

A Novel Role of Cysteinyl Leukotrienes to Promote Dendritic Cell Activation in the Antigen-Induced Immune Responses in the Lung

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Although the critical role of cysteinyl leukotrienes (cysLTs) in the inflammation, especially eosinophilic lung inflammation, in asthma has been well documented, their role in the early stage of Ag-specific immune response has not been completely clarified. In the present study, with a mouse model of asthma and in vitro studies we demonstrated that cysLTs potentiated dendritic cell (DC) functions such as Ag-presenting capacity and cytokine production. The cysLT-1 receptor antagonist (LTRA) strongly suppressed the activation of these DC functions and led to inhibition of subsequent not only Th2, but also Th1, responses in the early stage of immune response. Moreover, treatment with LTRA during the early stage of the immune response potently suppressed the development of Ag inhalation-induced eosinophilic airway inflammation, mucus production, and airway hyper-reactivity in vivo. Treatment with LTRA significantly increased PGE₂ production in the lung, and treatment with the cyclooxygenase inhibitor indomethacin abolished LTRA's suppressive effect on DCs and deteriorated the Th2 and Th1 responses and airway inflammation. With in vitro studies, we also confirmed that cysLTs production by DCs increased with LPS stimulation, and that LTRA directly suppressed the alloantigen-presenting capacity of DCs. These results suggested that cysLTs potentiate DC functions both in vivo and in vitro, and that LTRA could be beneficial to suppress the initial immune response in many immune-mediated disorders beyond asthma. *The Journal of Immunology*, 2004, 173: 6393–6402.

It is well known that eicosanoids play important roles in lung function and diseases (1). Leukotrienes (LTs)² are generated from arachidonic acid (AA) via the 5-lipoxygenase (5-LO) pathway. Although a few reports have recently demonstrated the important role of LTB₄ in T cell recruitment (2, 3), it seems that the major role of LTB₄ is neutrophil activation (4). In contrast, cysteinyl leukotrienes (cysLTs) have been shown to be potent bronchoconstrictors and important inflammatory mediators for asthma (4). Several cell types can produce cysLTs, including mast cells, basophils, eosinophils, macrophages, and dendritic cells (DCs) (5, 6). CysLTs can induce smooth muscle cell proliferation and contraction, increase vascular permeability, stimulate mucus secretion, and recruit eosinophils into lungs (4). Th2 cytokines and cysLTs up-regulate their production each other from mast cells in a positive feedback manner (7, 8). Of the two receptors for cysLTs, the cysLT₁ receptor is thought to be more important in its biochemical actions than cysLT₂ receptor (9). Many studies have elucidated the role of cysLTs or effects of the cysLT₁ receptor antagonist (LTRA) on lung eosinophilic inflammation or the remodeling of asthma in animal models (10, 11) or humans

(12–14). Contrary to the proinflammatory nature of LTs, lipoxin A₄, another AA metabolite via 5-LO pathway, is an anti-inflammatory mediator (15, 16).

Many studies have also reported that prostanoids, generated from AA via the cyclooxygenase (COX) pathway, play an important role in lung inflammation. In inflammatory allergic airway diseases, the overall anti-inflammatory role of prostanoids has been suggested using COX-deficient mice (17) or indomethacin-treated mice (18). Among prostanoids, PGD₂, PGF_{2α}, and thromboxane A₂ (TxA₂) seem to have detrimental effects in allergic lung diseases (18, 19). PGI₂ causes relaxation of isolated precontracted human bronchi, but has minimal effects on airway functions in vivo (18). Therefore, it seems that the overall anti-inflammatory role of prostanoids mainly depends on PGE₂ action. In fact, the anti-inflammatory effect of PGE₂ has been confirmed in many in vitro and in vivo experiments (20–24).

As described above, the critical role of eicosanoid, especially LTs, in the effector phase of inflammation have been well studied. In contrast, there have been only a few reports that studied the role of LTs in the early stage of immune response. Ag presentation from APCs to T cells and B cells initiate an Ag-induced immune response. Among APCs, DCs are the most potent APCs (25). Only a few papers have reported the role of cysLTs in DC functions. Robbani et al. (26) reported that cysLTs are necessary for DC migration. Recently, Machida et al. (27) reported that in vitro treatment with LTRA modifies the cytokine profile of DCs. However, other effects of cysLTs on DC function, such as APCs and the effects of cysLTs on DCs in vivo, have rarely been clarified.

In the present study we analyzed the role of cysLTs in an early stage of Ag sensitization with a mouse model of asthma and in vitro studies. The results demonstrated that cysLTs play a very important role in Ag-induced immune responses via directly potentiating DC function. We also found that treatment with LTRA completely

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Received for publication May 3, 2004. Accepted for publication September 13, 2004.

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² Abbreviations used in this paper: LT, leukotriene; AA, arachidonic acid; AHR, airway hyper-reactivity; BALF, bronchoalveolar lavage fluid; CMC, carboxymethyl-cellulose; COX, cyclooxygenase; cysLT, cysteinyl LT; Ind, indomethacin; LTRA, cysteinyl LT-1 receptor antagonist; LO, lipoxygenase; Mch, methacholine chloride; Mk, montelukast sodium; Penh, enhanced pause; Prl, pranlukast hydrate; Tx, thromboxane; Ind, indomethacin.

abolished this immune response by potently suppressing DC function, and that this effect might be exhibited through the production of PGE₂ in vivo. Moreover, we found that COX inhibitor treatment potentiated DC function and thus enhanced immune responses.

Materials and Methods

Mice

Male BALB/c mice and C57BL/6 mice (aged 6 wk) were obtained from Charles River Japan (Kanagawa, Japan). Male BALB/c-transgenic (DO11.10) mice and 5-LO deficient mice (aged 6–8 wk) were purchased from The Jackson Laboratory (Bar Harbor, ME). They were maintained under conventional animal housing conditions in a specific pathogen-free setting. All animal experiments conducted in this study were approved by the animal research ethics board of the University of Tokyo.

Study protocols in vivo

BALB/c mice were immunized with 2 µg of OVA (Sigma-Aldrich, St. Louis, MO)/2 mg of alum i.p. on days 0 and 11. Control mice received saline injection instead of the OVA/alum solution. Some of the OVA-sensitized mice were treated with pranlukast hydrate (Prl; 5 mg/kg as Prl low, or 50 mg/kg as Prl high; provided by Ono Pharmaceutical, Osaka, Japan), montelukast sodium (Mk; 1 mg/kg; purchased as Singulair tablets for humans; Banyu Pharmaceutical, Tokyo, Japan), or their vehicle (0.5% (w/v) carboxymethylcellulose (CMC) solution) via a gastric tube on days 11–17. Some of the saline control mice were also treated with Prl high (50 mg/kg). LTRA was made colloidal in distilled water containing 0.5% (w/v) CMC. Some mice treated with Prl (50 mg/kg) also received indomethacin (Ind; 4 mg/kg; Sigma-Aldrich) i.p. 30 min before Prl administration on days 11–17. Ind (80 mg) was diluted in 10 ml of 100% ethanol, and then 200 µl of this was added to 7.8 ml of PBS. On day 18, airway hyper-reactivity (AHR) to methacholine chloride (Mch; Sigma-Aldrich) was measured by the enhanced pause (Penh) system (Buxco, Troy, NY) as reported previously (28–30). In brief, at first the baseline value of Penh was measured after the saline inhalation. Then increasing concentrations of Mch were delivered by the nebulizer, the percent Penh compared with the baseline value was calculated, and AHR was examined by the change in Penh to Mch. We also used PC₂₀₀Mch as an indicator of AHR, as previously described (29). PC₂₀₀Mch (micrograms per milliliter) is the concentration of Mch that induced a 100% increase in Penh (200% over the baseline value), and it was calculated from the semilog scale dose-response curve. After measuring AHR, we obtained bronchoalveolar lavage fluid (BALF) from each mouse and blood samples as previously reported (29). Cell count and cell differentials of BALF were also determined as previously reported (29). Concentrations of IL-4, IL-5, and IFN-γ, IgE (BD Pharmingen, San Diego, CA) and IgG (Bethyl Laboratories, Montgomery, TX) were measured using an ELISA kit. To measure OVA-specific IgE and IgG in sera, plates were coated with 1 mg/ml OVA/0.1 M carbonate solution instead of capture Abs for IgE and IgG, then the ELISA kits described above were used. The titers of the samples were calculated by comparison with internal standards, which were obtained from the sera of six individual OVA-sensitized mice on day 18. These standards were calculated as 1000 U/ml. Concentrations of PGE₂ in BALF were measured using the competitive enzyme immunoassay system (Amersham Bioscience, Uppsala, Sweden). Concentrations of PGD₂, PGF_{2α}, PGI₂, and TxA₂ in BALF were also measured using the competitive enzyme immunoassay system (Cayman Chemical, Ann Arbor, MI). PGI₂ and TxA₂ were measured as 6-keto-PGF_{1α} and TxB₂, respectively. The data were analyzed with Microplate Manager III, version 1.45 (Bio-Rad, Tokyo, Japan).

