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J Immunol 2004; 172:1833-1838; doi: 10.4049/jimmunol.172.3.1833
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Cysteinyl Leukotrienes Regulate Dendritic Cell Functions in a Murine Model of Asthma

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Dendritic cells (DCs) act as APCs in the airway and play a critical role in allergy. Cysteinyl leukotrienes (cysLTs) synthesized from arachidonic acid are primary mediators of immediate asthmatic reaction. The aim of this study was to investigate the effects of cysLTs on Dermatophagoides farinae (Der f)-pulsed mouse myeloid DCs in inducing allergic airway inflammation in vitro and in vivo. Control DC (medium-pulsed), Der f-pulsed DC, cysLT-pulsed DC, Der f- and cysLT-pulsed DC, and Der f-pulsed and cysLT receptor antagonist (LTRA)-treated DC were prepared from murine bone marrow, and the production of cytokines was compared. Subsequently, these DCs were intranasally instilled into another group of naive mice, followed by intranasal Der f challenge to induce allergic airway inflammation in vivo. Der f-pulsed DC produced significantly higher amounts of IL-10 and IL-12 compared with control DC. Der f- and cysLT-pulsed DC further increased IL-10 production compared with Der f-pulsed DC. In contrast, treatment of Der f-pulsed DC with LTRA increased IL-12 and decreased IL-10. Intranasal instillation of Der f-pulsed DC resulted in airway eosinophilia associated with a significant rise in IL-5 levels in the airway compared with control DC. Pulmonary eosinophilia and excess IL-5 were further enhanced in Der f- and cysLT-pulsed DC-harboring mice. In contrast, Der f-pulsed and LTRA-treated DC significantly inhibited airway eosinophilia, reduced IL-5, and increased IFN-γ in the airway. Our results suggest that cysLTs play an important role in the development of allergic airway inflammation by regulating the immunomodulatory functions of DCs. The Journal of Immunology, 2004, 172: 1833–1838.
The procedures were reviewed and approved by the Nagasaki University School of Medicine committee on animal research. All experiments were repeated at least three times.

Reagents

Mite extract Dermapthoglyphoides farinae (Der f) was obtained from Cosmo Bio (Tokyo, Japan). The LPS concentration in the Der f preparations was <0.96 EU/mg Der f (Limulus amebocyte lysate test; E-Toxate; Sigma-Aldrich, St. Louis, MO). Recombinant mouse GC-CSF (rmGM-CSF) was purchased from R&D Systems (Oxford, U.K.). LTC₄, LTD₄, and LTE₄ were purchased from Sigma-Aldrich. Three kinds of leukotriene receptor antagonists (LTRA): Montelukast (Merck, Rahway, NJ), Pranlukast (Ooe Pharmaceutical, Osaka, Japan), and Zafirlukast (Astra Zeneka, London, U.K.), were obtained from each company. The concentrations of cysLTs, LTC₄, LTD₄, and LTE₄ in supernatants of cultured DCs were assayed by enzyme immunoassays (EIA; Cayman Chemical, Ann Arbor, MI). Concentrations of IL-12p70, IL-10, IL-5, and IFN-γ in culture supernatants and bronchoalveolar lavage fluid (BALF) samples were measured using ELISA (Endogen, Woburn, MA) using the methods described by the manufacturer. The detection limits for IL-12p40, IL-10, IL-5, and IFN-γ were 3, 12, 5, and 10 pg/ml, respectively.

