

**Effect of Respiratory Syncytial Virus Infection on Plasmacytoid Dendritic Cell-  
Regulation of Allergic Airway Inflammation**

**Short title: RSV on pDCs in asthma**

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## ABSTRACT

**Background:** Respiratory syncytial virus (RSV) can infect myeloid dendritic cells (mDCs) and regulate their function in the development of allergy. It has been widely reported that plasmacytoid DCs (pDCs) play a critical role in anti-viral innate immunity. In contrast, not much is known about the role of pDCs in the interaction between allergy and viral infection. The purpose of the present study was to investigate the effect of RSV infection on pDC function in the regulation of allergic airway inflammation in a murine model of *Dermatophagoides farinae* (*Derf*)-sensitized allergic asthma.

**Methods:** Splenic pDCs isolated from *Derf*-sensitized donor mice were infected with live RSV *ex vivo*. Subsequently, these pDCs were inoculated into the airways of *Derf*-sensitized recipient mice. Lung pathology, lung tissue cytokine profiles, the number of regulatory T cells (Treg) and mDCs, as well as the effects of IL-10 neutralization in the lung tissue of recipient mice were determined.

**Results:** Intranasal inoculation of *Derf*-sensitized pDCs significantly inhibited the development of allergic airway inflammation and both Th1 and Th2 immunity. Live RSV infection of these pDCs prior to inoculation interfered with their inhibitory effects through decreasing Treg and IL-10 and increasing mDCs.

**Conclusions:** In asthmatic airways, pDCs mediate tolerance to inhaled allergens

through the regulation of Treg, IL-10 and mDCs. RSV infection of pDCs potentially inhibits their immunotolerogenic effects and thus exacerbates allergic airway inflammation.

**Key words:** plasmacytoid dendritic cell; asthma; respiratory syncytial virus; *Dermatophagoides farinae*; regulatory T cells

## **Introduction**

Bronchial asthma is a chronic airway inflammatory disease characterized by reversible airflow obstruction and hyper-responsiveness to non-specific stimuli, and represents a type-2 helper T (Th2) cell-mediated immunologic lung disease.[1] The most common trigger of acute exacerbation of asthma is viral infection in both children and adults.[2,3] Although the exact underlying mechanism of virus-induced asthma exacerbation remains unknown, it is likely that viral infection exacerbates Th2-dominant allergic airway inflammation.[4] Respiratory syncytial virus (RSV) is a representative lower respiratory tract pathogen of childhood and may exacerbate asthma.[5,6] According to the so-called hygiene hypothesis[7], repeated viral infections in early childhood inhibit the development of allergic disease[8], whereas rival hypotheses propose that viral respiratory infections and mite allergen exposure promote the development of asthma.[9] An infant history of RSV bronchiolitis can result in the development of asthma in later childhood.[10-12] Therefore, the interaction between viral infections and allergic asthma remains unclear.

Dendritic cells (DCs) are the most potent antigen-presenting cells (APCs) in the airways and are involved in antigen uptake, processing, and presentation of antigenic fragments to T cells in the respiratory mucosa.[13,14] Furthermore, DCs

determine the type of T cell-mediated immune response (Th1 or Th2) and play a central role in initiating both allergy and infection.[15,16] Lung tissue contains a large number of DCs. Until recently, the precise lineage of DCs in lung tissue was poorly defined. It is now becoming clear that different DC subsets exist with functional specialization. In humans and mice, several subtypes of DCs, characterized by surface markers and function, have been described. Generally, DCs can be distinguished as either myeloid DCs (mDCs) or plasmacytoid DCs (pDCs).[17] Utilizing a murine model of allergic asthma and bone marrow-derived mDCs, we previously reported that the in vivo transfer of mDCs into airways caused allergic airway inflammation[18], that primary RSV infection ameliorated mDC-induced allergic airway inflammation[19] and that repeated RSV infection enhanced allergic airway inflammation via differential regulation of pulmonary mDC function.[20] It is likely that mDCs are collectively important for generating T cell division and priming and that RSV can directly infect mDCs to modulate their immunological properties. On the other hand, pDCs possess only a modest capacity to activate naïve T cells and constitute an essential component of innate immunity by secreting various cytokines and chemokines as well as by participating in the activation of natural killer cells.[21-23] pDCs have been demonstrated to be specialized in the production of high levels of IFN $\alpha/\beta$  in response to

various viruses both in humans and mice.[24-26] In contrast to their primary role in innate immunity, not much is known about the role of pDCs in allergy. It has been reported that pDCs have an essential immunoregulatory role in the lung that protects against the development of airway inflammation towards harmless antigens in mice.[23] To date, there have been few studies that have evaluated the role of pDCs in the interaction between respiratory viral infection and allergy.

The purpose of this study was to investigate the effect of RSV infection on pDC function in the regulation of allergic airway inflammation in a murine model of allergic asthma.

## **Methods**

### **Mice**

Female BALB/c mice, 4–6 weeks of age, free of specific pathogens, were purchased from Charles River Laboratories (Yokohama, Japan). They were housed under pathogen-free conditions at the Laboratory Animal Center for Biochemical Research, Nagasaki University School of Medicine. All experimental procedures were reviewed and approved by the Nagasaki University School of Medicine Committee on Animal Research. Experiments were repeated at least three times.

### **Virus**

The human RSV A2 strain was purchased from the American Type Culture Collection (ATCC, Rockville, MD), and the virus was propagated by infection of Hep-2 cells (ATCC) in a monolayer culture. For preparation of a large stock of the virus, Hep-2 cells were infected with the ATCC stock virus and the cell supernatant was collected 5 or 6 days post-infection. The supernatant was centrifuged at 2,000 rpm for 10 min at 4 °C to remove cellular debris. The clear supernatant was collected and stored at –70 °C. The infectivity of the virus stock ( $1.0 \times 10^4$  plaque-forming units RSV/mL) was adjusted as assessed by a quantitative plaque-forming assay. An aliquot of the viral

suspension was inactivated by heating for 30 min at 121 °C (heat-inactivated RSV; HI-RSV).