Effect of 5-LO blockade on PGE₂ production in the lung in Ag sensitization

C57BL/6 wild-type mice and 5-LO-deficient mice were also immunized with OVA on days 0 and 11 as described above. Control C57BL/6 mice received saline injection on days 0 and 11. On day 18, the PGE₂ concentration in BALF was measured using the enzyme immunoassay system.

RT-PCR

RNA was extracted from DCs obtained from the OVA-sensitized mice on day 11 by the acid-guanidium phenol chloroform method using Isogen (Nippon Gene, Toyama, Japan). RT-PCR for cysLT₁ receptor was conducted as previously described (31). The sense primer was 5'-CAA CGA ACT ATC CAC CTT CAC C-3', and the antisense primer was 5'-AGC CTT CTC CTA AAG TTT CCA C-3'. PCR products were electrophoresed in a 3% agarose gel. The results were visualized by ethidium bromide staining.

Medium for cell culture

Complete DMEM was used as the medium for cell incubation. Complete DMEM consisted of DMEM with glucose (4.5 g/L), pyridoxine HCl (4.0 mg/l), and sodium pyruvate (110 mg/l), which contained FBS (10%, w/v), HEPES buffer solution (0.01 M), MEM nonessential amino acid solution (0.1 mM), 2-ME (50 µM; Sigma-Aldrich), penicillin (100 U/ml), and streptomycin (100 mg/ml).

Animal preparation for ex vivo analyses

Male BALB/c mice were immunized with OVA/alum on day 0. On days 0–10, some mice were treated with Prl (50 mg/kg) or Prl plus Ind (4 mg/kg). Saline control mice received only saline injection on day 0. On day 11, spleens were collected and incubated at 37°C for 20 min after treatment with 0.1% (w/v) collagenase (Sigma-Aldrich)/complete DMEM solution. Then spleens were minced, and single-cell suspensions were prepared by passing through the cell strainer. RBCs were removed by hypotonic lysis using RBC lysing buffer (Sigma-Aldrich). After two washes in HBSS, splenocytes were used for experiments.

Cell proliferation

Throughout the present study, cell proliferation was measured by BrdU incorporation (Roche, Mannheim, Germany), following the manufacturer's protocol.

Spleen cell responses to OVA stimulation

Spleen cells (2.5 × 10⁶ cells/ml) were cultured in a 96-well, flat-bottom microtiter assay plate with several concentrations of OVA in an incubator (37°C, 5% CO₂, 90% humidity). Cell proliferation was measured on day 3. IL-2 production on day 4 was measured using ELISA kit (BD Pharmingen). On day 4, IL-5 and IFN-γ production by cells stimulated with 100 µg/ml OVA were measured by ELISA.

Effect of LTRA on CD4⁺ T cells

CD4⁺ T cells were selected from spleen cells of the mice prepared for ex vivo analyses on day 11. To obtain CD4⁺ T cells, we used monoclonal rat anti-mouse CD4 Abs conjugated with MACS colloidal superparamagnetic microbeads using LS separation columns (Miltenyi Biotec, Auburn, CA), following the manufacturer's protocol. The purity of CD4⁺ cells, confirmed by the flow cytometry, was >95%. DCs were also selected from spleen cells of the OVA-sensitized mice, using MACS CD11c microbeads following the manufacturer's protocol as previously reported (32). Then CD4⁺ T cells (2.5 × 10⁵ cells/ml) were cocultured with DCs (5 × 10⁴ cells/ml) under OVA stimulation (1000 µg/ml) in a 96-well, flat-bottom microtiter assay plate. On day 3, cell proliferation was measured. To examine the direct effect of LTRA on CD4⁺ T cells, CD4⁺ T cells (1 × 10⁶ cells/ml), obtained from the OVA-sensitized mice on day 11, were incubated with PMA (1 ng/ml; Sigma-Aldrich) and ionomycin (1 µg/ml; Sigma-Aldrich) with or without Prl (0.1 or 1 µM). After 2-day incubation, cell proliferation was measured.

Effects of LTRA on DC function in vivo

DCs were selected from spleen cells of the mice prepared for ex vivo analyses as described above. In some experiments, DCs were also obtained from mice treated with Mk (1 mg/kg) on days 0–10 after OVA sensitization on day 0. As in a previous report (32), the positively selected cells from spleens were routinely >85% CD11c⁺, and there was no significant difference in the purity of CD11c⁺ cells between each group of mice in every experiment. Positively selected DCs were incubated with mitomycin-C (10 µg/ml; Sigma-Aldrich) for 35 min at 37°C in a humidified atmosphere containing 5% CO₂, then washed four times with PBS. DCs (5 × 10⁴ cells/ml) obtained from each group of mice and CD4⁺ T cells (2.5 × 10⁵ cells/ml) obtained from congenic BALB/c mice immunized with OVA/alum were cocultured under OVA stimulation (1000 µg/ml). On day 3, cell proliferation was measured. We also examined the ability of DCs for cytokine production. IL-10 and IL-12p70 concentrations in the supernatant were measured (1 × 10⁶ cells/ml) by ELISA (BD Pharmingen) after a 2-day incubation with LPS (1 µg/ml).

