

ORIGINAL ARTICLE

Effects of *Lactobacillus rhamnosus* on asthma with an adoptive transfer of dendritic cells in miceH.-J. Kim¹, Y.-J. Kim¹, S.-H. Lee¹, M.-J. Kang¹, H.-S. Yu¹, Y.-H. Jung^{2,3}, E. Lee^{2,3}, J.-H. Seo⁴, J.-W. Kwon⁵, B.-J. Kim⁶, J. Yu², H.-M. Park⁷ and S.-J. Hong^{2,3}¹ Asan Institute for Life Sciences, University of Ulsan College of Medicine, Seoul, Korea² Department of Pediatrics, Asan Medical Center, Childhood Asthma Atopy Center, Seoul, Korea³ Research Center for Standardization of Allergic Disease, University of Ulsan College of Medicine, Seoul, Korea⁴ Department of Pediatrics, Korea Cancer Center Hospital, Seoul, Korea⁵ Department of Pediatrics, Seoul National University Bundang Hospital, Seongnam, Korea⁶ Department of Pediatrics, Inje University Haeundae Paik Hospital, Busan, Korea⁷ Department of Veterinary Internal Medicine, College of Veterinary Medicine, Konkuk University, Seoul, Korea**Keywords**

animal model, asthma, allergy, dendritic cell, mice, probiotics.

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Abstract**Aim:** This study was designed to investigate whether the protective effects of *Lactobacillus rhamnosus* (*Lcr35*) on allergic asthma are associated with the adoptive transfer of dendritic cells (DCs) and regulatory T cells (Tregs), using a mouse experimental model of asthma.**Methods and Results:** BALB/c mice were orally administered *Lcr35* or intravenously treated with *in vivo* *Lcr35*-treated DCs daily and were then sensitized and challenged with ovalbumin (OVA) in accordance with a model of asthma protocol. Both the oral application of *Lcr35* and intravenous administration of *Lcr35*-treated DCs suppressed all aspects of the asthmatic response, including bronchial hyperresponsiveness (BHR), total cell counts in the bronchoalveolar lavage (BAL) fluid, the production of OVA-specific immunoglobulin E (IgE), and pulmonary eosinophilic inflammation. The mechanism of action of *Lcr35* is related to Tregs, which suppress the Th2 response in the respiratory organs, and this is mediated by DCs in the mouse model of asthma.**Conclusions:** These results confirm that the mechanism underlying the effects of *Lcr35* on asthma involves the adoptive transfer of DCs.**Significance and Impact of the Study:** This finding broadens the possibility that *Lcr35* has potential as an alternative therapeutic approach to the treatment of human asthma.**Introduction**

Asthma is a chronic inflammatory disease of the airways and is associated with Th2-related allergic responses (Cohn *et al.* 2004). The prevalence of asthma and allergic diseases has increased dramatically in many Western countries during the last 30 years of the 20th century, and its incidence is now >10% in humans (Hong *et al.* 2004; Marks *et al.* 2005; Kim *et al.* 2012a; Lee *et al.* 2012). As a result, studies reporting the successful management of allergic asthma have gained considerable attention recently.

Lcr35 is included among the identified probiotics, which are nonpathogenic micro-organisms that confer a number of beneficial health effects in the host (Marteau 2006). Several studies have characterized the ability of probiotic strains to alter cytokine production in the gut and associated lymphoid tissue, demonstrating immunomodulatory effects against some allergic diseases (Forsythe *et al.* 2007; Kwon *et al.* 2010; Jang *et al.* 2012; Kim *et al.* 2012b). It was previously shown that the oral application of *Lcr35* attenuates the features of allergic asthma in mouse models (Yu *et al.* 2010; Jang *et al.* 2012).