### **Preparation of pDC from mite allergen-sensitized donor mice**

Two groups of donor mice were prepared: Control and *Dermatophagoides farinae* (*D. farinae*) allergen-sensitized (Df) mice. Control mice were injected twice intraperitoneally (i.p.) on days 1 and 14 with phosphate-buffered saline (PBS) and then challenged intranasally (i.n.) with 50 µL of PBS once a day on days 14–16. Df mice were immunized twice i.p. on days 1 and 14 with 0.5 mg/mouse of *D. farinae* allergen (LG-5339, Cosmo Bio, Tokyo, Japan) precipitated in aluminum hydroxide and then challenged i.n. with 50 µg/mL of *D. farinae* allergen once a day on days 14–16. Donor mice were sacrificed by cervical dislocation on day 17 followed by dissection of the spleen tissue. For splenic pDC isolation, spleen tissues were injected with 500 µL of Collagenase D (Boehringer-Mannheim, Mannheim, Germany) solution per spleen, the tissue was then cut into small pieces and incubated in Collagenase D solution for 30 min at 37 °C. Splenic cells were mechanically filtered through a 250-µm mesh, and further purified over a Ficoll (Amersham Biosciences, Uppsala, Sweden) gradient by suspending the cells in a 10-mL solution of 1.075 g/mL high density Ficoll and

centrifuging at 1,500 rpm for 10 min. Low density cells were collected, resuspended, and washed in RPMI-1640 (Gibco, Grand Island, NY, USA) supplemented with 1% fetal bovine serum (FBS) and 0.1% penicillin-streptomycin (Gibco) (hereafter referred to as cRPMI). Splenic pDCs, isolated from Control (contDC) and Df (DfDC) mice were purified by magnetic cell sorting (MACS Technology) using the Plasmacytoid Dendritic Cell Isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. Splenic cells were incubated with anti-CD3, anti-CD19, anti-CD11b and anti-CD49b antibody-MicroBeads and were passed through a negative selection LD column with a MidiMACS magnet. The collected unlabeled cells were incubated with anti-CD45R (B220) antibody-MicroBeads and passed through a positive selection LS column with a MiniMACS magnet. The viability of the cells was assessed, in triplicate, after 48 h by trypan-blue exclusion. The isolated splenic pDCs were sorted based on their expression of PDCA-1 and CD11c using specific antibodies (Miltenyi Biotec) and a BD FACS CantoII (Becton Dickinson, Oakville, Canada). The pDC population was  $\geq 90\%$  pure in three independent experiments.

### **In vivo transfer of pDCs to mite allergen-sensitized recipient mice**

Purified splenic pDCs from donor mice were washed and resuspended in cRPMI.

Isolated contDC or DfDCs were inoculated i.n. at a concentration of  $4 \times 10^5$  cells, in a volume of 100  $\mu\text{L}$ /mouse, into 6-week-old naïve female BALB/c mice on day 1. All mice were also immunized i.p. on day 1 with 0.5 mg/mouse of the *D. farinae* allergen precipitated in aluminum hydroxide. Mice were then challenged i.n. with either 50  $\mu\text{L}$  of PBS (contDC-PBS and DfDC-PBS groups of mice) or with 50  $\mu\text{L}$  of 50  $\mu\text{g}/\text{mL}$  of *D. farinae* allergen (contDC-Df and DfDC-Df groups of mice) once a day on days 14–16.

In a different set of experiments, DfDC-Df recipient mice were injected i.p. with 500  $\mu\text{g}$  anti-mouse IL-10R monoclonal antibody (1B1.3a; Becton Dickinson) (DfDC-Df- $\alpha\text{IL-10R}$  group) or isotype control antibody (rat IgG1; BD Biosciences) on day 1 together with the *D. farinae* allergen precipitated in aluminum hydroxide.

Bronchoalveolar lavage (BAL) was conducted using 1 mL of PBS in the immediate postmortem period at 17 days after receipt of the pDCs. The obtained BAL samples were centrifuged. Differential cell counts were performed using cytocentrifuged BAL samples stained with May-Grünwald-Giemsa. Lung tissues were also harvested for staining with hematoxylin and eosin (H&E) and periodic acid-Schiff stain (PAS).

Mononuclear cells (MNCs) were prepared from lung tissues of mice that were isolated as described previously [27]. In brief, lung tissues were chopped using sterile scissors, and digested at 37 °C in a water bath for 2 h in digestion buffer containing 1.5 mg/mL

collagenase A (type IA, Boehringer Mannheim, Mannheim, Germany), 0.02 mg/mL DNase I (type I, Boehringer Mannheim), and 0.75 mg/mL hyaluronidase (type I, Sigma). Digested lungs were filtered using a metal mesh to exclude cell pellets. The filtered digests were then washed three times with cRPMI 1640, followed by density gradient centrifugation to purify MNCs. These MNCs were cultured in the absence or presence of *D. farinae* for 48 h. The concentration of IFN- $\gamma$ , IL-5 and IL-10 in the supernatants were determined by ELISA (Quantikine, R&D Systems Inc., Minneapolis, MN, USA) using the procedure described in the instruction manual.

### **Effects of RSV infection on pDCs in vitro and in vivo**

In a different set of experiments, contDC and DfDC were infected with live RSV or HI-RSV [multiplicity of infection (MOI) of 1.0] for 2, 24 or 48 h. Quantitative real time PCR analysis of RSV L mRNA expression was performed using an ABI 7500 (Applied Biosystems) as described elsewhere.[25] IFN- $\alpha$  was detected in the pDC-culture supernatants by ELISA (Quantikine). Four groups of pDCs (contDC-sham-Df, DfDC-sham-Df contDC-RSV-Df and DfDC-RSV-Df) were prepared from the spleen tissues of Control and Df mice. The contDC-sham-Df and DfDC-sham-Df groups were sham-infected with culture medium and the contDC-RSV-Df and DfDC-RSV-Df groups

were infected with live RSV for 24 h, at which time the viability of the RSV infected pDCs was >98%. Thereafter, these pDCs, at a concentration of  $4 \times 10^5$  cells and in a volume of 100  $\mu$ L/mouse, were inoculated i.n. into four groups of 6-week-old female BALB/c mice on day 1. These mice were i.p. immunized twice (on days 1 and 14) with 0.5 mg/mouse of *Df* allergen precipitated in aluminum hydroxide. All groups of mice were then i.n. challenged with 50  $\mu$ g/mL of the *Df* allergen once a day on days 14–16. Lung pathology, BAL and cytokine profiles in lung MNCs were determined as described above. HI-RSV failed to significantly regulate pDC function both *in vitro* and *in vivo*.

#### **Flow cytometric analysis of Tregs, myeloid DCs and IL-10 producing cells.**

Lung MNCs were prepared from the contDC-sham-Df, DfDC-sham-Df and the live RSV-infected contDC-RSV-Df and DfDC-RSV-Df groups of mice and were resuspended at  $1 \times 10^6$  cells/mL. The cells were stained for expression of extracellular CD4 and CD25 and intracellular Foxp3 using the Mouse Treg Flow™ kit (Biolegend, San Diego, CA, USA). In a different set of experiments, the cells were stained for expression of extracellular CD4 and intracellular Foxp3 using PerCP-Cy5.5-labeled anti-murine CD4 antibodies and Alexa Fluor®488-labeled anti-murine Foxp3

antibodies (BD Pharmingen, San Diego, CA, USA). For intracellular IL-10 staining, MNCs were cultured in the presence of 10  $\mu$ g/mL Brefeldin A (Sigma, Buchs, Switzerland) for 4 h and were then stained with PE-labeled anti-murine IL-10 antibodies (BD Pharmingen).[28] mDCs were identified as cells that stained positive with fluorescent antibodies against CD11c (Miltenyi Biotec) and CD11b (BD Pharmingen) but negative with antibodies against GR-1 (BD Pharmingen). For each analysis, staining with the appropriate isotype control mAb was also performed. The value of the control antibody was subtracted from that obtained for each antibody used. All flow cytometry acquisitions were performed using a BD FACS CantoII (Becton Dickinson).

