

## Effects of various anti-asthmatic agents on mite allergen-pulsed murine bone marrow-derived dendritic cells

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### Summary

**Background** Dendritic cells (DCs) play an important role in the immune response and are critically involved in asthma.  $\beta_2$ -agonists could potentially exacerbate type 2 T helper (Th2) cell-mediated immune response.

**Objectives** To determine the effects of various anti-asthmatic agents on DCs function both *in vitro* and *in vivo*.

**Methods** Murine bone marrow-derived DCs were pulsed with mite allergen in the presence of pranlukast, salbutamol, salmeterol or fluticasone. These DCs were then inoculated intranasally into naïve mice to induce allergic airway inflammation *in vivo*.

**Results** Pranlukast reduced IL-10 and increased IL-12, while fluticasone reduced both IL-10 and IL-12 production by mite allergen-pulsed DCs. Allergic airway inflammation in pranlukast- and fluticasone-treated and mite allergen pulsed DCs-harboured mice was attenuated and such response was associated with inhibition of Th2 response in the airway. Salbutamol did not alter cytokine production, while salmeterol reduced IL-12 production by mite allergen-pulsed DCs. Lung pathology in  $\beta_2$ -agonist-harboured mice was comparable with those of mite allergen-pulsed DCs-harboured mice.

**Conclusions** Our results indicate that leukotriene receptor antagonists and corticosteroids inhibit DCs-induced Th2 skewed immune response, and that short- and long-acting  $\beta_2$ -agonists do not modify DCs-induced allergic airway inflammation.

**Keywords** airway inflammation, asthma,  $\beta_2$ -agonists, corticosteroids, cysteinyl leukotriene receptor antagonists, dendritic cells, IL-10, IL-12, mite allergen, mouse model

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### 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 T helper (Th2) cell-mediated immunologic lung disease [1]. Anti-inflammatory agents, especially inhaled corticosteroids (ICS), play a central role in the treatment of asthma. The international guidelines recommend the regular use of ICS as first line therapy [2].  $\beta_2$ -agonists, potent bronchodilators, are classified as either short- or long-acting based on their duration of action. Short-acting  $\beta_2$ -agonists (SABA) induce rapid bronchodilation and are used for the relief of asthma. On the other hand, long-acting  $\beta_2$ -agonists (LABA) cause long lasting bronchodilation and are used for the control of asthma. There is some concern regarding the use of  $\beta_2$ -agonists for the treatment of asthma. Since  $\beta_2$ -agonists are not anti-inflammatory agents, they may mask airway

inflammation [3]. In addition, several reports suggested that  $\beta_2$ -agonists might enhance Th2 response [4, 5].

Dendritic cells (DCs) are the most potent antigen-presenting cells in the airways [6]. DCs are distributed throughout the body and act as immune monitoring cells as well as initiate immune responses by presenting antigens to T cells [7]. Immature DCs actively capture and process antigens, and migrate to the draining lymph nodes, where they increase their ability to stimulate resting T cells, and thereby initiate various immune responses [8–10]. DCs alter the polarization of Th cells with the production of cytokines and co-stimulatory molecules [11–13]. They are critically involved in the development of Th2-mediated immune response in asthma [14–17]. A number of studies reported the effects of anti-asthmatic agents on airway inflammation [18–21], while a few examined the effects of anti-asthmatic agents on DCs. Interestingly DCs express histamine receptors [22], cysteinyl leukotriene (cysLT) receptors [23], and  $\beta_2$ -adrenergic receptors [24], which could be targets for anti-asthmatic drugs. Previous studies from our laboratories showed that LT receptor antagonists (LTRA) inhibited Th2 response in mite allergen-pulsed murine bone marrow-derived DCs [23]. In the present study, mite allergen-pulsed murine bone marrow-derived DCs were cultured in the presence of either SABA,

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LABA, LTRA or CS to determine the effects of these agents on the functions of DCs *in vitro*. To determine their *in vivo* effects, these DCs were intranasally transferred into the naïve mice to develop allergic airway inflammation.

## Material and methods

### Animals

Female BALB/c mice (5- and 10-week-old) were purchased from Charles River (Yokohama, Japan) and housed in a specific pathogen-free facility. The procedures were reviewed and approved by Nagasaki University School of Medicine Committee on Animal Research. All experiments were repeated at least three times and each experimental group consisted of two to three mice. Samples were evaluated individually.