The mechanism underlying the protective effects of probiotics against allergic diseases reportedly involves immune suppressive cytokines (IL-10, TGF- β), Tregs (Feleszko *et al.* 2007; Kwon *et al.* 2010; Jang *et al.* 2012; Kim *et al.* 2012b) and toll-like receptor 9 (Forsythe *et al.* 2007). However, it is still unknown how probiotics interact with the immune system. In peripheral tissues, contact with an antigen induces a DC maturation process and the migration of DCs to secondary lymphoid organs, where they induce the differentiation of naïve T cells (Lambrecht 2001). *Lactobacilli* have been reported to regulate mouse DC surface molecule expression and cytokine production (Christensen *et al.* 2002).

In our present study, we investigated the mechanism by which *Lcr35* exerts its effects on asthma to induce immune modulation via the adoptive transfer of DCs in a mouse model. To our knowledge, this is the first study to investigate the protective effects of the *Lactobacillus rhamnosus* in mouse model of asthma by adoptive transfer of dendritic cells. Our findings lend weight to the beneficial effects of probiotics on asthma in mice, and this supports the possibility of using probiotics as a therapeutic alternative in the treatment of human asthma.

Materials and methods

Mice

Female BALB/c mice were purchased from OrientBio (Seongnam, Korea) and were cared for and used in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) at Asan Medical Center and Ulsan University College of Medicine.

Probiotic culture

Lcr35 was obtained from Lyocentre[®] laboratory (Aurillac, France) and cultured in sterile *Lactobacillus* MRS broth (Difco, Sparks, MD, USA) at 37 °C, for 48 h. After cultivation, cells were collected by centrifugation at 5000 g for 10 min, washed twice with phosphate-buffered saline, and lyophilized. The lyophilized powder was reconstituted with sterile saline before use.

Adaptive transfer of DCs after *in vitro* stimulation and maturation marker analysis

Bone marrow-derived DCs were generated from BALB/c mice (6- to 8 weeks old). Briefly, bone marrow cells were cultured in Iscove's modified Dulbecco's medium supplemented with 10% foetal bovine serum (FBS), 1% L-glutamine, antibiotic-antimycotic and 10 ng ml⁻¹ recombinant murine granulocyte-macrophage colony-stimulating factor

(GM-CSF) (R&D systems, Minneapolis, MN, USA). The culture medium was replaced with fresh medium containing GM-CSF on days 3 and 6. On day 6, DCs were purified by positive selection over a magnetic cell-sorting column (MACS), using microbead-conjugated anti-CD11c MAb (Miltenyi Biotec, Auburn, CA, USA). After checking the purity, the freshly purified DCs were prepared at 1×10^6 cells 10^{-1} ml⁻¹ (nontreated DCs) and stimulated with *Lcr35* (1×10^7 CFU) for 48 h *in vitro* (*Lcr35*-treated DCs). Finally, the cells were collected and injected intravenously as *Lcr35*-treated or nontreated DCs (per 1×10^6 200^{-1} μ l⁻¹), 5 days prior to the first sensitization. To analyse the maturity of the DCs, the cell-surface levels of CD80 and CD86 were measured by flow cytometry after the same above procedure *in vitro* in nontreated and *Lcr35*-treated DCs.

Experimental design

Female BALB/c mice ($n = 5$ for each group) aged 6 weeks were sensitized using an ovalbumin mixture (OVA; 10 μ g; grade V, Sigma Inc., St. Louis, MO, USA) and alum (2.25 mg; Imject[®], Pierce, Rockford, IL, USA) intraperitoneally (i.p.). The mixture was administered a second time, 1 week after the first sensitization. Seven days after the second sensitization, the mice were given an inhalation of 1% OVA, administered using an ultrasonic sprayer (Nesasonic UN-511, Alfresa, Osaka, Japan), for 30 min every day for three successive days (OVA challenge). The mice received *Lcr35* (1×10^9 colony-forming units 600^{-1} ml⁻¹ per mouse, daily) orally, beginning 1 week prior to the primary sensitization and continuing until the endpoint of the study. Two other groups of mice received *Lcr35*-treated or nontreated DCs intravenously. The negative control group was injected with saline alone (instead of OVA) for both the sensitization and airway challenge, and the positive control group received nothing during the sensitization with OVA.