### **Statistical Analysis**

The results are expressed as the mean  $\pm$  SEM. Differences between groups were examined for statistical significance using repeated-measures analysis of variance with a Bonferroni multiple comparison test. A p value of less than 0.05 was considered significant.

## Results

### **In vivo transfer of pDC-attenuated allergic airway inflammation in association with inhibition of Th1 and Th2 cytokines**

The effect of transfer of pDCs, isolated from control or Df mice, on allergic airway inflammation following challenge was examined. Mite allergen sensitized recipient mice harboring contDC (contDC-Df group) developed allergic airway inflammation characterized by peribronchovascular eosinophilic inflammation and goblet cell hyperplasia, as well as pulmonary eosinophilia determined by BAL. Transfer of DfDC significantly attenuated mite allergen-induced allergic airway inflammation (Figures 1A and B). The concentration of IFN- $\gamma$ , IL-5 and IL-10 in pulmonary MNCs isolated from these mice, and cultured in the absence or presence of the *Derf* allergen, was determined by ELISA (Figure 2). In comparison with contDC-PBS mice, contDC-Df mice produced significantly higher amounts of IFN- $\gamma$  and IL-5. In the DfDC-Df mice, production of both IFN- $\gamma$  and IL-5 was significantly inhibited compared to contDC-Df mice. Additionally, the concentration of IL-10, an immunoregulatory cytokine, was significantly enhanced in DfDC-Df mice compared to contDC-Df mice. Administration of the IL-10R monoclonal antibody before pDC transfer into DfDC-Df mice (DfDC-Df- $\alpha$ IL-10R group) significantly increased the number of pulmonary

eosinophils (Figure 1) and the level of IFN- $\gamma$  and IL-5 (Figure 2), while these parameters were still significantly lower compared to those in contDC-Df mice. In contrast to the effect of the IL-10R monoclonal antibody, administration of an isotype antibody into DfDC-Df mice failed to show significant effects on either the pathology or the cytokine profiles (data not shown). The combined data suggest that allergen-sensitized pDCs inhibited allergic airway inflammation and both Th1 and Th2 immunity, at least partially via induction of IL-10.

### **RSV replicated in pDCs and induced IFN- $\alpha$ production**

To determine the susceptibility of splenic pDCs to RSV infection, ContDCs and DfDCs were exposed to either live RSV or HI-RSV (MOI of 1.0) for 2, 24 or 48 h. RSV L-gene mRNA expression was detected within 2 h ( $1020 \pm 304.4$  copies), peaked at 24 h ( $3420 \pm 640.5$  copies) and had decreased by 48 h ( $760 \pm 175.3$  copies), as assessed by quantitative real time PCR. In accordance with these PCR results, live RSV infection induced IFN- $\alpha$  production 24 h post-infection at a significantly higher level than HI-RSV infection ( $105.9 \pm 16.4$  pg/mL for live RSV vs.  $13.6 \pm 5.4$  pg/mL for HI-RSV,  $p < 0.05$ ). The levels of IFN- $\alpha$  were comparable between contDC and DfDC. Therefore, murine pDCs can be infected with live RSV and produce anti-viral IFN as described

elsewhere.[29-32]

### **Regulation of the in vivo function of mite allergen-sensitized pDCs by RSV**

Live RSV- or sham-infected-pDCs were inoculated i.n. into mite allergen-sensitized donor mice followed by i.n. Df challenge. Representative photographs of the lung pathology as well as the BAL cell counts of these mice are shown in Figures 3A and B.

In accordance with the earlier results, contDC-sham-Df mice developed allergic airway inflammation and DfDC-sham-Df mice showed ameliorated allergic airway inflammation. The lung pathology and different cellular components of BAL of contDC-RSV-Df mice were similar to those in contDC-sham-Df mice. In marked contrast to the DfDC-sham-Df mice, in which allergic airway inflammation was attenuated, DfDC-RSV-Df mice showed allergic airway inflammation similar to that in contDC-sham-Df mice. Cytokine profiles in lung MNCs revealed that the DfDC-sham Df mice showed significant reductions in IFN- $\gamma$  and IL-5 and a significant increase in IL-10 compared to the contDC-sham-Df mice (Figure 4). ContDC-RSV-Df mice showed a significant increase in IFN- $\gamma$  and a decrease in IL-10 but failed to alter IL-5 production compared to contDC-sham-Df mice. Compared to contDC-sham-Df mice, the DfDC-RSV-Df mice demonstrated significant increases in IFN- $\gamma$  and IL-5 but failed

to alter IL-10 production. To determine the mechanisms by which pDCs induce immune tolerance and the effects of RSV infection, the number of Treg, mDCs and the level of intracellular IL-10 in the lung tissues of recipient mice were determined by FACS analysis (Figure 5). Intranasal transfer of DfDC significantly increased the number of Treg and decreased that of mDCs compared to that of contDC. Furthermore, RSV infection significantly decreased Treg and increased mDCs in the lung tissue of DfDC-inoculated mice. Staining of intracellular IL-10 revealed that most CD4<sup>+</sup>IL-10<sup>+</sup> T cells were Foxp3<sup>-</sup> (Figure 5C). The pDCs *per se* failed to produce IL-10 following *Derf* or RSV stimulation (data not shown). The combined data suggest that RSV infection may inhibit the immunoregulatory effects of pDCs through reductions in IL-10 and Treg and an increase in mDCs, thereby leading to enhancement of Th1 and Th2 immunity, resulting in enhanced allergic airway inflammation.

## **Discussion**

We have previously shown that RSV infection inhibits mDC function in the development of allergic airway inflammation.[19] The present study further characterized the effect of RSV infection on the other important DC subset, the pDCs. The results indicated that pDCs inhibit the development of allergic airway inflammation and that RSV infection interferes with the pDC-inhibitory effect via inhibition of immunotolerance wherein Treg play a partial but critical role. Thus, RSV may differently regulate DC function in the development of allergic airway inflammation; RSV ameliorates or exacerbates allergic airway inflammation by regulation of mDCs or pDCs, respectively.