Effect of LTRA on Th1-biased response

Male BALB/c mice were immunized with 2 µg of OVA/0.5 ml of CFA (Difco, Detroit, MI) i.p. and treated with or without Prl or Ind as described above. Saline control mice received only saline injection on day 0. On day 7, spleen cells were collected. IL-5 and IFN-γ production by spleen cells after 4 days OVA (10 µg/ml) stimulation, and IL-10 and IL-12p70 production by

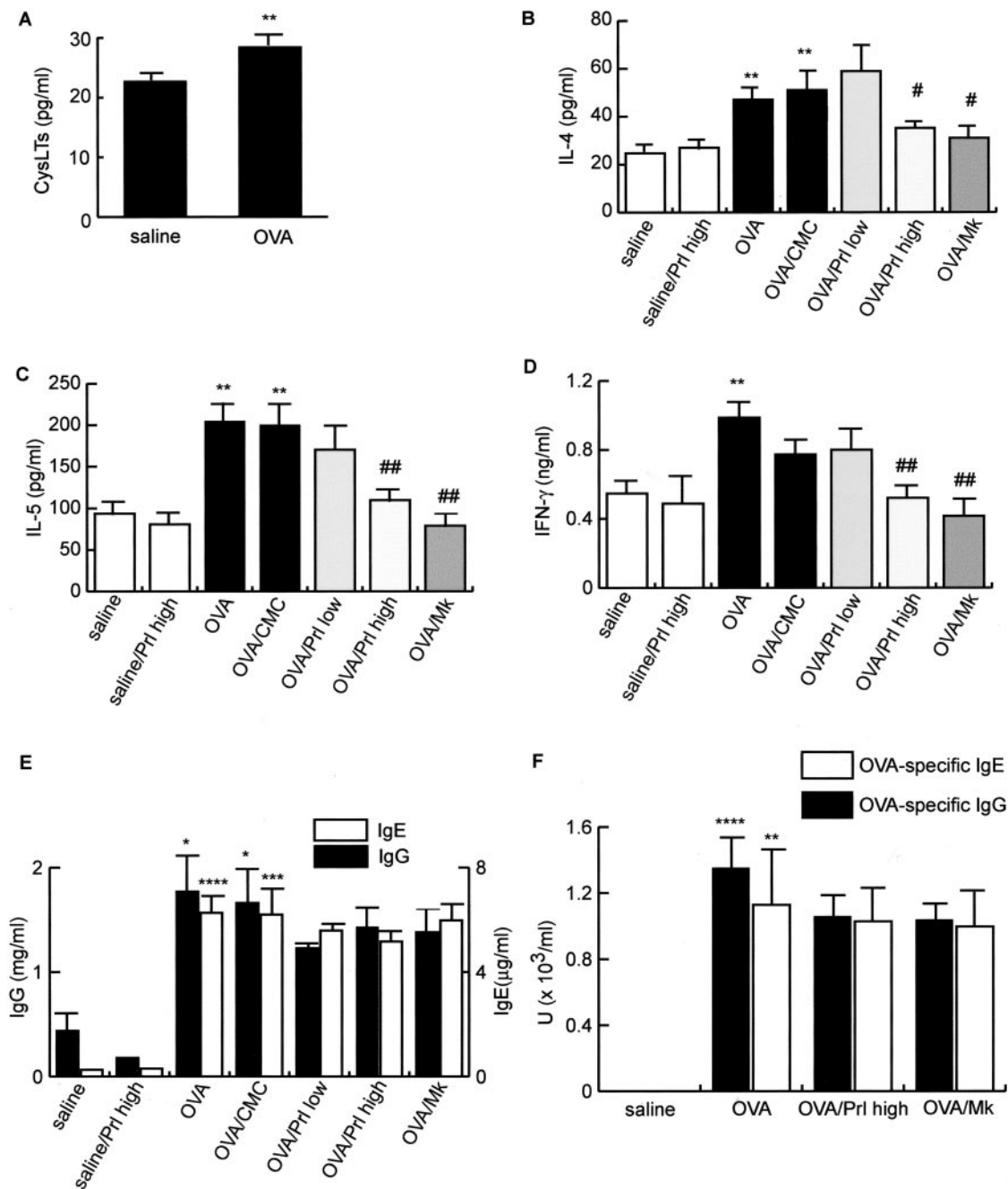


FIGURE 1. LTRAs suppress the immune responses in the lung induced by systemic OVA sensitization alone. Mice were immunized with 2 μ g of OVA/alum on days 0 and 11 (OVA). Some of the OVA-sensitized mice were treated with Prl (5 mg/kg (OVA/Prl low) or 50 mg/kg (OVA/Prl high)), Mk (1 mg/kg; OVA/Mk), or their vehicle, CMC (OVA/CMC), on days 11–17. Saline control mice received saline injections on days 0 and 11 (saline), and some of the saline control mice were treated with Prl (50 mg/kg; saline/Prl high). On day 18, BALF and sera were obtained. *A*, CysLTs production in the lung after saline injections ($n = 14$) and OVA sensitization ($n = 14$). *B–D*, The concentration of cytokines in BALF. *B*, IL-4. *C*, IL-5. *D*, IFN- γ . *E* and *F*, Igs in the sera. Total (*E*) and OVA-specific (*F*) IgG (■) and IgE (□). Data are the mean $\pm$ SEM for six to eight animals in *B–F*. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$ (vs saline mice). #, $p < 0.05$; ##, $p < 0.01$ (vs OVA mice).

DCs after 2 days LPS (1 μ g/ml) stimulation were measured as described above.

Effect of LTRA on DC function in vitro

At first, DCs obtained from the OVA mice on day 11 were cultured with or without LPS (1 μ g/ml) for 2 days. Then the cysLT concentration in the supernatant was measured by cysLTs ELISA kit (Cayman Chemical). DCs (1×10^6 cells/ml) obtained from the OVA-treated mice on day 11 were also cultured with LPS (1 μ g/ml) for 2 days with or without Prl (1 μ M) after mitomycin C treatment. Some of DCs incubated with Prl were also treated with Ind (2 μ g/ml). After a 2-day incubation, DCs (1×10^5 cells/ml) from

BALB/c (H-2D^d) mice were cocultured with CD4⁺ T cells (1×10^6 cells/ml) obtained from allogenic C57BL/6 (H-2D^b) naive mice. After a 3-day coculture, cell proliferation was measured. DCs obtained from the OVA mice on day 11 were also cultured with LPS (1 μ g/ml) for 2 days in the same manner as the culture of DCs for allogenic MLR without mitomycin C treatment. Then PGE₂ concentrations in the supernatant were measured.

Effects of LTRA in the early phase of Ag-induced immune responses on the induction of eosinophilic airway inflammation

Mice were immunized with OVA and were treated with Prl and/or Ind on days 11–17 as described for the in vivo study protocols. Then mice were

challenged with 3% OVA in PBS delivered by the nebulizer for 10 min every day on days 18–20. Control mice received saline injection on days 0 and 11 and were challenged with PBS on days 18–20. On day 21, AHR was measured, and BALF and lung samples were obtained and subjected to analyses.

Effect of LTRA treatment in the early phase of immune responses on DC function in the lung

On day 21, DCs in the lung of mice treated as described above were positively selected as previously reported (32). In brief, lung tissues were minced and then treated with 0.033% (w/v) collagenase/complete DMEM solution for 90 min. Then, single-cell suspensions of lung tissues were obtained, and CD11c⁺ cells were positively selected using MACS CD11c microbeads as described above. The positively selected cells from lung tissues were routinely ~70% CD11c⁺, and there was no significant difference in the purity of CD11c⁺ cells between the groups of mice. Positively selected lung DCs were treated with mitomycin-C as described above and cocultured at the indicated concentrations with CD4⁺ T cells (1×10^6 cells/ml) positively selected from spleens of DO11.10 mice. After 3-day coculture, cell proliferation was measured.

Statistical analysis

Data were evaluated using two-way ANOVA, followed by Student's *t* test for comparison between two groups. A value of $p < 0.05$ was considered significant.