Measurement of bronchial hyperresponsiveness

Twenty-four hours after the final OVA challenge, bronchial hyperresponsiveness (BHR) was measured in conscious unrestrained mice using a barometric whole-body plethysmograph (one chamber plethysmography; All Medicus, Anyang, Korea), as described previously (Yu *et al.* 2010).

Bronchoalveolar lavage and determination of the cellular composition of the BAL fluid

After the measurement of BHR, the mice were anaesthetized. Bronchoalveolar lavage (BAL) was performed as

described previously (Yu *et al.* 2010). To count the cells in the BAL fluid, cytospin slides were prepared and stained with Wright stain. The different cell types were identified on the basis of their morphology under a light microscope. Five sections per slide were evaluated.

Quantification of immunoglobulin, cytokine and chemokine levels

Serum was collected upon sacrifice 24 h after the last 1% OVA challenge. Serum immunoglobulins were quantified by sandwich ELISA, as described previously (Yu *et al.* 2010). Murine IL-4, IFN- γ and CCL11 (eotaxin-1) (R&D Systems) were assayed by ELISA in accordance with the manufacturer's instructions.

Histopathologic analysis of lung

For the histologic evaluation of lung tissues, left lung tissue from each mouse was embedded in paraffin, sectioned at 5 μ m, and stained with haematoxylin and eosin (H&E) to assess eosinophilic infiltration. Inflammation was scored by two independent investigators in a blind manner. To evaluate lung tissues histologically, lung samples were prepared as described previously (Tournoy *et al.* 2000). Inflammation was again scored by two independent investigators in a blind manner. The degree of peribronchial and perivascular inflammation was evaluated on a subjective scale of 0–3 as described. Cellular infiltration was assessed in five randomly selected fields using a Zeiss Axiophot microscope (Carl Zeiss Inc., Thornwood, NY, USA) at a 100 $\times$ magnification.

Flow cytometry

Mouse Tregs were collected from the mesenteric lymph nodes (MLNs). Mouse regulatory T cell staining kits with FITC-labelled anti-CD4, PE-labelled anti-CD25, APC-labelled anti-Foxp3 and each isotype control (eBioscience, San Diego, CA, USA) were used for analysis of CD4, CD25 and Foxp3 expression as directed by the manufacturer. The cell-surface levels of CD80 and CD86 on DCs *in vitro* were also measured by FACS. PE-labelled anti-CD80, FITC-labelled anti-CD86 and each isotype control (eBioscience, San Diego, CA) were used. Staining was analysed by flow cytometry using a FACS Calibur with CellQuest software (BD Biosciences, Mountain View, CA, USA).

Statistical analysis

Results are expressed as the mean $\pm$ SEM, and statistical significance was calculated (GraphPad Prism 5, La Jolla, CA, USA) using one-way ANOVA (Tukey's Multiple

Comparison Test). A *P*-value of < 0.05 was considered to be significant.

Results

The adoptive transfer of Lcr35-treated DCs inhibits the asthmatic phenotype in a mouse model of this disorder

To determine whether the inhibitory effect of *Lcr35* is mediated by DCs, mice were injected with *Lcr35*-treated DCs, and then sensitized and challenged with OVA.

The BHR elicited in response to aerosolized methacholine was measured using Penh values. Both groups receiving *Lcr35*, via the oral application of *Lcr35* (*Lcr35* PO; 4.94 ± 0.78) or by injection of *Lcr35*-treated DCs (*Lcr35*-treated DC; 5.87 ± 0.85), showed significant suppression of BHR. Penh values determined for the maximum methacholine dose (50 mg ml⁻¹) were compared with those of the positive control (OVA) group (9.48 ± 0.86 ; $P < 0.01$; $P < 0.05$, Fig. 1) or of the group that received an injection of nontreated DCs (Nontreated DC; 9.95 ± 1.32 $P < 0.05$; $P < 0.01$, Fig. 1a). Penh values for mice that received intravenous, nontreated DCs were similar to those of the OVA-positive control group.