Intranasal inoculation of mite allergen-pulsed murine bone marrow-derived mDCs enhances allergic airway inflammation and IL-5 and IFN- $\gamma$  production in lung draining lymph nodes, indicating that mDCs promote allergy.[18,19] In contrast, depletion of pDCs during allergen inhalation resulted in the enhancement of Th2-mediated allergic airway inflammation. Furthermore, adoptive transfer of pDCs prior to allergen sensitization prevented allergic airway inflammation and suppressed the generation of effector T cells induced by mDCs.[23] These data suggest that pDCs have a protective role against allergy. In the present study, splenic pDCs isolated from mite

allergen-sensitized mice also inhibited allergic airway inflammation. Our preliminary unpublished observations showed that; i) pDCs isolated from PBS-challenged *Derf*-alum sensitized mice failed to cause significant effects in recipient mice, ii) pDCs isolated from *Derf*-sensitized and challenged mice failed to cause significant effects in *Aspergillus fumigatus* allergen-sensitized and challenged recipient mice and iii) in vitro sensitization of control pDCs with *Derf* allergen could not show similar effects to those observed with pDCs from allergen sensitized and challenged mice. These observations confirmed that the immunological effects of pDCs observed in the present study required systemic allergen sensitization along with local allergen challenge and that they were allergen specific. The cytokine profile of lung MNCs demonstrated that mite allergen-sensitized pDCs inhibit IL-5 and IFN- $\gamma$  production but increase IL-10 production. In addition, neutralization of IL-10 in the recipient lung significantly, but incompletely blocked the inhibitory effects of pDCs on the development of allergic airway inflammation. Since IL-10 has been linked to the development of Treg, which can be induced by pDCs [32], we had originally expected that a pDC-induced increase in Treg might be the source of the IL-10. Indeed, flow cytometric analysis revealed that intranasal transfer of mite allergen-sensitized pDCs enhanced the number of Treg in the lung tissues of recipient mice. However, unexpectedly, intracellular IL-10 staining

demonstrated that CD4<sup>+</sup>Foxp3<sup>-</sup>, but not CD4<sup>+</sup>Foxp3<sup>+</sup> cells were the source of the IL-10. In accordance with these results, adoptive transfer of Treg suppressed allergic inflammation via a mechanism dependent upon IL-10, wherein the IL-10 was induced by bystander CD4<sup>+</sup> cells rather than by the Treg themselves.[33] Although a direct association between Treg and IL-10 is yet to be determined these results suggest that one of the mechanisms by which pDCs inhibit allergic airway inflammation appears to be by the induction of both Treg and IL-10.

Few studies have evaluated the interaction between RSV infection and allergen sensitization at the level of pDCs. In this study, once they were infected with live RSV, the pDCs lost their ability to induce Treg and IL-10 in the lung tissues of recipient mice and thus lost their ability to inhibit development of allergic airway inflammation. However, neutralization of IL-10 in the recipient lung significantly, but incompletely, restored the inhibitory effects of pDCs in allergic airway inflammation. Although the number of pulmonary Treg significantly decreased in RSV-infected DfDC-transferred mice compared to that in sham-infected DfDC-transferred mice, it was still significantly higher than that in sham-infected, control DfDC-transferred mice. Nonetheless, both Th1 and Th2 cytokine production was significantly enhanced in lung tissue of RSV-infected DfDC-transferred mice compared to sham-infected control

DfDC-transferred mice. Collectively, these results suggest that RSV infection of pDCs not only interferes with Treg induction but also enhances adaptive immunity through another pathway. Our search for a second pathway focused on the regulation of mDCs by pDCs. It is known that, following viral infection pDCs produce IFN- $\alpha$ , which is a growth and maturation factor for mDCs.[32,34-36] Furthermore, pDCs have been reported to suppress eosinophilic inflammation by inhibiting the function of mDCs.[37] Intranasal inoculation of pDCs significantly decreased the number of mDCs in recipient mice, but significantly increased mDCs following RSV infection. Thus, the innate IFN- $\alpha$  response against RSV by pDCs might promote mDC proliferation resulting in the enhancement of adaptive immunity as previously described for the adjuvant action of poly IC.[38]

In conclusion, these results indicate that pDCs are potent inhibitors of the development of allergic airway inflammation and that these inhibitory effects are reduced following RSV infection. The present study indicates that the interaction between pDCs and RSV may modulate pDC-effects on immunity and that these pDC-effects include induction of IL-10, Treg and a decrease in the number of mDCs. These effects of pDCs may synergize with other as yet unknown cellular and molecular mechanisms to modulate immunity. In addition, the present study not only sheds light

on the mechanism of viral infection and allergy, but also suggests a new therapeutic modality. Therapies targeting the function of pDCs may be useful for generation of a vaccine against the development of asthma.

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## Figure legends

**Figure 1. Lung pathology and differential cell counts in bronchoalveolar lavage of mice inoculated with pDCs.** Mice were inoculated with splenic pDCs (CD3<sup>-</sup>CD19<sup>-</sup>CD11b<sup>-</sup>CD49b<sup>-</sup>B220<sup>+</sup>CD11c<sup>+</sup>PDCA1<sup>+</sup>) and, following sacrifice, lung tissues were obtained and stained with H&E and PAS. Representative photographs of each group (n=8, from three independent experiments, each experimental group consisted of 2–3 mice) are shown (A). Original magnification ×400. Differential cell counts in BAL (B). The bars represent the mean ± SEM (n=8 for each group). Differences between groups were examined for statistical significance using repeated-measures analysis of variance with a Bonferroni multiple comparison test. \*\*p<0.01 and \*p<0.05 vs. contDC-PBS. ††p<0.01 and †p<0.05 vs. contDC-Df. #p<0.05 vs. DfDC-Df.

**Figure 2. Cytokine profiles of lung tissue of pDC-inoculated mice.** Pulmonary MNCs were isolated from the indicated five groups of BALB/c mice inoculated with pDCs. These MNCs were cultured in the absence or presence of the *Derf* allergen, and the concentration of IFN- $\gamma$ , IL-5 and IL-10 was determined by ELISA. The bars represent the mean (n=8 for each group) ± SEM. Differences between groups were

examined for statistical significance using repeated-measures analysis of variance with a Bonferroni multiple comparison test. \*\* $p < 0.01$  and \* $p < 0.05$  vs. contDC-PBS. †† $p < 0.01$  and † $p < 0.05$  vs. contDC-Df. # $p < 0.05$  vs. DfDC-Df.

**Figure 3. Lung pathology and differential cell counts in bronchoalveolar lavage of mice inoculated with RSV-infected pDCs.** Mice were inoculated with RSV-infected pDCs. Lung pathology was determined microscopically (A) and BAL was analyzed (B) as described in the legend to Figure 1. The bars represent the mean  $\pm$  SEM (n=8 for each group). Differences between groups were examined for statistical significance using repeated-measures analysis of variance with a Bonferroni multiple comparison test. \*\* $p < 0.01$  and \* $p < 0.05$  vs. contDC-sham-Df. †† $p < 0.01$  and † $p < 0.05$  vs. DfDC-sham-Df.

**Figure 4. Cytokine profiles of lung tissue of RSV-infected pDC-inoculated mice.** Pulmonary MNCs were isolated from the indicated four groups of BALB/c mice inoculated with RSV- or sham-infected pDCs. These MNCs were cultured in the absence or presence of *Derf* allergen, and the concentration of IFN- $\gamma$ , IL-5 and IL-10 was determined by ELISA. The bars represent the mean (n=8 for each group)  $\pm$  SEM.