Generation of DCs

Murine bone marrow-derived DCs were generated as described by Lutz et al. (27). Briefly, femurs and tibiae of female 10-week-old BALB/c mice were removed and purified from the surrounding muscle tissues. Then both ends were cut, and the marrow was flushed with PBS. After washing, leukocytes were suspended at a density of 2 × 10⁷ cells/ml in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% FCS (Invitrogen) and 1% penicillin-streptomycin (Invitrogen; hereafter referred to as cRPMI) and were cultured in 100-mm dishes. In the next step we prepared 11 groups of DCs (medium-pulsed DC (Control-DC); Der f-pulsed DC (Der f-DC); LTC₄, LTD₄, or LTE₄-stimulated DC (LTC₄-DC, LTD₄-DC, and LTE₄-DC, respectively); Der f-pulsed and LTC₄-, LTD₄-, or LTE₄-stimulated DC (Der f-LTC₄-DC, Der f-LTD₄-DC, and Der f-LTE₄-DC, respectively); and Der f-pulsed and treated with Pranlukast, Montelukast, or Zafirlukast DC (Der f-Prf-DC, Der f-Mon-DC, and Der f-Zaf-DC, respectively)). On day 3 another 10 ml of cRPMI containing 20 ng/ml rmGM-CSF was added to the plates. On days 6 and 8, half the culture supernatants were replaced with fresh medium containing 5 ng/ml rmGM-CSF. On day 10 the media were collected and centrifuged, then incubated with 0.1 mg/ml Der f for 24 h, except for Control-DC, LTC₄-DC, LTD₄-DC, and LTE₄-DC. LTRA1 (1 × 10⁻⁸ M; Pranlukast, Montelukast, or Zafirlukast) was added to DCs on days 8 and 10. These agents were first dissolved in ethanol and 1 N NaOH, then dissolved in the cRPMI at 1 × 10⁻⁶ M. Groups containing cysLTs (LTC₄-DC, LTD₄-DC, and LTE₄-DC) were stimulated with 100 ng/ml LTC₄, LTD₄, or LTE₄ on day 10. The selected concentrations of LTRAs and cysLTs were based on preliminary experiments and our published report (29). In all experiments incubation was conducted in a humidified 5% CO₂ atmosphere at 37°C.

RT-PCR

Total RNA was isolated from DCs with TRIzol (Life Technologies, Gaithersburg, MD) using the method recommended by the supplier. cDNA was synthesized from 2 μg of total RNA using a SuperScript One-Step RT-PCR System with platinum Tag DNA polymerase (Invitrogen) and was amplified using 200 ng of cDNA, with primers complementary to the published sequences of murine 5-LO, FLAP, LTC₄ synthase (Sigma-Genosys, Hokkaido, Japan), cysLT1 receptor, or β-actin (Amersham Pharmacia Biotech, Tokyo, Japan) (30–32). Thirty-five cycles were performed (15 s at 94°C for denaturation, 30 s at 60°C for annealing, and 1 min at 72°C for extension). After amplification, portions of the PCR products were electrophoresed on a 3% agarose gel and visualized using ethidium bromide. Controls in which the RT step was omitted confirmed that the PCR products reflected mRNA levels rather than contaminating genomic DNA.

Immunization with Ag-pulsed DCs and secondary exposure to Ag

On day 11, 1 × 10³ cells/50 μl in each group of DCs (Control-DC, Der f-DC, LTC₄-DC, LTD₄-DC, LTE₄-DC, Der f-LTC₄-DC, Der f-LTD₄-DC, Der f-LTE₄-DC, Der f-Prf-DC, Der f-Mon-DC, or Der f-Zaf-DC) were intranasally instilled into another group of naive BALB/c mice (5 wk old; n = 8 in 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 (BAL) and lung pathology

Twenty-four hours after the final Der f challenge, mice were sacrificed, and BAL was conducted in the immediate postmortem 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-micron sections were stained with H&E.

Statistical analysis

Results are expressed as the mean ± SEM. Differences between groups were examined for statistical significance using repeated-measures ANOVA with Bonferroni multiple comparison test. A value of p < 0.05 denoted the presence of a statistically significant difference.

Results

Murine bone marrow-derived DCs express mRNAs for cysLT1 receptor, 5-LO, FLAP, and LTC₄ synthase, and produce cysLTs

RT-PCR demonstrated that murine bone marrow-derived DCs expressed mRNAs for cysLT1 receptor, 5-LO, FLAP, and LTC₄ synthase (Fig. 1a). In comparison with Control-DC, Der f-DCs produced significantly higher amounts of LTC₄ and LTD₄ (Fig. 1b). LTE₄ could not be detected. These results indicated that murine bone marrow-derived DCs expressed cysLT1 receptor, 5-LO, FLAP, and LTC₄ synthase mRNAs, and the Der f pulse increased cysLT production.