The total cell counts in BAL fluid of both the *Lcr35* PO ($7.44 \pm 0.74/10^{-5}$ ml) and the *Lcr35*-treated DC ($5.85 \pm 0.47/10^{-5}$ ml) groups exhibited significant suppression of eosinophilic inflammation compared with the OVA group ($13.00 \pm 2.42/10^{-5}$ ml; $P < 0.05$; $P < 0.001$, Fig. 1b). However, the differences between *Lcr35*-treated DC and nontreated DC ($10.65 \pm 1.08/10^{-5}$ ml) groups were not significant.

With respect to the eosinophil-specific cell count in the BAL fluid, the *Lcr35* PO ($0.41 \pm 0.12/10^{-5}$ ml) and *Lcr35*-treated DC ($0.23 \pm 0.05/10^{-5}$ ml) groups showed significant suppression compared with the OVA group ($2.71 \pm 1.16/10^{-5}$ ml; $P < 0.05$; $P < 0.05$, Fig. 1c). In addition, the *Lcr35*-treated DC groups also showed a significant increase in eosinophils when compared with the nontreated DC group ($10.65 \pm 1.08/10^{-5}$ ml; $P < 0.05$, Fig. 1c), suggesting that the adoptive transfer of *Lcr35*-treated DCs suppresses airway eosinophilic inflammation in a mouse model of asthma.

The systemic immune response was assessed by measuring the serum OVA-specific IgE levels. The administration of *Lcr35*-treated DCs (18.61 ± 2.32 ng ml⁻¹) significantly suppressed these levels in serum compared with OVA treatment (37.83 ± 4.88 ng ml⁻¹; Fig. 1d, $P < 0.05$), but no significant suppression was observed in the nontreated DC group (34.33 ± 6.71 ng ml⁻¹).

The histopathology of *Lcr35* PO and *Lcr35*-treated DC groups showed a decreased infiltration of inflammatory cells in the lung compared with the OVA group, whereas