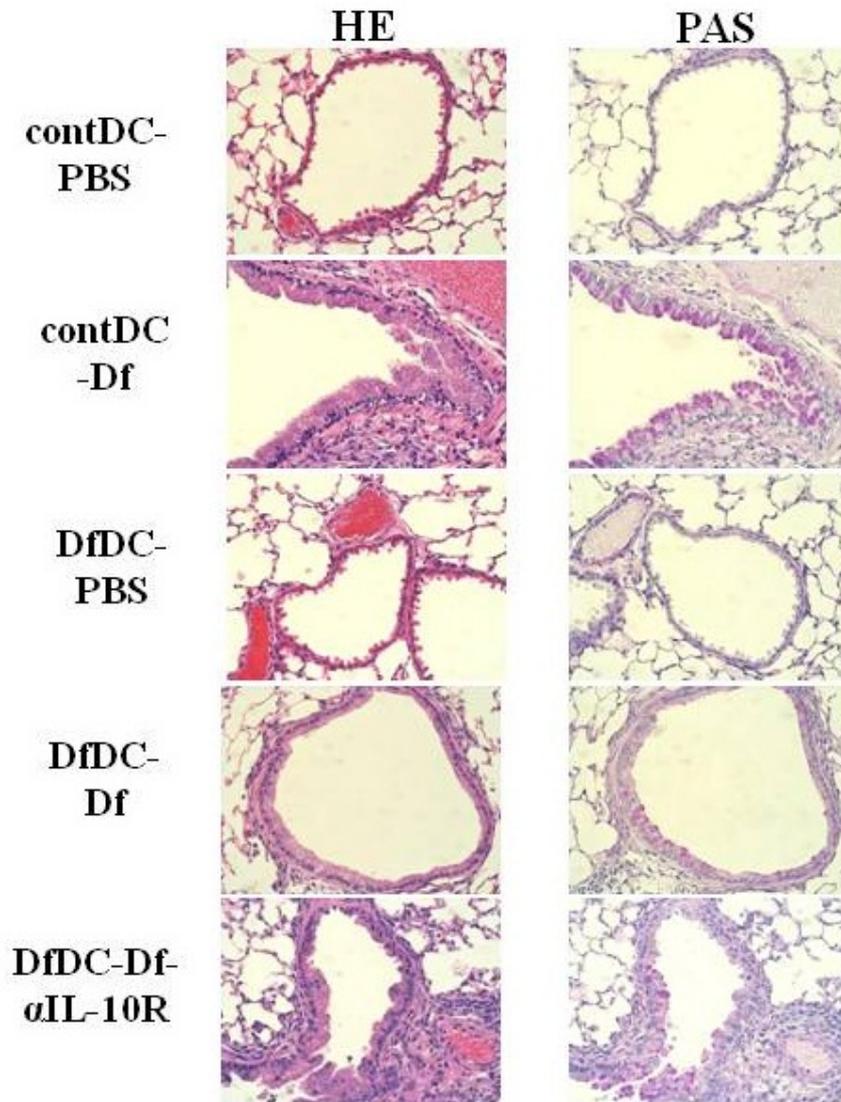
Differences between groups were examined for statistical significance using repeated-measures analysis of variance with a Bonferroni multiple comparison test.

\*\*p<0.01 and \*p<0.05 vs. contDC-sham-Df. ††p<0.01 and †p<0.05 vs. DfDC-sham-Df.

**Figure 5. Number of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells, mDCs and IL-10<sup>+</sup> populations in**

**lung.** Pulmonary MNCs were isolated from the indicated four groups of BALB/c mice that had been inoculated with pDCs. The number of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells (upper) and CD11c<sup>+</sup>CD11b<sup>+</sup>GR-1<sup>-</sup> mDCs (middle), as well as the number of IL-10<sup>+</sup> cells in CD4<sup>+</sup>Foxp3<sup>-</sup> and CD4<sup>+</sup>Foxp3<sup>+</sup> cells (lower) in the lung was then assessed. The bars represent the mean (n=8 for each group) ± SEM. Differences between groups were examined for statistical significance using repeated-measures analysis of variance with a Bonferroni multiple comparison test. \*p<0.05 vs. contDC-sham-Df. †p<0.05 vs. DfDC-sham-Df.

Figure 1. **A**



**B**

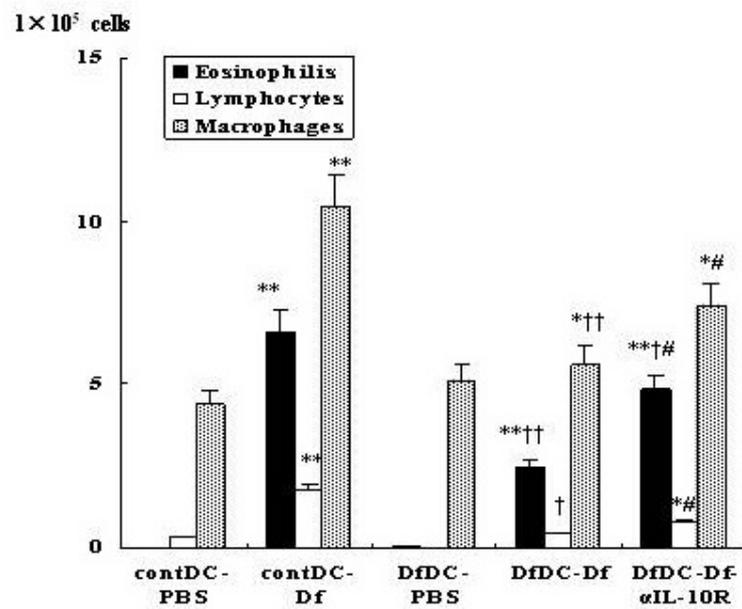


Figure 2.