### Reagents

Mite extract *Dermatophagoides farinae* (Der f) was obtained from Cosmo Bio (Tokyo, Japan). Lipopolysaccharide (LPS) concentration in the Der f preparations was  $<0.96$  EU/mg Der f (Limulus amoebocyte lysate test; E-Toxate; Sigma-Aldrich, St Louis, MO, USA). Recombinant mouse granulocyte/macrophage colony-stimulating factor (rmGM-CSF) was purchased from R&D Systems (Oxford, UK). Salbutamol was purchased from Sigma. Salmeterol and fluticasone (GlaxoSmithKline, Brentford, UK) and Pranlukast (Ono Pharmaceutical, Osaka, Japan), were obtained from the respective suppliers. All drugs were dissolved in 99% ethanol. Preliminary experiments showed that the solvent did not alter cell viability or cytokines production. Concentrations of IL-12p40, IL-10 and IFN- $\gamma$  in DCs culture supernatants and IL-5 and IFN- $\gamma$  in bronchoalveolar lavage fluid (BALF) samples were measured using ELISA (Endogen, Woburn, MA, USA) using the methods described by the manufacturer. The detection limits for IL-12p40, IL-10, IL-5 and IFN- $\gamma$  were 3, 12, 5, and 10 pg/mL, respectively.

### Generation of DCs

Murine bone marrow-derived DCs were generated as described previously [23]. Briefly, femurs and tibiae of female, 10-week-old BALB/c mice were removed and purified from the surrounding muscle tissue. Then both ends were cut and the marrow flushed with phosphate-buffered saline (PBS). After washing, leucocyte were suspended at a density of  $2 \times 10^6$  cells/mL in RPMI 1640 (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS) (Invitrogen Corp.) and 1% penicillin–streptomycin (Invitrogen Corp.) (hereafter referred to as cRPMI) and were cultured in 100 mm dishes. We prepared six groups of DCs (medium-pulsed DC (Control-DC), Der f-pulsed DC (Der f-DC), and Der f-pulsed and treated with either pranlukast or salbutamol or salmeterol or fluticasone DC (Der f-Prl-DC, Der f-Salbu-DC, Der f-Salme-DC and Der f-Flu-DC, respectively)). At day 3, another 10 mL of cRPMI containing 20 ng/mL rmGM-CSF was added to the plates. At days 6 and 8, half of the culture supernatants were replaced with fresh medium containing 5 ng/mL rmGM-CSF. At day 10, the

media were collected, and then DCs were incubated with 0.1 mg/mL of Der f for 24 h with the exception of Control-DC. The cell number and viability were repeatedly determined by trypan blue staining at days 8 and 10 in order to adjust the cell number in each drug-treated group.  $1 \times 10^{-6}$  M of each drug (pranlukast, salbutamol, salmeterol or fluticasone) was added to DCs at days 8 and 10. The drug concentration used in this study was selected based on the results of previous studies [4, 23]. In all experiments, incubation was carried out in a humidified 5% CO<sub>2</sub> atmosphere at 37 °C.

In a preliminary experiment DCs were cultured with Der f allergen extract in the absence (medium) or presence of solvents (99% ethanol), and the results of all experiments showed no significant differences between groups.

### Immunization with antigen-pulsed DCs and secondary exposure to antigen

On day 11,  $1 \times 10^5$  cells/50  $\mu$ L of each group of DCs (Control-DC, Der f-DC, Der f-Prl-DC, Der f-Salbu-DC, Der f-Salme-DC or Der f-Flu-DC) were intranasally instilled into another group of naïve BALB/c mice (5-week-old,  $n = 8$  for each). Thereafter, these mice were intranasally challenged with 0.05 mg of Der f once daily for 5 consecutive days (days 21–25).

### Bronchoalveolar lavage and lung pathology

Twenty-four hours after the final Der f challenge, mice were killed and 1 mL of PBS was used for BAL was conducted in the immediate post-mortem period. The obtained BAL fluid was centrifuged and supernatants were frozen until analysis. Differential cell counts were performed on cytocentrifuged BALF stained with May–Grünwald–Giemsa. After BAL, 4% paraformaldehyde-PBS fixative was gently infused through the lavage catheter. The resected lungs were fixed for an additional 24 h and paraffin embedded. Four-micrometre sections were stained with haematoxylin and eosin (H&E).

### Statistical analysis

Results are expressed as mean  $\pm$  standard error of the mean (SEM). Differences between groups were examined for statistical significance using repeated-measures (ANOVA) with Bonferroni's multiple comparison test. A *P*-value less than 0.05 denoted the presence of a statistically significant difference.