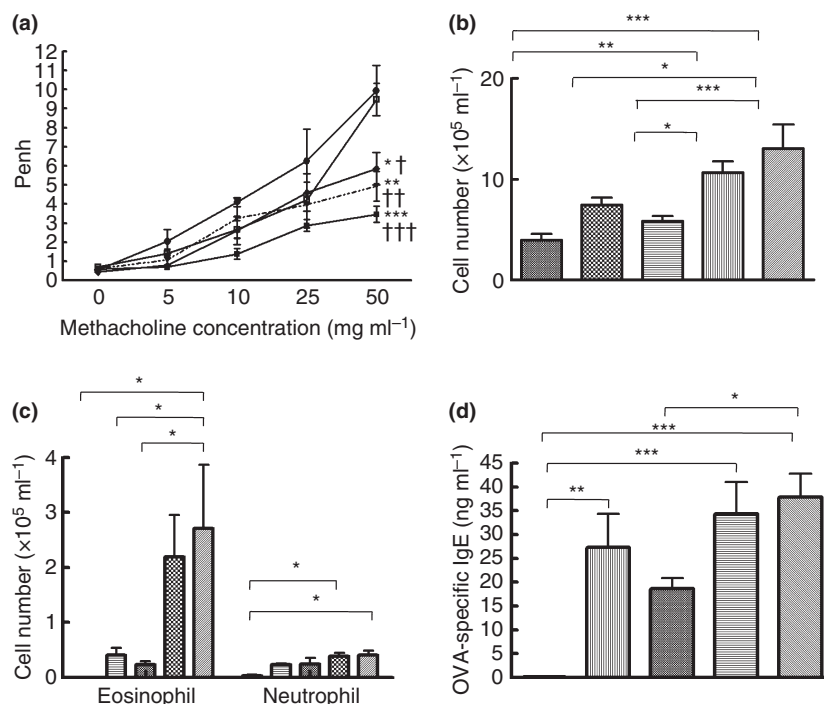


Figure 1 Effects of the adoptive transfer of *Lcr35*-treated DC on asthmatic phenotypes in a mouse model of asthma. (a) Bronchial hyperresponsiveness (BHR). (■) Saline; (▼) *Lcr35* PO; (◆) *Lcr35*-treated DC; (●) Non-treated DC; (□) OVA. (b) Total (■) Saline; (▼) *Lcr35* PO; (◆) *Lcr35*-treated DC; (●) Non-treated DC; (□) OVA and (c) differential cell counts. (■) Saline; (▼) *Lcr35* PO; (◆) *Lcr35*-treated DC; (●) Non-treated DC; (□) OVA. (d) Serum OVA-specific IgE levels. (■) Saline; (▼) *Lcr35* PO; (◆) *Lcr35*-treated DC; (●) Non-treated DC; (□) OVA. OVA group ($n = 5$): mice sensitized with intraperitoneal injections of OVA on days 0 and 7 were then challenged with aerosolized OVA on days 14–16; Saline group ($n = 5$): mice were sensitized and then challenged with saline following the same time schedule as that described for OVA group mice; *Lcr35* PO group ($n = 5$): mice that received *Lcr35* (1×10^9 colony-forming units/600 ml/mouse/day) orally from day 7 to the endpoint of the study were then sensitized and challenged with OVA; *Lcr35*-treated DC group ($n = 5$): mice that were injected intravenously with *Lcr35*-treated DCs on day 5 were then sensitized and challenged with OVA; Nontreated DC group ($n = 5$): mice that were injected intravenously with untreated DCs on day 5 were then sensitized and challenged with OVA. *Compared with OVA; †Compared with nontreated DCs in BHR; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

the nontreated DC group showed no improvement in inflammation (Fig. 2a). This observation was consistent with the lung inflammation score, for which the *Lcr35*-treated DC group (0.92 ± 0.07) as well as the *Lcr35* PO group (1.16 ± 0.10) showed significant suppression compared with the OVA group (1.95 ± 0.08 ; Fig. 2b, $P < 0.001$). In addition, the difference between *Lcr35*-treated DC and nontreated DC (1.65 ± 0.08) groups was statistically significant (Fig. 1f, $P < 0.001$). These results demonstrate that the adoptive transfer of *Lcr35*-treated DCs suppresses pulmonary inflammation in the mouse model of asthma.

Adoptive transfer of *Lcr35*-treated DC increased the percentage of CD4⁺CD25⁺Foxp3⁺ Tregs in MLNs

To investigate whether treatment with *Lcr35* increased the number of Tregs in mice, the percentage of CD4⁺CD25⁺Foxp3⁺ Tregs in the MLNs was determined. Oral *Lcr35* administration ($15.83 \pm 0.72\%$) increased

the percentage of CD4⁺CD25⁺Foxp3⁺ Tregs in MLNs compared with OVA treatment ($12.38 \pm 0.50\%$; Fig. 3, $P < 0.05$). Interestingly, *Lcr35*-treated DC administration significantly increased the percentage of CD4⁺CD25⁺Foxp3⁺ Tregs compared with nontreated DCs ($11.89 \pm 0.88\%$; Fig. 3, $P < 0.05$), suggesting that the adoptive transfer of *Lcr35*-treated DCs may increase the percentage of CD4⁺CD25⁺Foxp3⁺ Tregs in a manner similar to that of the oral administration of *Lcr35*.

The adoptive transfer of *Lcr35*-treated DCs suppresses the Th2 response in BAL fluid

To investigate whether treatment with *Lcr35* affected the Th1 and Th2 response in the mouse, IL-4, IFN- γ and CCL11 (eotaxin-1) were assayed in BAL fluid by ELISA. IL-4, a Th2-related cytokine, was not detected in any sample (data not shown). CCL11 (eotaxin-1, a Th2-related chemokine) was significantly suppressed in both *Lcr35*-treated groups compared with the OVA and