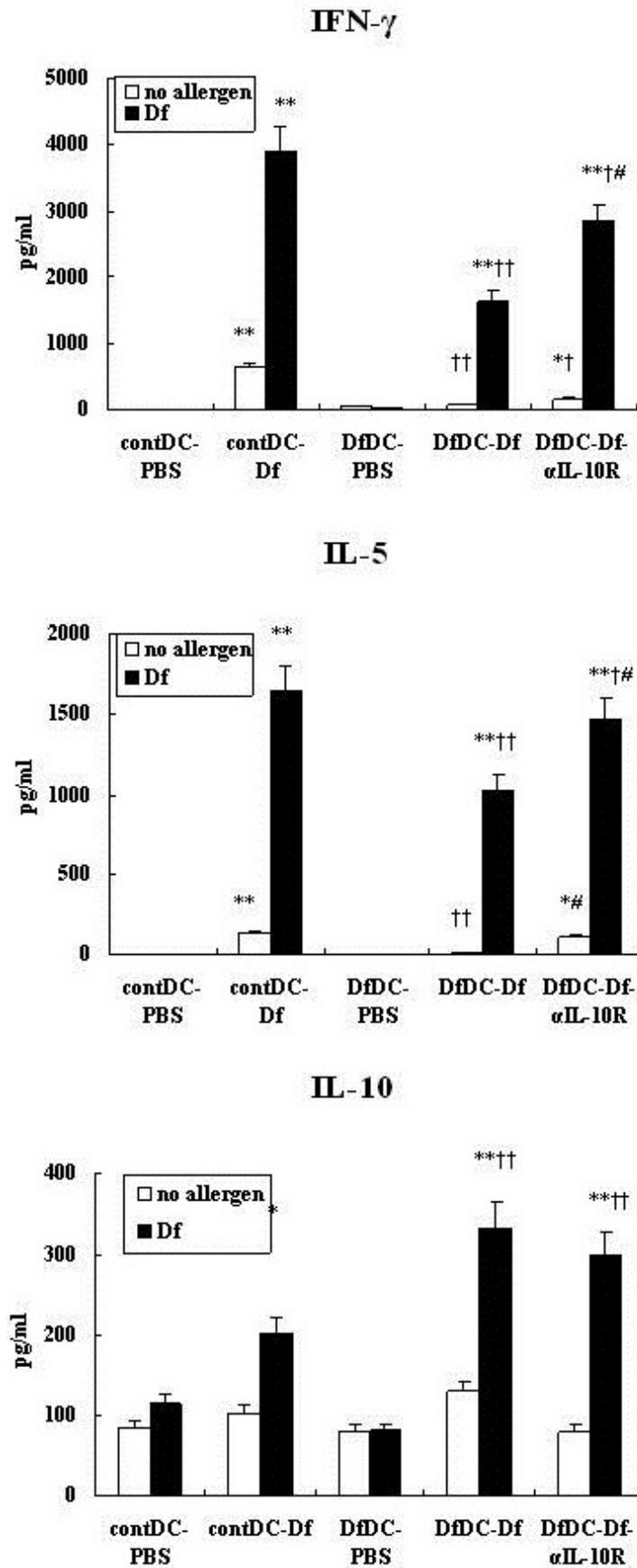


Figure 3.

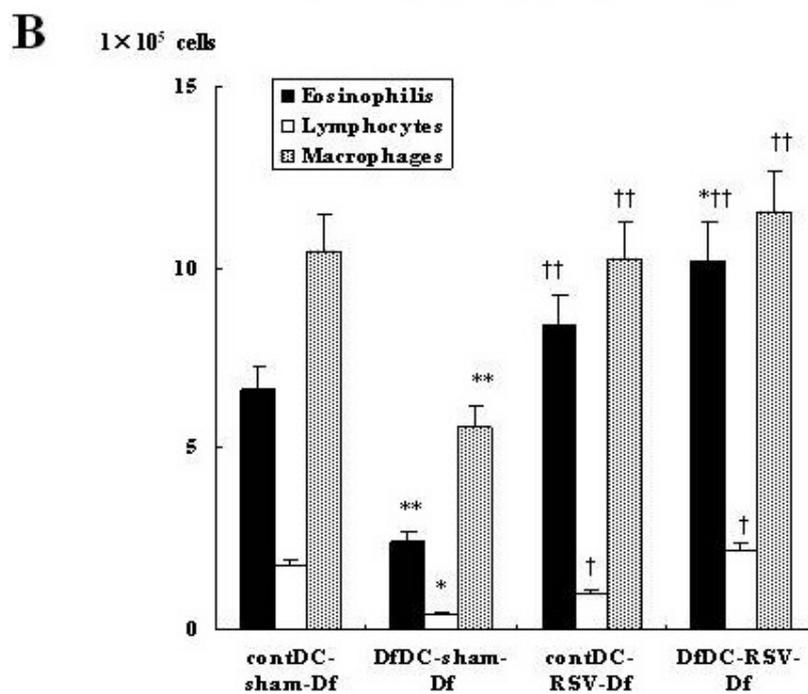
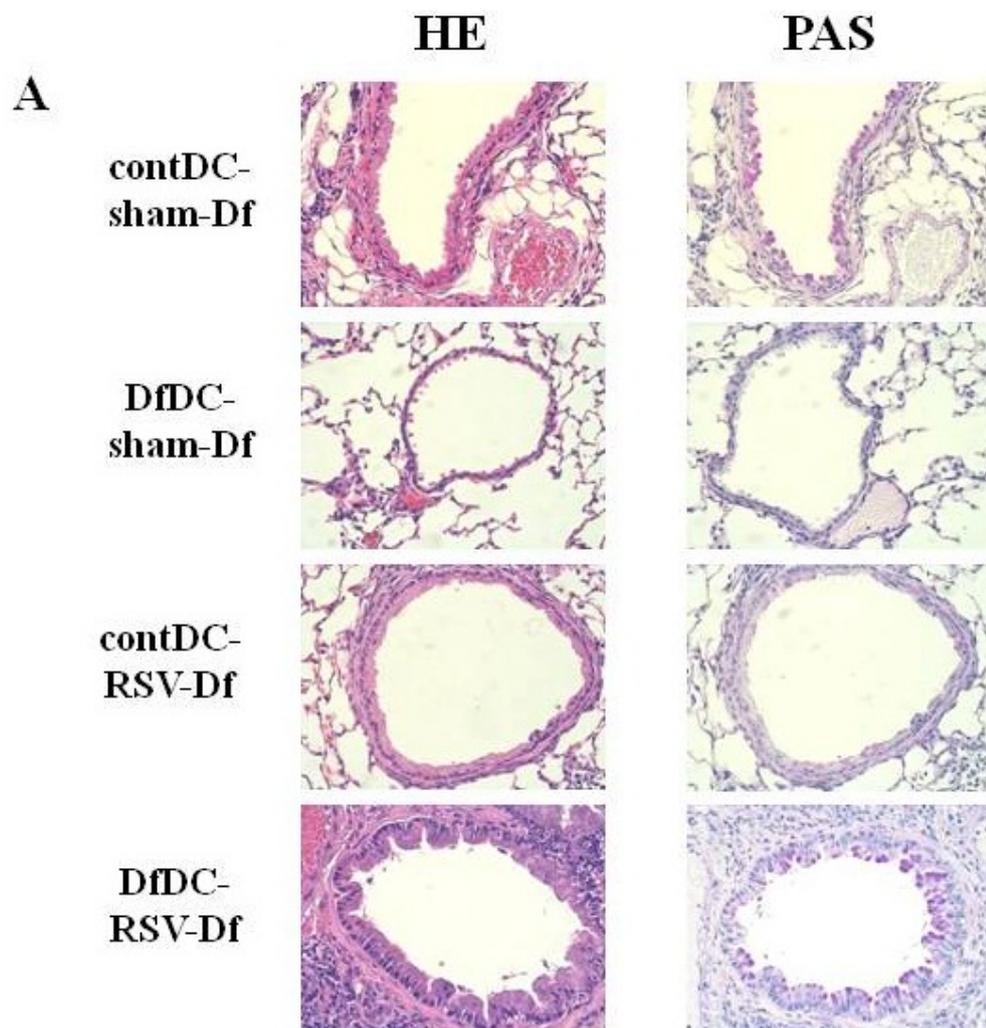


Figure 4.