Results

Role of cysLTs in Ag-induced immune response

We previously reported that systemic Ag sensitization alone, without Ag inhalation, could induce AHR and increase cytokine production in the lung (29). In the present study we also used this protocol with systemic Ag sensitization alone to examine the role of cysLTs on the early phase of the Ag-induced immune response. Systemic OVA sensitization significantly increased cysLT production in the lung (Fig. 1A). In addition, the OVA-treated mice showed significantly enhanced Th2 and Th1 cytokine production in the lung compared with saline-treated mice (Fig. 1, B–D). Both high dose Prl (OVA/Prl high, 50 mg/kg) and Mk (OVA/Mk, 1 mg/kg) significantly decreased cytokine production in the lung induced by OVA sensitization, whereas treatment with low dose Prl (OVA/Prl low, 5 mg/kg) failed to decrease it. The IL-13 concentration in BALF was very low on day 18 in our system, and there was no significant difference among the four groups (data not shown). We also confirmed that CMC, the vehicle for LTRA, did not affect cytokine production (OVA/CMC), and that Prl treatment did not affect the baseline cytokine level in the lung (saline/Prl high). Based on these findings, we conducted the following experiments using Prl at a concentration of 50 mg/kg. In BALF findings, we observed slight, but not significant, increases in total cell, macrophage, and neutrophil numbers in OVA-treated mice compared with saline-treated mice (Table I). Very few eosinophils were detected on day 18 in each group. In contrast, lymphocytes increased significantly in OVA-treated mice compared with those in saline-treated mice. LTRAs significantly reduced lymphocyte number in the lung. Macrophage and neutrophil numbers were also suppressed by LTRA treatment.

There was no significant difference in either total or OVA-specific IgE and IgG concentrations in the sera between the OVA-treated mice and LTRA-treated mice, which suggested that LTRA exhibited its effects not through suppression of IgE production (Fig. 1, E and F).

Role of cysLTs in AHR induced by systemic Ag sensitization

As we have previously reported (29), an OVA-specific immune response provoked by systemic sensitization alone significantly enhanced AHR. LTRA significantly suppressed this AHR induction (Fig. 2).

Role of PGE₂ in LTRA treatment

To examine whether blockade of the cysLT₁ receptor might shift the AA pathway from the 5-LO pathway toward the COX pathway, we measured the prostanoid concentration in BALF. OVA sensitization induced a marked increase in the PGE₂ concentration in BALF compared with that in the saline control group (Fig. 3A). The PGE₂ concentration in BALF further increased in both OVA/Prl mice and OVA/Mk mice. Treatment with Prl after saline injections or with CMC by itself did not alter PGE₂ production in the lung (Fig. 3B). We also confirmed that LT blockade increased PGE₂ production in the lung after systemic OVA sensitization using 5-LO-deficient mice (Fig. 3C). PGD₂, PGF₂α, PGI₂, and TxA₂ in the lung did not increase after Ag sensitization (Table II). Then we studied the role of PGE₂ in LTRA treatment by inhibiting its production with a nonselective COX inhibitor, Ind. As shown above, Prl and Mk seemed to have almost the same effects, so we conducted the following experiments using Prl as a representative of LTRA. Some mice received Ind plus Prl (OVA/Prl/Ind) or Ind alone (OVA/Ind) daily on days 11–17. The PGE₂ concentration in the lung significantly decreased in the OVA/Prl/Ind mice and the OVA/Ind mice compared with that in OVA mice or OVA/Prl mice (Fig. 3D). IL-4 and IL-5 concentrations in BALF were significantly higher in OVA/Prl/Ind mice than in OVA/Prl mice (Fig. 3, E and F). Production of IFN-γ in the lung also tended to increase in OVA/Prl/Ind mice compared with that in OVA/Prl mice (Fig. 3G). We also confirmed that the addition of Ind to treatment with Prl significantly restored AHR to a level similar to that in OVA mice or OVA/Ind mice (Fig. 3H). In these experiments most of the effect of LTRA was abolished by additional treatment with Ind. These results implied that LTRA might exhibit its effect via increasing PGE₂ production.

Effect of cysLTs on CD4⁺ T cells

Next, we examined the effect of LTRA on cell activation using *ex vivo* analyses. Cell proliferation and cytokine production by splenocytes obtained from OVA-treated mice significantly increased compared with those of splenocytes from saline-treated mice (Fig. 4, A–D). Prl treatment significantly reduced these responses to OVA stimulation, whereas they were significantly restored by additional treatment with Ind. Prl treatment also significantly reduced the proliferation of CD4⁺ T cells that were

Table I. Bronchoalveolar lavage cell findings^a

	Total Cells (/ml)	Macrophage (/ml)	Lymphocyte (/ml)	Neutrophil (/ml)
Saline	37,500 ± 18,708	37,112 ± 18,694	238 ± 351	150 ± 281
OVA	53,333 ± 16,857	51,417 ± 16,477	1,465 ± 654 ^b	451 ± 510
OVA/Prl	35,000 ± 16,583	34,253 ± 16,623	577 ± 406 ^c	170 ± 144
OVA/Mk	37,916 ± 11,227	37,007 ± 11,185	606 ± 382 ^c	303 ± 375

^a Data are the mean ± SD from six animals per group.

^b $p < 0.01$ vs saline mice.

^c $p < 0.05$ vs OVA mice.

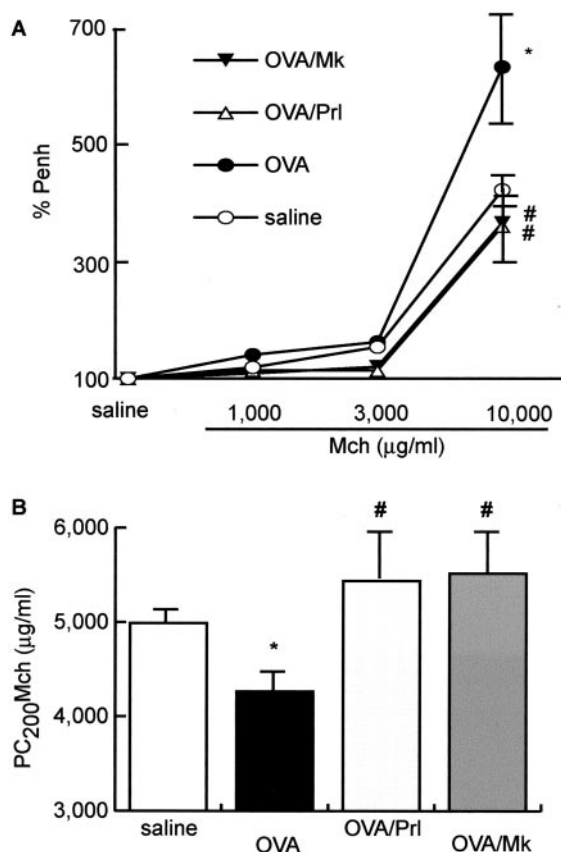


FIGURE 2. LTRA reduces AHR enhanced by systemic OVA sensitization alone. Mice were sensitized with OVA/alum on days 0 and 11 and were treated with Prl (50 mg/kg) or Mk (1 mg/kg) on days 11–17. The control mice received saline injections on days 0 and 11. On day 18, AHR to Mch was measured. At first, the baseline Penh value was measured after the saline inhalation. Then increasing concentrations of Mch (1,000, 3,000, and 10,000 µg/ml) were delivered by the nebulizer and the percent Penh compared with the baseline value was calculated. AHR was examined by the change in Penh to Mch (A) or by PC₂₀₀Mch, which was the concentration of Mch that induced a 100% increase in Penh (200% over the baseline value; B). Data are the mean ± SEM for six animals per group. *, $p < 0.05$ (vs saline mice). #, $p < 0.05$ (vs OVA mice).

cocultured during OVA stimulation with DCs obtained from the OVA mice, and Ind treatment also significantly restored this suppression of CD4⁺ T cell proliferation (Fig. 4E). These results revealed that LTRA potently inhibited CD4⁺ T cell priming in the Ag-induced immune responses. In contrast, addition of Prl had no direct effect on the proliferation of CD4⁺ T cells stimulated with PMA and ionomycin (Fig. 4F). In another experiment we did not find any effect of LTD₄ on CD4⁺ T cell cytokine production (data not shown). Generally, cysLT₁ receptor mRNA or protein is rarely present in T cells (33). We also confirmed, using PCR, that CD4⁺ T cells had little expression of cysLT₁ receptor mRNA (data not shown). These results indicated that LTRA inhibited CD4⁺ T cell activation in an indirect manner. Therefore, we next examined the effect of LTRA on DC, the most potent APC.