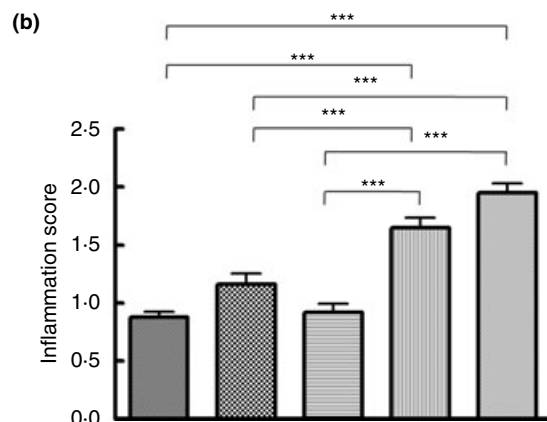
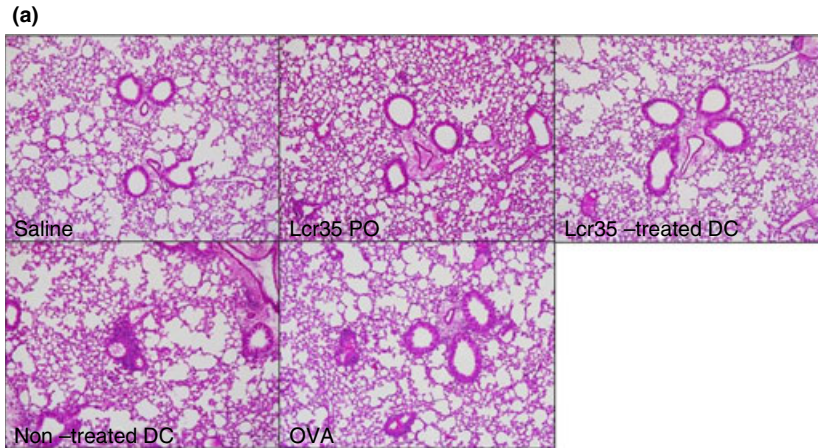


Figure 2 Effects of the adoptive transfer of *Lcr35*-treated DCs on lung inflammation in a mouse model of asthma. (a) Histopathology of lung ($\times 100$, H&E staining). (b) Inflammation score of lung. Groups were as described in Fig. 1. *** $P < 0.001$. (▨) Saline; (▩) Lcr35 PO; (▧) Lcr35-treated DC; (▪) Non-treated DC; (▤) OVA.

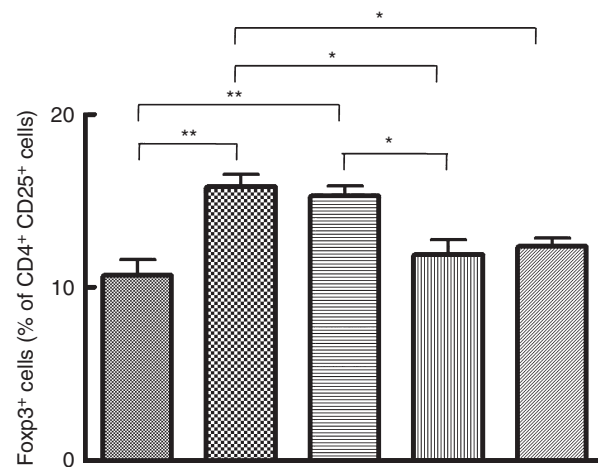


Figure 3 Effects of the adoptive transfer of *Lcr35*-treated DCs on the percentage of MLN CD4⁺CD25⁺Foxp3⁺ Tregs in a mouse model of asthma. Groups were as described in Fig. 1. * $P < 0.05$; ** $P < 0.01$. (▨) Saline; (▩) Lcr35 PO; (▧) Lcr35-treated DC; (▪) Non-treated DC; (▤) OVA.

nontreated DC groups (Fig. 4). However, the IFN- γ levels in BAL fluid were found not to significantly differ between groups (Fig. 4).

Lcr35 up-regulates maturation surface marker expression in DCs *in vitro*

To investigate whether treatments with *Lcr35* affected the maturation of DCs, the level of CD80 and CD86, maturation surface markers in these cells, was analysed by flow cytometry. The levels of both surface markers in DCs were increased by treatment with *Lcr35* for 48 h *in vitro* compared with nontreated cells (Fig. 5).