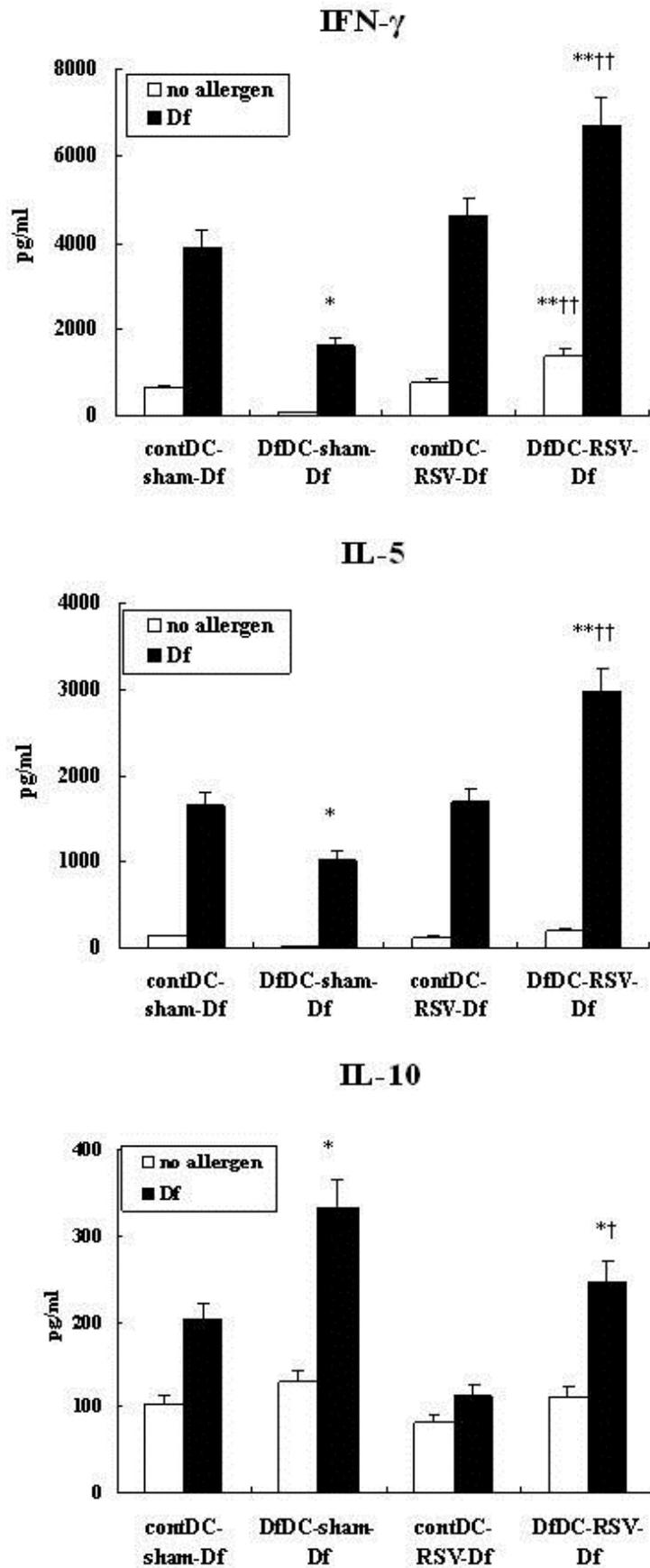
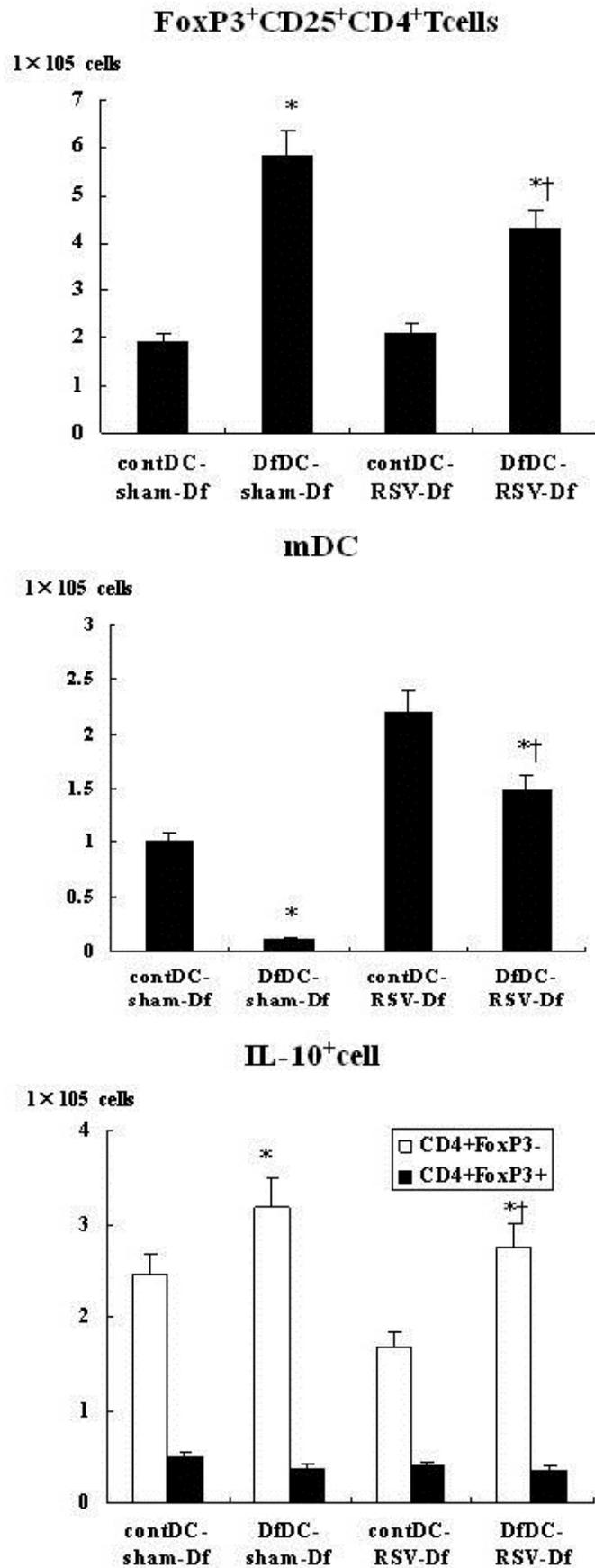


Figure 5.



## **References**

1. Barnes PJ. The cytokine network in asthma and chronic obstructive pulmonary disease. *J Clin Invest* 2008;118:3546-3556.
2. Lemanske RF Jr. Viruses and asthma: inception, exacerbation, and possible prevention. *J Pediatr*. 2003 Feb;142:S3-7.
3. Nicholson KG, Kent J, Ireland DC. Respiratory viruses and exacerbations of asthma in adults. *BMJ*. 1993;307:982-86.
4. Message SD, Johnston SL. The immunology of virus infection in asthma. *Eur Respir J*. 2001;18:1013-25.
5. Gern JE. Viral respiratory infection and the link to asthma. *Pediatr Infect Dis J*. 2004;23:S78-86.
6. Simoes EA. Respiratory syncytial virus infection. *Lancet* 1999;354:847-52.
7. Von Mutius E. Allergies, infections and the hygiene hypothesis-the epidemiological evidence. *Immunobiology*. 2007;212:433-39.
8. Renz H, Herz U. The bidirectional capacity of bacterial antigens to modulate allergy and asthma. *Eur Respir J*. 2002;19:158-71.
9. Murray CS, Simpson A, Custovic A. Allergens, viruses, and asthma exacerbations. *Proc Am Thorac Soc*. 2004;1:99-104.

