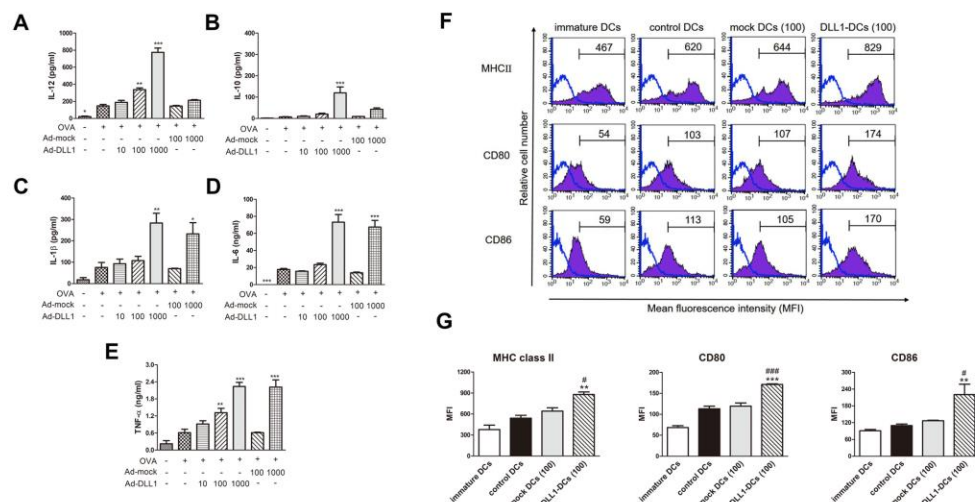
Figure 5 Effects of DLL1-DC treatment on airway function and inflammation. (A) Adoptive transfer of DLL1-DCs suppressed the development of AHR in OVA-induced asthma. One day after the last OVA challenge, airway reactivity to increasing concentrations of methacholine (1~32 mg/mL) was measured by invasive body plethysmography. Results are expressed as the pulmonary resistance (R_L) in the ratio of lung resistance after PBS nebulization. (B) Effect of DLL1-DCs on airway inflammatory cells. After measuring the pulmonary function parameters, each group of mice was sacrificed, and BALF was collected. Cells from BALF were counted and classified as macrophages, eosinophils, neutrophils, and lymphocytes. Results are expressed as the mean $\pm$ SEM of six mice in each group. * $p < 0.05$ vs. the PC group. # $p < 0.05$, ## $p < 0.01$ vs. the control-DC group. + $p < 0.05$ vs. the mock-DC group. (C) Lung sections were stained with hematoxylin/eosin (H&E) to measure inflammatory cells. (D) Lung sections were stained with periodic acid-Schiff (PAS) to measure mucus production around the airway.

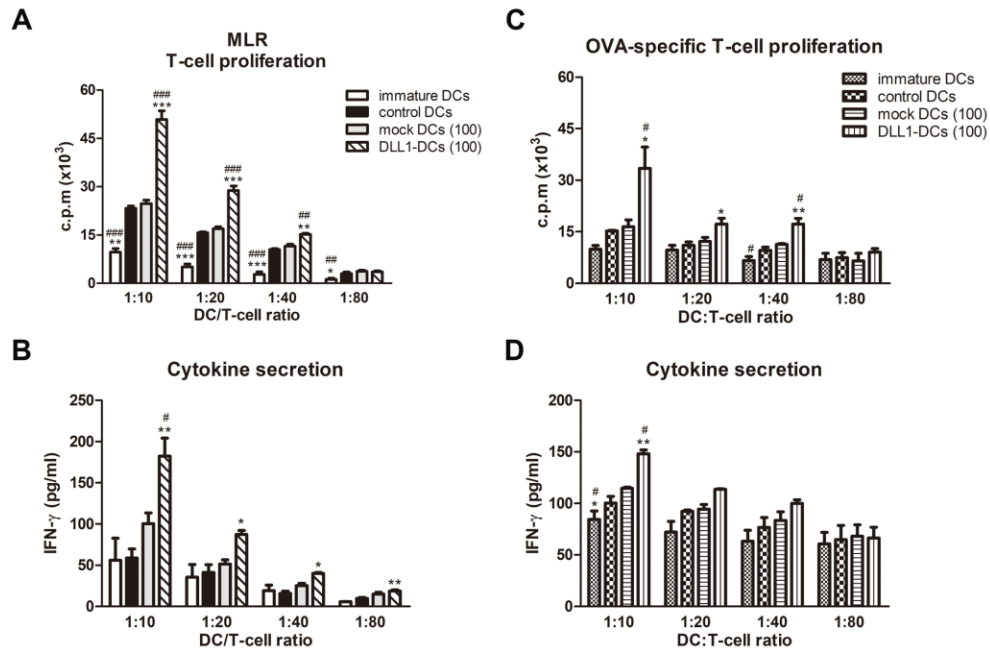
Figure 6 Effect of DLL1-DCs on cytokine and chemokine production in BALF and OVA-specific splenocyte culture supernatants. (A-F) Effects of DLL1-DCs on cytokines (IL-4, IL-5, IL-10, IL-13, and IFN- γ), and eotaxin production in BALF. After measuring pulmonary function parameters, each group of mice was immediately sacrificed, and the BALF was collected and analyzed for eotaxin, IL-4, IL-5, IL-13, and IFN- γ contents by ELISAs. (G-J) Effects of DLL1-DCs on levels of IL-4, IFN- γ , TNF- α , and IL-10 production by OVA-specific T cells. Splenocytes (10^7 cells/well) from each group were stimulated with 50 μ g/ml OVA *in vitro*. After 72 h of incubation,