Discussion

This study investigated the beneficial effects, and the mechanisms underlying these impacts, of *Lcr35* using the adoptive transfer of DCs in a mouse model of asthma. The administration of *Lcr35*-treated DCs suppressed asthma phenotypes including BHR, airway total cell and inflammatory (eosinophils) cell counts, lung inflammation and serum immunoglobulin. These results demonstrate that *Lcr35* induces immune modulation in mice through innate immune cells, such as DCs.

Microbial exposure activates the innate immune system [e.g. toll-like receptors (TLRs)], and subsequently induces

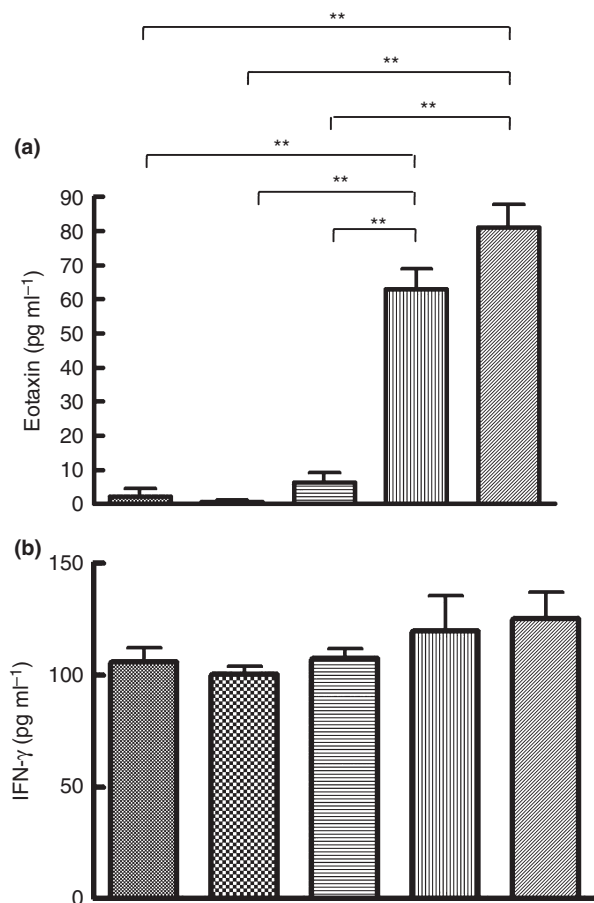


Figure 4 Effects of the adoptive transfer of *Lcr35*-treated DCs on the Th1/Th2 response in a mouse model of asthma. (a) The level of CCL11 (eotaxin-1) in the BAL fluid. (b) The level of IFN-γ in BAL fluid. Groups were as described in Fig. 1. ***P* < 0.01. (□) Saline; (▨) *Lcr35* PO; (▤) *Lcr35*-treated DC; (▥) Non-treated DC; (▧) OVA.

T cell differentiation to Th1 cells, which competes for Th2-related allergic diseases (e.g. asthma, atopic dermatitis; Renz *et al.* 2006). In this regard, beneficial probiotic organisms have been highlighted as a topic of some interest in relation to allergic disease. In fact, many studies have been conducted using probiotics to investigate the mechanisms of allergic diseases in humans and other animals (Feleszko *et al.* 2007; Kwon *et al.* 2010; Yu *et al.* 2010; Jang *et al.* 2012).

Dendritic cells interact with microbes using pattern recognition receptors, including TLRs (Hart *et al.* 2004). Signals from DCs determine whether tolerance or an active immune response occurs after a particular antigen-activated immune receptor is stimulated (Banchereau and Steinman 1998), and thereafter influence whether a Th1 or Th2 immune response predominates. In our present study, the expression of CCL11 (eotaxin-1) was found to be significantly suppressed in BAL fluid following DC treatment with *Lcr35*, both *in vitro* and orally. Hence, the suppression of the Th2 immune response by *Lcr35* treatment appears from our study results to be mediated by signals from DCs.