Effect of cysLTs on DC function in vivo

First we confirmed that DCs had cysLT₁ receptor mRNA (Fig. 5A). To examine DC function as an APC, we cocultured DCs from each group of mice after mitomycin-C treatment with CD4⁺ T cells from OVA-sensitized congenic mice under OVA stimulation and measured cell proliferation. Prl treatment in vivo significantly

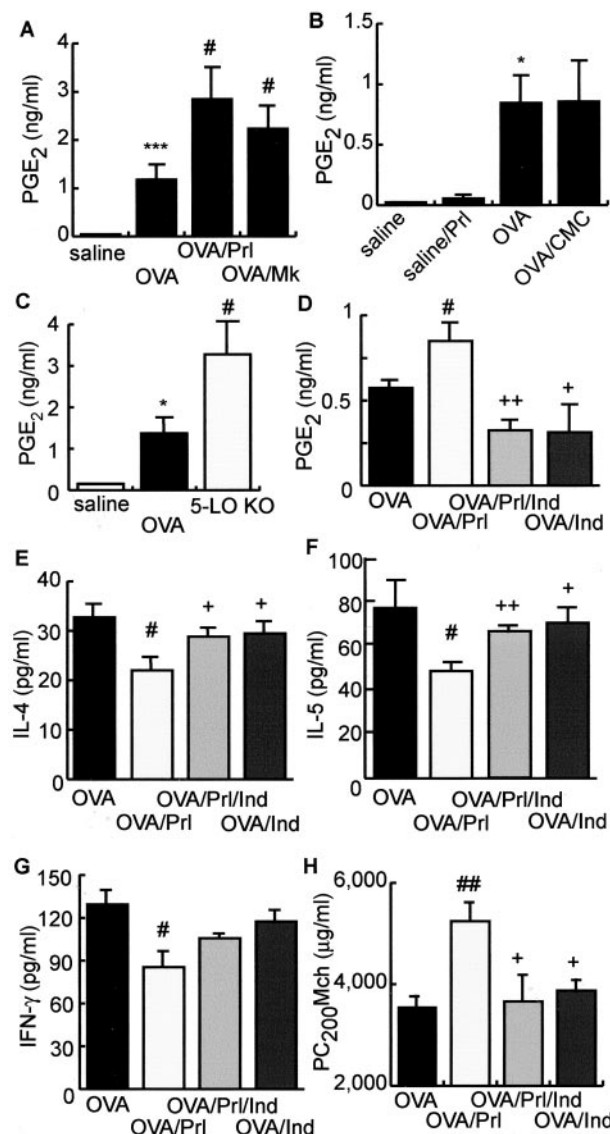


FIGURE 3. LT blockade increases PGE₂ production in the lung, and COX inhibitor significantly reverses the suppressive effect of LTRA. A, BALB/c mice were sensitized with OVA on days 0 and 11. The control mice received saline injections on days 0 and 11. Some of the OVA-sensitized mice were treated with Prl (50 mg/kg) or Mk (1 mg/kg). On day 18, the PGE₂ concentration in BALF was measured. B, Treatment with Prl after saline injections or with CMC by itself did not alter PGE₂ production in the lung. C, Wild-type C57BL/6 mice and 5-LO-deficient mice were sensitized with OVA on days 0 and 11. The control C57BL/6 mice were treated with saline instead of OVA. On day 18, the PGE₂ concentration in BALF was measured. D–H, Some of the OVA-sensitized BALB/c mice (■) were treated with Prl (□), Prl plus Ind (OVA/Prl/Ind; □), or Ind alone (OVA/Ind; ■) on days 11–17. On day 18, after examination of AHR, PGE₂ and cytokine production in the lung was measured. D, PGE₂. E, IL-4. F, IL-5. G, IFN-γ. H, AHR was examined by PC₂₀₀Mch. Data are the mean ± SEM from 12 animals per group in A and from four to six mice per group in B–H. *, $p < 0.05$; ***, $p < 0.001$ (vs saline mice). #, $p < 0.05$; ##, $p < 0.01$ (vs OVA mice). +, $p < 0.05$; ++, $p < 0.01$ (vs OVA/Prl mice).

suppressed DC function as an APC, which Ind treatment significantly restored (Fig. 5B). Similar to the findings for Ag-presenting capacity, IL-10 and IL-12p70 production by DCs during LPS stimulation were significantly suppressed by Prl, and Ind restored this suppression (Fig. 5, C and D). These results demonstrated that Prl potently inhibited DC function in vivo and might exhibit its effects

Table II. Prostanoid concentrations in BALF on day 18^a

	PGD ₂ (pg/ml)	PGF _{2α} (pg/ml)	PGI ₂ (pg/ml)	TxA ₂ (pg/ml)
Saline	ND	26.7 ± 13.0	21.7 ± 31.2	ND
OVA	ND	24.6 ± 6.7	20.5 ± 15.7	ND
OVA/Prl	ND	23.6 ± 6.0	13.7 ± 5.8	ND
OVA/Mk	ND	28.2 ± 6.7	16.7 ± 15.8	ND

^a Data are the mean ± SD from six animals per group. There were no significant differences in the prostanoid concentrations described above between the group of mice. PG₂ and TxA₂ were measured as 6-keto-PGF_{1α} and TxB₂, respectively. ND, not detectable.

on DCs through PGE₂ action. One paper has reported the possibility that Prl can block not only cysLT₁ receptor, but also cysLT₂ receptor (31). However, in vivo treatment with Mk, which blocks only cysLT₁ receptor (31), also significantly suppressed cytokine production by DCs after LPS stimulation to almost the same level as Prl treatment in vivo (Fig. 5, C and D). Thus, these suppressive effects of Prl on DCs seem to be due to blockade of the cysLT₁ receptor.

Effect of cysLTs on Ag-induced Th1-biased immune response

As described above, LTRA treatment in vivo significantly suppressed the Th1 response as well as the Th2 response induced by OVA/alum sensitization (Figs. 1 and 4). Therefore, we examined the effect of LTRA on the Th1-biased immune response induced by OVA/CFA (Fig. 6). OVA/CFA sensitization significantly increased IFN-γ production from spleen cells after ex vivo OVA stimulation (Fig. 6A). We also confirmed that spleen cells from the OVA/CFA-sensitized mice did not increase IL-5 production after OVA stimulation compared with spleen cells from saline-treated mice (data not shown). OVA/CFA sensitization also enhanced cytokine production by DCs after LPS stimulation (Fig. 6, B and C) compared with saline injection alone. Prl treatment significantly reduced these effects of OVA/CFA sensitization, and additional Ind treatment canceled this suppressive effect. These results indicated that cysLTs played an important role in the Th1-biased immune response as well.

Direct effect of LTRA on DC function in vitro

First we elucidated whether DCs could directly produce cysLTs. DCs obtained from the spleens of OVA-sensitized mice on day 11 produced a certain amount of cysLTs, and the production was enhanced upon LPS stimulation (Fig. 7A). Then we examined the effect of LTRA treatment in vitro on DC function. DCs obtained from OVA-treated mice on day 11 were incubated with or without Prl and/or Ind for 2 days under LPS stimulation, then DC functions were examined. Prl treatment in vitro significantly reduced the alloantigen-presenting capacity of DC, and Ind plus Prl treatment significantly restored it (Fig. 7B). Prl treatment in vitro did not alter PGE₂ production by DCs with LPS stimulation (Fig. 7C). LTRA did not up-regulate the PGE₂ production by DCs themselves. Considering this result, although LTRA's suppressive effect on DCs in vivo might be exhibited through up-regulation of PGE₂, as described above, this effect via PGE₂ was not mediated by DCs themselves in an autocrine manner.