10. Wu P, Dupont WD, Griffin MR, Carroll KN, Mitchel EF, Gebretsadik T, et al. Evidence of a causal role of winter virus infection during infancy in early childhood asthma. *Am J Respir Crit Care Med* 2008; 178:1123-29.
11. Schauer U, Hoffjan J, Bittscheidt J, Köchling A, Hemmis S, Bongartz S, et al. RSV bronchiolitis and risk of wheeze and allergic sensitization in the first year of life. *Eur Respir J* 2002;20:1277-83.
12. Sigurs N, Bjarnason R, Sigurbergsson F, Kjellmen B. Respiratory syncytial virus bronchiolitis in infancy is an important risk factor for asthma and allergy at age 7. *Am J Respir Crit Care Med* 2000;161:1501-07.
13. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature*. 1998 Mar 19;392:245-52.
14. Werling D, Koss M, Howard CJ, Taylor G, Langhans W, Hope JC. Role of bovine chemokines produced by dendritic cells in respiratory syncytial virus-induced T cell proliferation. *Vet Immunol Immunopathol* 2002;87:225-33.
15. Van Rijt LS, Lambrecht BN. Role of dendritic cells and Th2 lymphocytes in asthma: lessons from eosinophilic airway inflammation in the mouse. *Microsc Res Tech* 2001;53:256-72.
16. Klagge IM, Schneider-Schaulies S. Virus interactions with dendritic cells. *J Gen*

*Virology* 1999;80:823-33.

17. Steinman RM. Some interfaces of dendritic cell biology. *APMIS* 2003;111:675-97.

18. Machida I, Matsuse H, Kondo Y, Kawano T, Saeki S, Tomari S, et al. Cysteinyl leukotrienes regulate dendritic cell functions in a murine model of asthma. *J Immunol* 2004;172:1833-38.

19. Kondo Y, Matsuse H, Machida I, Kawano T, Saeki S, Tomari S, et al. Regulation of mite allergen-pulsed murine dendritic cells by respiratory syncytial virus. *Am J Respir Crit Care Med* 2004;169:494-98.

20. Hirose H, Matsuse H, Fukahori S, Tsuchida T, Tomari S, Kawano T, et al. Effects of repeated respiratory syncytial virus infections on pulmonary dendritic cells in a murine model of allergic asthma. *Int Arch Allergy Immunol.* 2008;147:197-205.

21. Liu YJ. IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. *Annu Rev Immunol.* 2005;23:275-306.

22. Cervantes-Barragan L, Züst R, Weber F, Spiegel M, Lang KS, Akira S, et al. Control of coronavirus infection through plasmacytoid dendritic-cell-derived type I interferon. *Blood.* 2007 Feb 1;109:1131-7.

23. De Heer HJ, Hammad H, Soullie T, Hijdra D, Vos N, Willart MA, et al. Essential role of lung plasmacytoid dendritic cells in preventing asthmatic reactions to harmless

inhaled antigen. *J Exp Med* 2004;200:89-98.

24. Dalod M, Hamilton T, Salomon R, Salazar-Mather TP, Henry SC, Hamilton JD, et al. Dendritic cell responses to early murine cytomegalovirus infection: subset functional specialization and differential regulation by interferon alpha/beta. *J Exp Med*. 2003 Apr 7;197(7):885-98.

25. Wang H, Peters N, Schwarze J. Plasmacytoid dendritic cells limit viral replication, pulmonary inflammation, and airway hyperresponsiveness in respiratory syncytial virus infection. *J Immunol*. 2006 Nov 1;177:6263-70.

26. Smit JJ, Rudd BD, Lukacs NW. Plasmacytoid dendritic cells inhibit pulmonary immunopathology and promote clearance of respiratory syncytial virus. *J Exp Med*. 2006 May 15;203:1153-9.

27. Kawano T, Matsuse H, Kondo Y, Machida I, Saeki S, Tomari S, et al. Cysteinyl leukotrienes induce nuclear factor kappa  $\beta$  activation and RANTES production in a murine model of asthma. *J Allergy Clin Immunol*. 2003 112:369-74.

28. Boudousquié C, Pellaton C, Barbier N, Spertini F. CD4<sup>+</sup>CD25<sup>+</sup> T cell depletion impairs tolerance induction in a murine mode of asthma. *Clin Exp Allergy*. 2009;39(9):1415-26.

29. Jewell NA, Vaghefi N, Mertz SE, Akter P, Peebles RS Jr, Bakaletz LO, et al.

Differential type I interferon induction by respiratory syncytial virus and influenza A virus in vivo. *J Virol.* 2007;81:9790-800.

30. Boogaard I, van Oosten M, van Rijt LS, Muskens F, Kimman TG, Lambrecht BN, et al. Respiratory syncytial virus differentially activates murine myeloid and plasmacytoid dendritic cells. *Immunology.* 2007;122:65-72.

31. Zuniga EI, Mcgavern DB, Prunedo-Paz JL, Teng C, Oldstone MB. Bone marrow plasmacytoid dendritic cells can differentiate into myeloid dendritic cells upon virus infection. *Nat Immunol.* 2004;5:1227-34.

32. Moseman EA, Liang X, Dawson AJ, Panoskaltsis-Mortari A, Krieg AM, Liu YJ, et al. Human plasmacytoid dendritic cells activated by CpG oligodeoxynucleotides induce the generation of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells. *J Immunol* 2004;173:4433-42.

33. Kearley J, Barker JE, Robinson DS, Lloyd CM. Resolution of airway inflammation and hyperreactivity after in vivo transfer of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells is interleukin 10 dependent. *J Exp Med.* 2005;202:1539-47.

34. Smit JJ, Lindell DM, Boon L, Kool M, Lambrecht BN, Lukacs NW. The balance between plasmacytoid DC versus conventional DC determines pulmonary immunity to virus infections. *PLoS ONE* 2008;3:e1720.

35. Schwarze J. Lung dendritic cells in respiratory syncytial virus bronchiolitis. *Pediatr*

Infect Dis J 2008;27:89-91.

36. Grayson MH, Ramos MS, Rohlfing MM, Kitchens R, Wang HD, Gould A, et al. Controls for lung dendritic cell maturation and migration during respiratory viral infection. *J Immunol.* 2007;179:1438-48.

37. Köhl J, Baelder R, Lewkowich IP, Pandey MK, Hawlisch H, Wang L, et al. A regulatory role for the C5a anaphylatoxin in type 2 immunity in asthma. *J Clin Invest.* 2006 Mar; 116(3):783-96.

38. Longhi MP, Trumpfheller C, Idoyaga J, Caskey M, Matos I, Kluger C, et al. Dendritic cells require a systemic type I interferon response to mature and induce CD4<sup>+</sup> Th1 immunity with poly IC as adjuvant. *J Exp Med.* 2009;206:1589-1602.