Cytokines production from DCs

Fig. 2 shows the production of IL-12 and IL-10 by each of the 11 groups of DCs. In comparison with the Control-DC, Der f-DC produced higher amounts of IL-12 and IL-10. Neither IL-12 nor IL-10 could be detected after either LTC₄, LTD₄, or LTE₄ stimulation alone. In contrast, LTD₄ added to Der f-DC (Der f-LTD₄-DC)
DC) produced higher amounts of IL-10 compared with Der f-DC, Der f-LTC\textsubscript{4}-DC, and Der f-LTE\textsubscript{4}-DC. IL-12 production was not significantly different among Der f-LTC\textsubscript{4}-DC, Der f-LTD\textsubscript{4}-DC, and Der f-LTE\textsubscript{4}-DC. Three types of LTRAs (Der f-Prl-DC, Der f-Mon-DC, and Der f-Zaf-DC) inhibited IL-10 and significantly increased IL-12 production by Der f-DC. The cell 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).

CysLTs exacerbated allergic airway inflammation in mice

To investigate whether in vitro functional changes in DCs also occur in vivo, we conducted additional experiments in which each of the 11 groups of DCs (Control-DC, Der f-DC, LTC\textsubscript{4}-DC, LTD\textsubscript{4}-DC, LTE\textsubscript{4}-DC, Der f-LTC\textsubscript{4}-DC, Der f-LTD\textsubscript{4}-DC, Der f-LTE\textsubscript{4}-DC, Der f-Prl-DC, Der f-Mon-DC, and Der f-Zaf-DC) was instilled intranasally into another group of naive BALB/c mice, followed by

**FIGURE 2.** IL-12 and IL-10 concentrations in culture supernatants of DCs. On day 11, culture media of DCs were collected and centrifuged. Cytokines in culture supernatants were measured by ELISA. Results are expressed as the mean \(\pm\) SEM (\(n = 8\) for each group). \(*, p < 0.01\) vs Control-DC; \(\dagger, p < 0.05\) vs Der f-DC.

**FIGURE 3.** Lung pathology in mice inoculated with DCs. Lung tissues were obtained from mice intranasally instilled with DCs and stained with H&E. Representative microphotographs from each group are shown. 

- a, Mouse harboring Control-DC; b, mouse harboring Der f-DC; c, mouse harboring LTD\textsubscript{4}-DC; d, mouse harboring DF-LTD\textsubscript{4}-DC; e, mouse harboring DF-Prl-DC. Original magnification, \(\times 400\).
inflammation, characterized by peribronchovascular eosinophilic infiltration and goblet cell hyperplasia (Fig. 3c), whereas mice inoculated with Der f-LTD4-DC exhibited enhanced airway inflammation (Fig. 3d), and lung tissues of mice inoculated with Der f-Prl-DC (Fig. 3e) were almost similar to those of the control DC (Fig. 3a). Allergic airway inflammation was evaluated quantitatively by BALF analysis, and the results confirmed those of lung pathology (Fig. 4).

Discussion

The present study examined the effects of Der f and cysLTs on the function of DCs. First, murine bone marrow-derived DCs were pulsed with Der f, and then in vitro production of cytokines was analyzed with and without treatment with exogenous cysLTs or specific cysLTs receptor antagonist. Subsequently, these DCs were instilled into the airway of naive mice, followed by Der f exposure, and then lung pathology and BAL cytokines profile were compared to determine whether in vitro functional changes in DCs by cysLTs could be actually reflected in vivo. It had been reported that murine bone marrow-derived DCs produce PGs and LTB4 (18). The present study is the first to show that murine bone marrow-derived DCs produce cysLTs in response to aeroallergens and express cysLT1 receptor, 5-LO, FLAP, and LTC4 synthase.