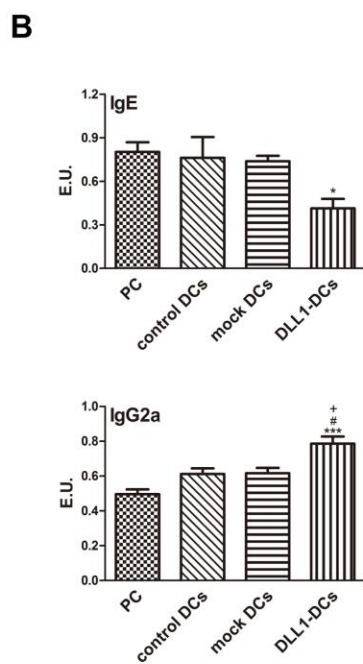
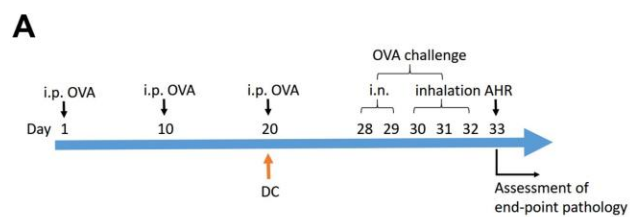
culture supernatants were collected, and cytokine contents were measured by ELISAs. Results are expressed as the mean $\pm$ SEM of six mice in each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. the PC group. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. the control-DC group. + $p < 0.05$, ++ $p < 0.01$ vs. the mock-DC group.