## Results

### In vitro effects of anti-asthmatic agents on cytokine production by DCs

Figure 1 shows the production of IL-12 and IL-10 by each of the six groups of DCs. In comparison with the Control-DC, Der f-DC produced significantly larger amounts of IL-12 and IL-10. Der f-Prl-DC significantly inhibited IL-10 and increased IL-12 production compared with Der f-DC. Both Der f-Salbu-DC and Der f-Salme-DC produced comparable IL-10 levels compared with Der f-DC. The production levels

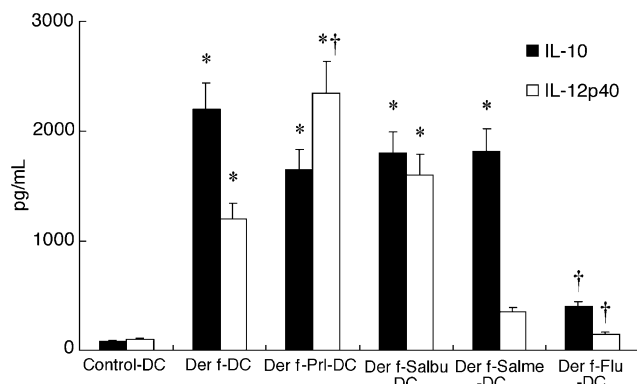


**Fig. 1.** IL-12 and IL-10 concentrations in culture supernatants of dendritic cells (DCs). At day 11, the DC culture media were collected and centrifuged. Cytokines were measured by ELISA. Der f DC produced IL-10 and IL-12. Pranlukast increased IL-12. Fluticasone reduced IL-10 and IL-12. Results are expressed as mean  $\pm$  SEM ( $n = 8$  in each group). \* $P < 0.01$  vs. Control-DC, † $P < 0.05$  vs. Der f-DC.

of IL-12 from Der f-Salbu-DC were similar to those of Der f-DC, while Der f-Salme-DC produced significantly less IL-12 in comparison with Der f-DC. Der f-Flu-DC produced significantly lower amounts of IL-10 and IL-12 compared with Der f-DC. Cells viability of all DCs groups was  $> 90\%$ . Levels of IFN- $\gamma$  were below the detection limit of our assay in all groups of DCs (data not shown).

#### *In vivo effects of anti-asthmatic agents on DC-induced allergic airway inflammation*

To investigate whether *in vitro* functional changes in DCs also occur *in vivo*, we conducted further experiments wherein each of the six groups of DCs (Control-DC, Der f-DC, Der f-Prl-DC, Der f-Salbu-DC, Der f-Salme-DC and Der f-Flu-DC) was instilled intranasally into another group of naïve BALB/c mice, followed by intranasal challenge with Der f allergen to develop allergic airway inflammation. Figure 2 shows representative lung pathology of six groups of DCs-transferred mice. Mice harbouring Der f-DC developed allergic airway inflammation characterized by peribronchovascular eosinophilic infiltration and goblet cell hyperplasia (Fig. 2b). In contrast, mice harbouring Der f-Prl-DC had no airway inflammation (Fig. 2c), while mice inoculated with Der f-Salbu-DC and Der f-Salme-DC developed allergic airway inflammation (Fig. 2d,e), and lung tissues of mice inoculated with Der f-Flu-DC (Fig. 2f) were almost similar to those of the Control-DC (Fig. 2a). Allergic airway inflammation was evaluated quantitatively by BALF analysis and the results confirmed those of lung pathology (Fig. 3).

#### *Cytokine profiles in BALF*

To determine the cytokine profiles in the airway, we determined the concentrations of IL-5 and IFN- $\gamma$  in BALF of the six groups of mice by ELISA (Fig. 4). IL-5 concentrations were below the detection limit in the Control-DC harbouring mice. In contrast, the mean concentration of IL-5 in BALF of mice instilled with Der f-DC was significantly higher than the Control-DC. Der f-Prl-DC and Der f-Flu-DC harbouring mice exhibited significantly less IL-5 in BALF compared with Der f-DC harbouring mice,