Early LTRA treatment prevents subsequent lung inflammation

Finally, we examined the effect of treatment with LTRA during the early stage of sensitization on subsequent lung inflammation induced by Ag inhalation. Treatment with Prl only during the period of systemic sensitization significantly reduced AHR (Fig. 8A), eosinophilic lung inflammation (Fig. 8, B and C), and mucus pro-

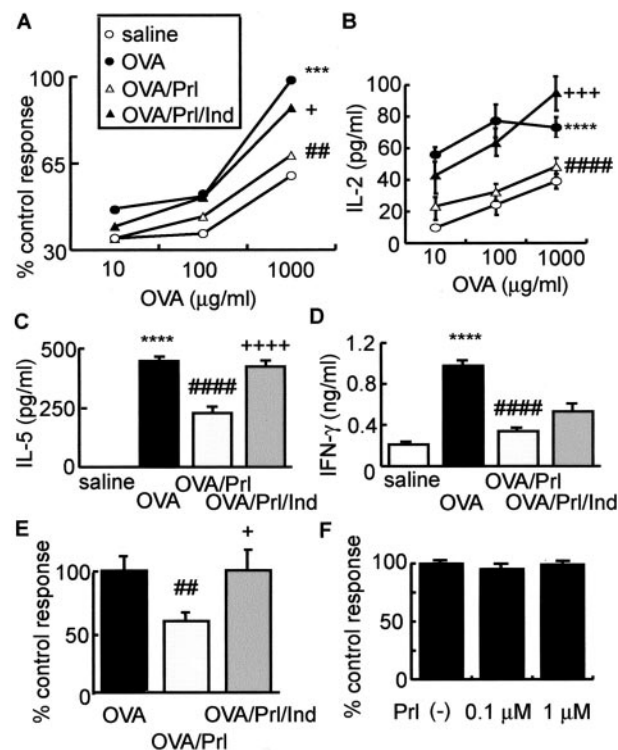


FIGURE 4. LTRA inhibits T cell priming induced by systemic OVA sensitization. A–D, Mice were immunized with OVA/alum on day 0 and were treated with Prl and/or Ind on days 0–10. The control mice received saline injection on day 0. On day 11, spleen cells were obtained from saline mice (○ in A and B; □ in C and D), OVA mice (● or ■), OVA/Prl mice (△ or □), and OVA/Prl/Ind mice (▲ or □). A and B, The cells were incubated with OVA at several concentrations. Then cell proliferation (A) and IL-2 production (B) were measured. A, Data are expressed as a percentage of the response compared with that of spleens from the OVA mice at 1000 μg/ml OVA. C and D, IL-5 (C) and IFN-γ (D) production by the cells were measured after 4 days of OVA (100 μg/ml) stimulation. E, CD4⁺ T cells obtained from each group of mice were cocultured with DCs obtained from OVA mice during OVA (1000 μg/ml) treatment for 3 days. Then CD4⁺ T cell proliferation was measured. F, CD4⁺ T cells obtained from the OVA-sensitized mice on day 11 were stimulated with PMA and ionomycin with various concentration of Prl in medium. After 2-day incubation, cell proliferation was measured. E and F, Data are expressed as a percentage of the response compared with that of CD4⁺ T cells from the OVA mice (E) or CD4⁺ T cells without Prl (F). Data are the mean ± SEM from nine wells per group. ***, *p* < 0.001; ****, *p* < 0.0001 (vs saline mice). ##, *p* < 0.01; ####, *p* < 0.0001 (vs OVA mice). +, *p* < 0.05; +++, *p* < 0.001; +++++, *p* < 0.0001 (vs OVA/Prl mice).

duction (Fig. 8D) induced by OVA inhalation. Treatment with Prl before OVA inhalation also prevented eosinophilic lung inflammation induced by longer OVA inhalation for 6 days (data not shown). We examined the effect of treatment with Prl during the sensitization phase on DC function in the effector phase. DCs were obtained from lung tissues of each group of mice after 3 days of OVA inhalation, and then were cocultured with CD4⁺ T cells obtained from the congenic DO11.10 mice (Fig. 8E). Pretreatment with Prl during the sensitization phase significantly suppressed the Ag-presenting capacity of DCs in the lung, even in the effector phase. Ind treatment significantly reversed these effects of Prl. These results suggest that treatment with LTRA in the early stage of sensitization could prevent future development of various characteristic features of asthma via potentially suppressing DC function, and that these effects of LTRA might be exhibited through up-regulation of PGE₂.

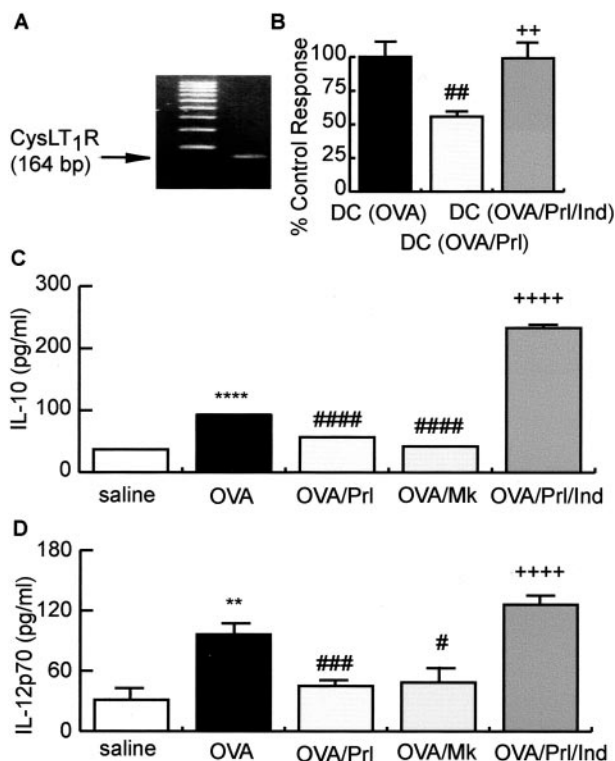


FIGURE 5. LTRA treatment in vivo suppresses DC functions such as Ag-presenting capacity and cytokine production, which were enhanced by systemic OVA sensitization. *A*, mRNA expression of *cysLT₁* receptor on DCs. *B–D*, Mice were sensitized and treated with Prl and/or Ind as described in Fig. 4. Some of the OVA-sensitized mice were also treated with Mk on days 0–10. On day 11, DCs were positively selected from spleens of each group of mice. *B*, LTRA treatment in vivo suppresses DC function as APCs. Mitomycin C-treated DCs from each group of mice and CD4⁺ T cells from OVA-sensitized congenic BALB/c mice were cocultured for 3 days under OVA stimulation (1000 μ g/ml). Then CD4⁺ T cell proliferation was measured. The data are expressed as a percentage of the response compared with that induced by DCs from the OVA mice. *C* and *D*, LTRA also suppresses cytokine production by DCs. IL-10 (*C*) and IL-12p70 (*D*) production by DCs after 2-day incubation with LPS was measured. Data are the mean $\pm$ SEM from nine wells per group in *B* and from six wells per group in *C* and *D*. **, $p < 0.01$; ****, $p < 0.0001$ (vs DCs from saline mice). #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$; ####, $p < 0.0001$ (vs DCs from OVA mice). ++, $p < 0.01$; +++, $p < 0.0001$ (vs DCs from the OVA/Prl mice).