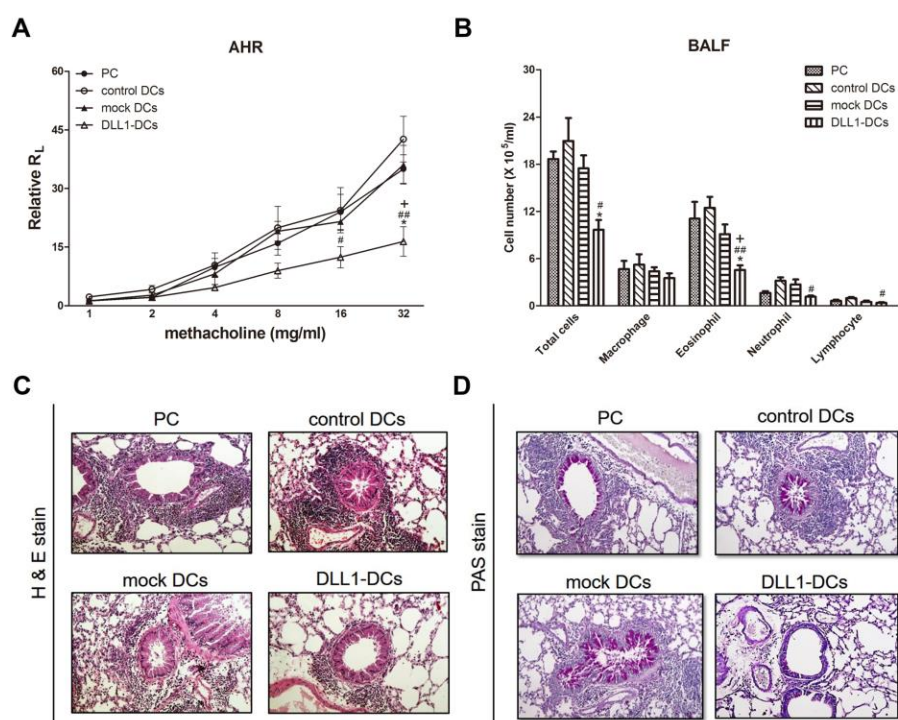
ACCEPTED MANUSCRIPT

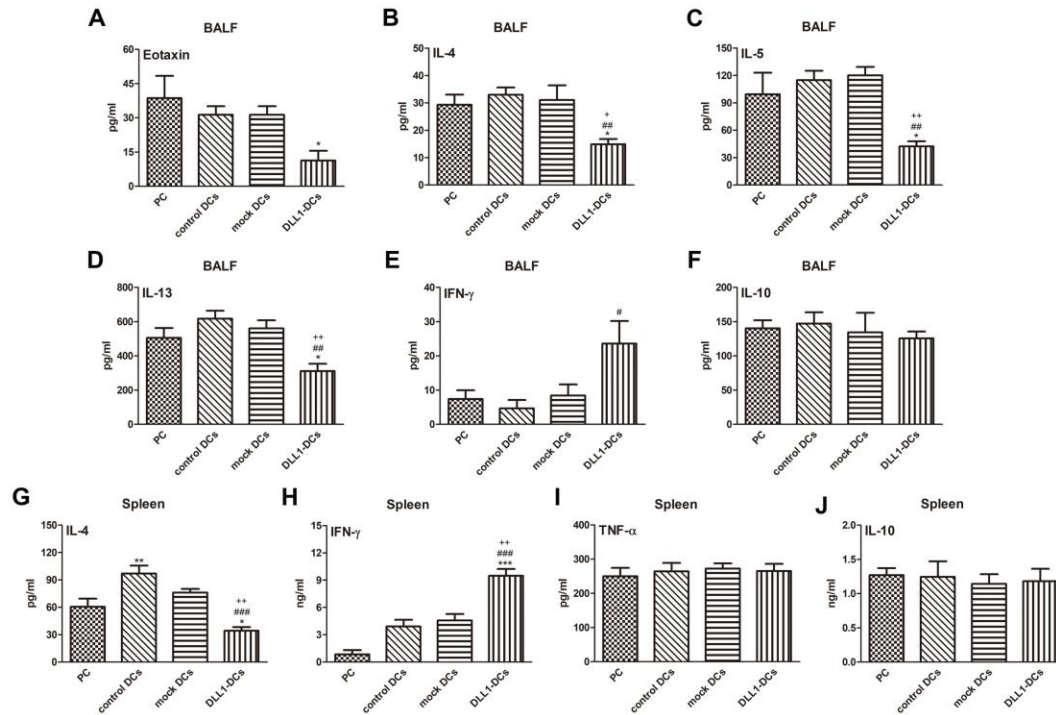


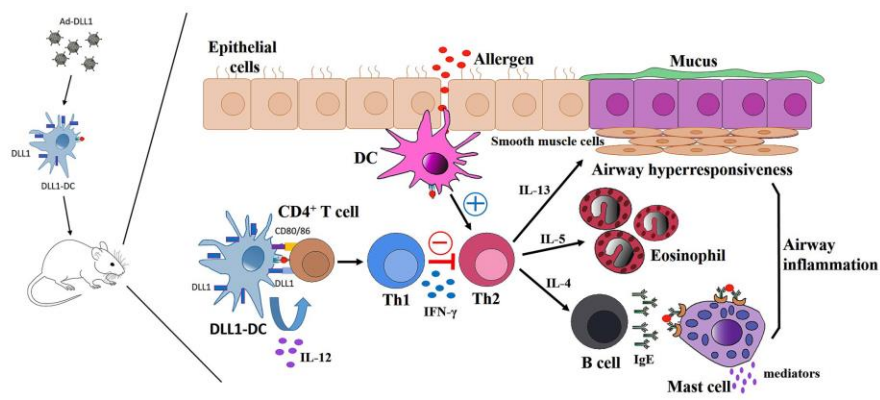












Highlights

1. DLL1-overexpressing DCs (DLL1-DCs) exerted a fully mature phenotype.
2. DLL1-DCs enhanced T cell proliferation and Th1-cell polarization *in vitro*.
3. DLL1-DC treatment downregulated anti-OVA IgE levels in OVA-induced allergic asthma.
4. DLL1-DC treatment decreased the severity of AHR and airway inflammation.
5. DLL1-DC treatment altered the Th1/Th2 ratio and ameliorated Th2-driven asthma.