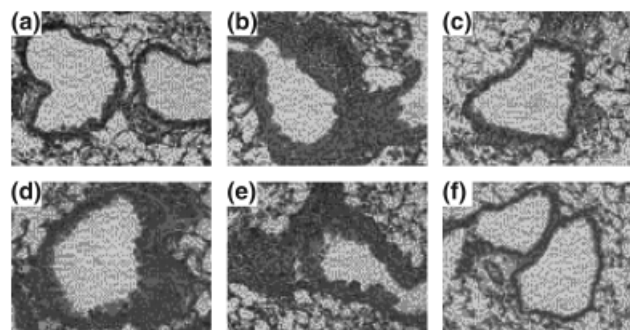


**Fig. 2.** Lung pathology in mice inoculated with dendritic cells (DCs). Mice were instilled intranasally with DCs and then sacrificed and lung tissues were obtained, then stained with H&E. Representative photographs of each group ( $n = 8$ , from three independent experiments, each experimental group consisted of two to three mice) are shown. (a) Mouse harbouring Control-DC, (b) mouse harbouring Der f-DC, (c) mouse harbouring Der f-Prl-DC, (d) mouse harbouring Der f-Salbu-DC, (e) mouse harbouring Der f-Salme-DC and (f) mouse harbouring Der f-Flu-DC. Original magnification  $\times 400$ .

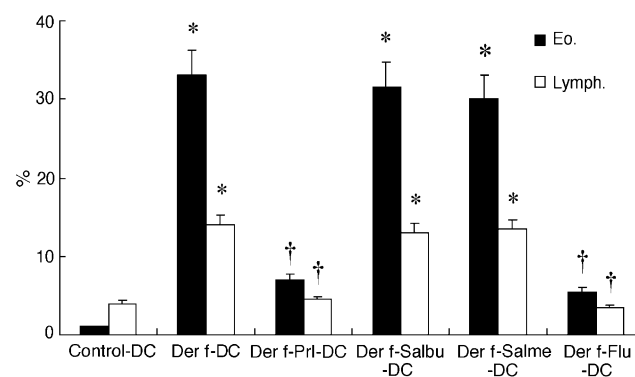


**Fig. 3.** Percentages of eosinophils (Eo.) and lymphocytes (Lym.) in bronchoalveolar lavage fluid (BALF). Bronchoalveolar lavage (BAL) was performed 24 h after the final Der f allergen exposure. Differential cell counts were based on May-Grünwald-Giemsa. Results are expressed as mean  $\pm$  SEM ( $n = 8$  in each group). \* $P < 0.01$  vs. mice harbouring Control. † $P < 0.05$  vs. mice harbouring Der f-DC.

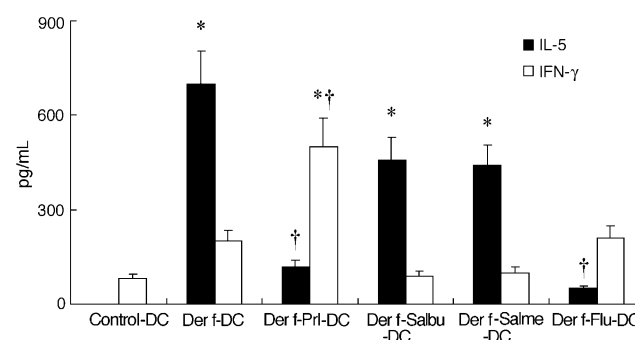


**Fig. 4.** Bronchoalveolar lavage fluid (BALF) concentrations of IL-5 and IFN- $\gamma$ . BALF was obtained 24 h after the final allergen exposure and assayed for IL-5 and IFN- $\gamma$  by ELISA. IL-5 of Der f-DC mice was higher than the Control-DC. Results are expressed as mean  $\pm$  SEM ( $n = 8$  in each group). \* $P < 0.01$  vs. mice harbouring Control-DC. † $P < 0.05$  vs. mice harbouring Der f-DC.

while Der f-Salbu-DC and Der f-Salme-DC harbouring mice showed comparable IL-5 levels in their BALF relative to Der f-DC harbouring mice. Interestingly, the concentration of IFN- $\gamma$  in BALF was significantly higher in Der f-Prl-DC

inoculated mice than in Der f-DC mice. Collectively, Der f-DC induced Th2 response in the airway and both LTRA and CS inhibited this Th2 response. Neither SABA nor LABA showed significant effects on the immune response in the airway.

## Discussion

We had previously shown that LTRA inhibits mite allergen-pulsed DCs to develop Th2 response both *in vitro* and *in vivo* [23]. The present study further characterized the effects of other important anti-asthmatic agents, e.g., SABA, LABA and CS, on DCs function. The present study was initially planned based on previous reports [4, 5, 25] that  $\beta_2$ -agonists might rather exacerbate the Th2 response. The results indicated that CS as well as LTRA inhibited DCs-induced Th2 response. Both SABA and LABA failed to inhibit Th2 response, however they did not exacerbate the Th2 response.