Discussion

The results of the present study clearly demonstrate that *cysLTs* plays a pivotal role not only in the effector phase of airway inflammation, but also in the early stage of immune response to an Ag via potentiating DC function. LTRA potentially suppressed DC function, and thus indirectly inhibited CD4⁺ T cell activation and subsequent allergic inflammation in the airway. To date, the role of *cysLTs* in immune responses has rarely been elucidated. APCs to T and B cells initiate an Ag-induced immune response, and DCs are the most potent APCs (25). Only a few papers have reported the role of *cysLTs* in DC functions (26, 27), and the effects of *cysLTs* on DC functions, such as Ag-presenting capacity, have rarely been clarified in vitro or in vivo. In the current study we examined the direct effect of *cysLTs* on DC function both in vivo and in vitro, and the significance of its regulation in modulating immune response.

OVA sensitization alone significantly increased *cysLT* production in the lung (Fig. 1*A*). Several cell types can produce *cysLTs*,

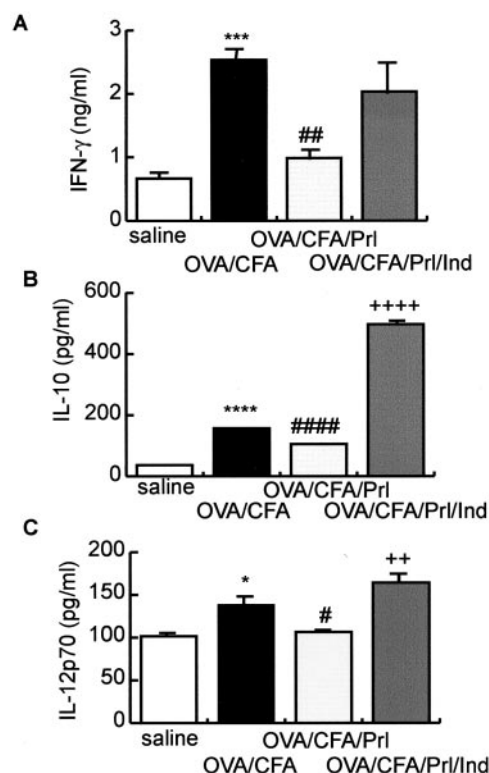


FIGURE 6. LTRA reduces Th1-biased immune response and DC function induced by OVA/CFA. On day 0, mice were immunized with OVA/CFA. Some of the sensitized mice were treated with Prl (OVA/CFA/Prl) or Ind (OVA/CFA/Ind) on days 0–6. The control mice received saline injection on day 0. On day 7, spleen cells were collected. Then IFN- γ production (*A*) by whole spleen cells during OVA (10 μ g/ml) stimulation and IL-10 (*B*) and IL-12p70 (*C*) production by positively selected splenic DCs during LPS stimulation were measured. Data are the mean $\pm$ SEM from three wells per group. *, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$ (vs saline mice). #, $p < 0.05$; ##, $p < 0.01$; ####, $p < 0.0001$ (vs OVA/CFA mice). ++, $p < 0.01$; +++, $p < 0.0001$ (vs OVA/CFA/Prl mice).

including mast cells, basophils, eosinophils, macrophages, and DCs (5, 6). In our in vivo experiments, the cells most responsible for *cysLTs* production could be macrophages, because cells in BALF mainly consisted of macrophages (Table I). Mast cells, the number of which is very low in the lung during the early stage of systemic sensitization, might also be responsible for *cysLT* production, because they can potentially release some chemical mediators including *cysLTs* (4).

Our results revealed that LTRA potentially suppressed Th1-type immune responses as well as Th2-type responses (Figs. 1 and 4). We also confirmed this result with a Th1-biased immune response induced by OVA/CFA (Fig. 6). There have been some reports that emphasized the importance of Th1-type cells in the development of Th2-type immune responses (34–36). In clinical situations, we frequently found that Th1-type inflammation, such as viral infection, induces an exacerbation of asthma. Our results in the present study suggest that LTRA might be very effective in preventing asthma exacerbation preceded by viral infection. Moreover, our results suggested that LTRA could be beneficial to suppress even Th1-mediated immune disorders, such as rheumatoid arthritis and multiple sclerosis, as well as Th2-mediated disorders, such as asthma. This requires further investigation.

Generally, *cysLT₁* receptor mRNA and protein are rarely present in T cells (33). We also confirmed that LTD₄ (data not shown) and LTRA (Fig. 4*F*) had no direct effect on T cells. Based

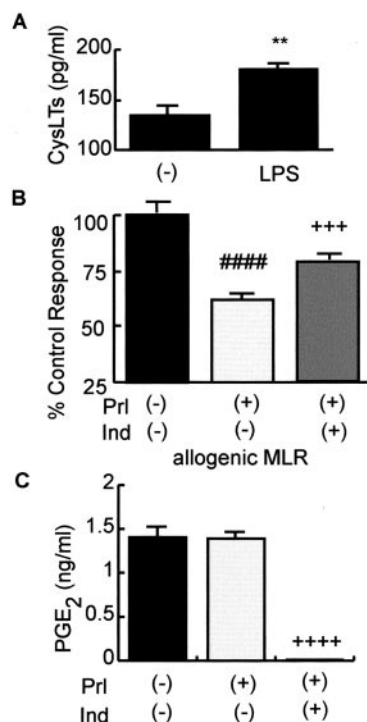


FIGURE 7. LTRA treatment in vitro directly suppresses the Ag-presenting capacity of DCs. DCs were obtained from the OVA-sensitized mice on day 11. **A**, CysLTs production by DCs was measured after 2-day incubation with or without LPS. **B** and **C**, DCs were cultured under LPS stimulation with no treatment (■) or with Prl (□) or Prl and Ind (□) for 2 days. **B**, DC function as an APC. After 2-day incubation, DCs were cocultured with CD4⁺ T cells from allogeneic naive C57BL/6 mice for 3 days. Then cell proliferation was measured, and the data are expressed as a percentage of the response compared with that induced by DCs from the OVA mice. **C**, After a 2-day incubation with LPS, PGE₂ production by DCs was measured. Data are the mean $\pm$ SEM from nine wells per group in **A** and from six wells per group in **B** and **C**. **, $p < 0.01$ (vs DCs without LPS stimulation). ####, $p < 0.0001$ (vs DCs with LPS stimulation without any treatment). +++, $p < 0.001$; +++++, $p < 0.0001$ (vs DCs treated with Prl).

on these results, we next examined the effect of LTRA on DCs. DCs expressed cysLT₁ receptor mRNA (Fig. 5A). LTRA treatment in vivo potently suppressed DC functions, such as Ag-presenting capacity and cytokine production (both IL-10 and IL-12), which OVA sensitization potentiated (Fig. 5, B–D; Fig. 6, B and C; and Fig. 8E). LTRA also directly suppressed DC functions in vitro (Fig. 7). These results clearly revealed that cysLTs directly affects DCs and potentiate their functions, and that LTRA potently inhibited T cell priming in the early stage of Ag-induced immune responses via suppression of DC functions. Machida et al. (27) have recently reported that in vitro treatment with LTRA decreased IL-10 production and increased IL-12 production from bone marrow-derived DCs, and that transfer of DCs treated with LTRA in vitro suppressed Ag-inhalation induced eosinophilic lung inflammation via increasing IFN- γ production in the lung. Contrary to their results, we demonstrated that in vivo treatment with LTRA significantly reduced not only IL-10, but also IL-12, production from splenic DCs to produce potent suppression of Th1-type as well as Th2-type immune responses. The reason for the discrepancy between the two reports remains unclear, but one possibility is that they adopted a quite different model from the one we used (27). Our findings that LTRA suppressed IL-12 production from DCs and the Th1-type immune response are supported by those