Probiotics are recognized by DCs, which are pivotal in maintaining immunological homeostasis in the gut, and as a result CD4⁺Foxp3⁺Tregs are increased in the MLNs (Coomes and Powrie 2008; Kwon *et al.* 2010). Our present findings also reveal a significant increase in MLN CD4⁺CD25⁺Foxp3⁺Tregs in response to *Lcr35* administered either orally or intravenously via *Lcr35*-treated DCs in the form of adoptive transfer. Previous studies have shown that probiotics induce DC maturation, as demonstrated by the expression of the surface markers CD40, CD80 and CD86 (Weiss *et al.* 2011). In our present

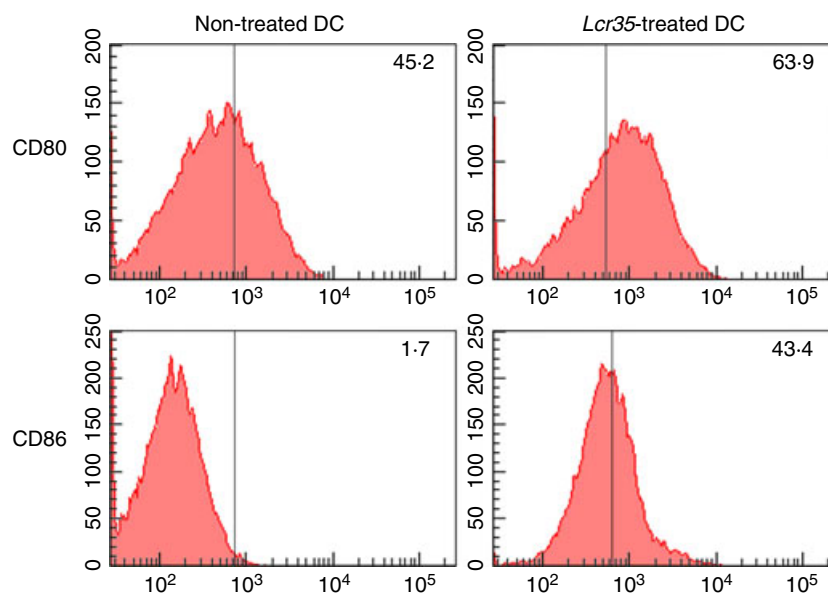


Figure 5 Effects of the adoptive transfer of *Lcr35*-treated DCs on maturation surface marker expression *in vitro*. *Lcr35*-treated DC: purified DCs prepared at 1×10^6 cells 10 ml^{-1} , then stimulated with *Lcr35* (1×10^7 CFU) for 48 h *in vitro*; Nontreated DC: only purified DCs were prepared.

experiments, *Lcr35*-treated DCs also showed increased CD80 and CD86 expression as found in previous studies *in vitro* and showed beneficial effects on biological markers *in vivo* in the mouse model of asthma. Some probiotics have been reported to induce Tregs via the modulation of DC function with maturation *in vitro* (Van Overtvelt *et al.* 2010). Our current results indicate that the maturation of DCs by exposure to *Lcr35* *in vitro* can induce a Treg population in the MLN and suppress allergic responses in the mouse.

The adoptive method described here investigated the possibility of using DCs, innate immune cells stimulated with probiotics, as an intervention for asthma, and confirmed the capacity of probiotics to stimulate innate immunity and, thereby, induce systemic immune modulation. Some studies have used the adoptive transfer of DCs in mice to identify the mechanism of probiotic action in relation to other diseases (Chen *et al.* 2009; Kwon *et al.* 2010), not for asthma. Our present study is the first to investigate the use of adoptive transfer of *Lcr35*-treated DCs as a possible new treatment for asthma.

In conclusions, probiotics can stimulate innate immunity to modulate the systemic immune response by suppressing the Th2 response by Tregs associated with the maturation of DCs. These findings indicate that the effects of probiotics can be mediated by DCs and may widen the field of potential therapeutic alternatives for human asthma.

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Conflict of interest

None declared.

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