We also tested the production of two immunomodulatory cytokines (IL-10 and IL-12) to investigate the *in vitro* functions of DCs. IL-12 is a critical cytokine that induces naïve T cells to Th1 phenotype [11, 13, 26, 27], while the role of IL-10, a murine prototypical Th2 cytokine, in the differentiation of Th cells is controversial [28, 29]. In agreement with our previous report, LTRA increased IL-12 in Der f-DC, resulting in the inhibition of Th2 favoured phenotype. CS significantly inhibited both cytokines from murine bone marrow-derived DCs, as described previously [30, 31]. In human DC, salbutamol, a SABA, inhibits IL-12 production [4] but failed to inhibit murine bone marrow-derived DCs in the present study. Thus, the response of DCs to various agents might be species-dependent. In contrast to SABA, salmeterol, a LABA, significantly inhibited IL-12, suggesting that LABA may enhance Th2 response. However, unexpectedly, representative lung pathology and the BALF cellular profile of Der f-Salme-DC-harboured mice exhibited inflammatory changes resembling those observed in Der f-DC-harboured mice. The cytokine profiles in BALF of Der f-Salme-DC-harboured mice were also comparable with Der f-DC-harboured mice. Thus, inhibition of IL-12 production by DC does not seem to play a critical role in our system. In this regard, it has also been reported that diminished IL-12 production by human peripheral blood monocyte-derived DCs is not involved in the development of Th2 skewed immune deviation [32]. In addition to the cytokine profiles, these anti-asthmatic agents might affect other phenotypes of DCs in a different manner, including the expression of co-stimulatory markers and chemokine receptors. In fact, LTRA reduced mite allergen-induced up-regulation of CD80 and CD86 in murine bone marrow-derived DCs (our unpublished observation). The present study examined the effects of cysLTs on DCs function to initiate immune response. CysLTs also act on Langerhans cells and cause their migration to accessory lymph nodes by modulating the response to chemokines [33]. Thus, it will be an interesting issue for future research to determine whether cysLTs-pulsed DC are also differentially responsive to chemokines.

LTRA and CS inhibited allergic airway inflammation by differently regulating DCs function. LTRA reduced IL-10 and increased IL-12 in Der f-DC, while CS reduced both. In

mice instilled with DCs, both agents inhibited IL-5 production in the airway, while only the former significantly increased IFN- $\gamma$  production. Although the underlying mechanism of this phenomenon remains unclear, it might partially explain the synergistic effects of LTRA and ICS observed in clinical asthma [34].

$\beta_2$ -agonists are potent bronchodilators and are representative anti-asthmatic agents. The international guidelines recommend SABA as reliever in all severity and LABA as the first line add-on therapy to inhaled steroid in moderately persistent asthma [2]. Since  $\beta_2$ -agonists are not anti-inflammatory agents, several groups have raised concern regarding possible exacerbation of airway inflammation when these agonists are used alone [35–38]. Furthermore, several reports suggest that  $\beta$ -agonists might rather induce a Th2 response. Salbutamol induced Th2 response in human cord blood but not adult peripheral blood T cells [5]. Salbutamol may be an effective therapeutic agent for multiple sclerosis, a Th1 disease, by inhibiting Th1 response [25]. In addition to its effect on DCs, salbutamol increases histamine and IL-4 release from murine mast cell line [39]. On the other hand, several authors reported the anti-inflammatory properties of  $\beta_2$ -agonists [40, 41]. Thus, the impact of  $\beta_2$ -agonists on the immune system remains unclear and it is important to determine whether  $\beta_2$ -agonists exacerbate Th2 response at DCs level. The present study shed light on this issue and found that  $\beta_2$ -agonists, both SABA and LABA, did not inhibit or exacerbate the induction of Th2 skewed immune response by DCs.

In conclusion, we have demonstrated in the present study that LTRA and ICS inhibited Th2 skewed immune response induced by DCs. Neither SABA nor LABA modulated DCs-induced allergic airway inflammation. For the treatment of asthma, a Th2-mediated chronic inflammatory disease,  $\beta_2$ -agonists should be used in combination with anti-inflammatory agents, especially ICS as recommended in the NIHBI/WHO guideline [2].

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