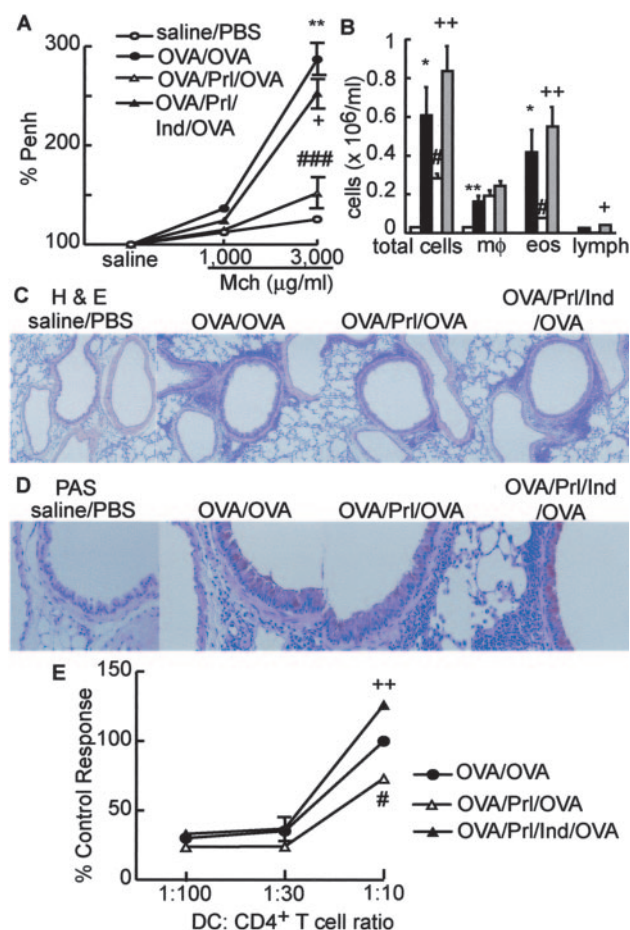


FIGURE 8. Treatment with LTRA in the early stage of systemic sensitization inhibits development of subsequent airway inflammation induced by Ag inhalation via suppression of the Ag-presenting capacity of DCs. Mice were sensitized with OVA and treated with Prl and/or Ind on days 11–17 as described in Fig. 2, then challenged with OVA on days 18–20 (■, OVA/OVA ($n = 5$); □, OVA/Prl/OVA ($n = 5$); □, OVA/Prl/Ind/OVA ($n = 5$)). The saline control mice received saline injections on days 0 and 11 and were challenged with PBS on days 18–20 (□, saline/PBS, $n = 3$). **A**, AHR on day 21 was assessed by change in percent Penh after Mch inhalation compared with the baseline Penh. **B**, BALF cell findings on day 21. **C** and **D**, Lung samples obtained on day 21 were stained with H&E (**C**) or PAS (**D**). Original magnifications, $\times 40$ for **C** and $\times 200$ for **D**. Mucus production is stained red in **D**. **E**, Effect of LTRA treatment during the sensitization phase on the Ag-presenting capacity of DCs in the lung in the effector phase. After 3 days of OVA inhalation, on day 21 lung DCs were obtained and then cocultured at several concentrations with CD4⁺ T cells obtained from DO11.10 mice. After 3-day coculture, cell proliferation was measured in triplicate, and the data are expressed as a percentage of the response compared with that induced by DCs from the OVA/OVA mice at a DC/CD4⁺ T cell ratio of 1:10. Data are the mean $\pm$ SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (vs saline/PBS mice). #, $p < 0.05$; ###, $p < 0.001$ (vs OVA/OVA mice or lung DCs obtained from OVA/OVA mice). +, $p < 0.05$; ++, $p < 0.01$ (vs OVA/Prl/OVA mice or lung DCs obtained from OVA/Prl/OVA mice).

data reported by Schultz et al. (37), who found that, using LTC₄ transporter multidrug resistance protein 1-deficient mice, blockade of the cysLT pathway decreased IL-12 and IFN- γ production against *Streptococcus pneumoniae* infection.

Eicosanoids interfere with each other's production both in vivo (23) or in vitro (38). Systemic OVA sensitization alone increased PGE₂ production in the lung, and LTRA treatment further enhanced it (Fig. 3A). We also confirmed that in the early stage of

systemic Ag sensitization, blockade of the LT pathway significantly increased PGE₂ production in the lung, using 5-LO-deficient mice (Fig. 3C). Harizi et al. (38) previously reported that LTB₄ does not affect COX expression and PGE₂ production from macrophages. In addition, in our *in vivo* experiments, the cells most responsible for PGE₂ production seem to be macrophages, because cells in BALF mainly consisted of alveolar macrophages (Table I), and PGE synthase mainly exists in macrophages, at least in mice (39). Taken together, it seemed that the increase in PGE₂ production in 5-LO-deficient mice in the present study was mainly due to the blockade of cysLTs, although 5-LO deficiency blocks both cysLTs and LTB₄ pathways. These results indicated that cysLTs might suppress PGE₂ production through cysLT receptor signal. The overall action of PGE₂ *in vivo* (23, 24) and *in vitro* (20–22) seems to be immunosuppressive. In the present study Ind treatment before Prl treatment *in vivo* significantly decreased PGE₂ production in the lung and ameliorated the allergic immune responses, including CD4⁺ T cell and DC activation, that had been potently suppressed by LTRA treatment (Figs. 2–6). This suggested that LTRA would exhibit its inhibitory effect on allergic immune responses through PGE₂ up-regulation, at least *in vivo*. In contrast, LTRA treatment did not up-regulate PGE₂ production by DCs in LPS stimulation *in vitro* experiments (Fig. 7C). Therefore, it seemed that, *in vivo*, LTRA suppressed DC function not by direct up-regulation of PGE₂ production from DCs themselves, but by up-regulation of PGE₂ production by other cells, such as macrophages.

Some studies have also focused on the importance of the pre- or early inflammatory phase without apparent lung inflammation for developing future asthma (40, 41). We finally demonstrated that treatment with LTRA in an early stage of systemic sensitization before the development of airway inflammation significantly suppressed the intensity of characteristic features of asthma such as AHR, eosinophilic inflammation, and mucus production that were later provoked by repeated Ag inhalation (Fig. 8). In addition, treatment with LTRA in the early stage of sensitization exhibited a suppressive effect on DC function even in the effector phase (Fig. 8E). This result suggested that in a clinical situation, treatment with LTRA in an early stage of systemic sensitization before completion of lung inflammation in childhood might potently prevent the individuals from developing asthma. In clinical settings, there is a trend dictating that LTRA should be administered to infants to prevent future exacerbation of asthma. The present findings offer experimental evidence to this trend.

In conclusion, we demonstrated a novel role of cysLTs in the immune system. In addition, our results offer a new possibility of LTRA in clinical use and propose a possible mechanism to provoke aspirin-induced asthma.

Acknowledgments

We thank Dr. Takao Shimizu (Department of Biochemistry and Molecular Biology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan) for critical review of this study and this manuscript. We thank E. Kasukawa, I. Makino, and A. Tobimatsu for their technical assistance. We also thank Ono Pharmaceutical (Osaka, Japan) for measuring the cysLT concentration in BALF and for their kind provision of Prl.

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