The production of two immunomodulatory cytokines (IL-10 and IL-12) was used to investigate the in vitro functions of DCs in this study. IL-12 represents a critical cytokine that induces naive T cell to Th1 phenotype (1, 8, 10, 33), whereas the role of IL-10, a murine prototypical Th2 cytokine, in the differentiation of Th cells is controversial. IL-10 enhances the formation of Th2 cells by down-regulating IL-12 production by DCs (34). In contrast, IL-10-treated DCs inhibit OVA-specific immune responses in naive and sensitized mice (35). In general, however, at least at the DC level, it is likely that DCs induce a Th1 response in high IL-12 and low IL-10, whereas they induce a Th2 response in low IL-12 and high IL-10 (8, 10). Similar to PGE2 and histamine, both of which do not affect the functions of immature DC, but can modulate LPS-stimulated mature DCs (14–17), the present study showed that LTD4 per se failed to alter IL-12 production from DCs, but they enhanced Der f-pulsed mature DCs to induce a Th2 response via augmentation of IL-10 production. These results were further supported by the experiments using the specific cysLT receptor antagonist, in which Der f-LTRAs-DC increased the production of IL-12 and reduced IL-10 secretion. Considered together, these results indicate that LTD4 is a critical enhancer of Der f-pulsed DC to induce a Th2 response by regulating immunoregulatory cytokines. Ghaemmaghami et al. (36) showed that a proteolytically active major dust mite allergen (Der p1), but not inactive allergen, biases the Th cell subset development in favor of Th2 via the cleavage of CD40 in DCs. LTD4 also enhances OVA-pulsed DCs to induce a Th2 response both in vitro and in vivo (our unpublished observations). Thus, the proteolytic activity of Ag does not seem to be required for LTD4 to bias the immune response to Th2. Interestingly, among cysLTs, LTD2 represents the most dominant cysLT produced by DCs, and only LTD4 could enhance IL-10 production from Der f-DC in vitro, resulting in amelioration of Der f-DC-induced allergic airway inflammation in vivo. In HEK-293
cells expressing the CysLT1 receptor, the agonist potency for calcium influx was LTD4 > LTC4 > LTE4 (37), suggesting that LTD4 is the most potent cysLT to cause biological effects. However, in asthmatics, inhaled LTD4 does not cause airway eosinophilia, whereas LTE4 does (38). Thus, the effects of cysLTs on allergy may depend on cell types and species.

Lambrecht et al. (39) showed that intratracheal injection of CFSE-labeled DCs (1 × 10^6 cells) was followed by the recovery of these cells in BALF (14.1% of cells), draining mediastinal lymph nodes (0.012%) within 12 h after instillation. Although our mice were instilled with a smaller number of DCs (1 × 10^5 cells), this number of DCs was sufficient to cause allergic airway inflammation, as shown in lung pathology (Fig. 3). The results of these in vivo experiments were consistent with those of in vitro experiments and demonstrated that mice instilled with Der f-pulsed DC developed allergic airway inflammation concomitant with increased production of IL-5, a Th2 cytokine, in the airway; more importantly, the addition of LTD4 further increased airway eosinophil count and IL-5 production. In contrast, mice instilled with Der f-pulsed DC treated with LTRA to antagonize endogenous cysLTs showed a reduction in airway eosinophil count and IL-5 production, but increased IFN-γ, a Th1 cytokine. Hence, the in vitro functional changes noted in Der f-pulsed DC by cysLTs in vitro were supported by these in vivo experiments.

Exposure to mite allergen and respiratory viral infections induce cysLTs production from a number of constitutive and inflammatory cells in asthmatic airways, including epithelial cells, mast cells, and eosinophils (40, 41). Using a mouse model of asthma, previous studies showed that CysLTs primarily act as chemical mediators to worsen asthma by constriction of airway smooth muscles, increased vascular permeability, and accumulation of activated eosinophils, and they play an important role in airway remodeling (42, 43). In addition, the present study clearly showed an immunological effect for cysLTs on the development and exacerbation of asthma, i.e., cysLTs might directly alter airway DCs to initiate a Th2 response. Currently, LTRA is a recommended medication for asthma (44). LTRA may show an immunoregulatory effect on human DCs. This number of DCs was sufficient to initiate a Th2 response. Currently, LTRA is a recommended medication for asthma (44). LTRA may show an immunoregulatory effect for cysLTs on the development and exacerbation of asthma (44). LTRA may show an immunoregulatory effect on human DCs. The present study examined the effects of cysLTs on DCs functions to initiate and bias the immune response. CysLTs also exert effects on Langerhans cells to migrate accessory lymph nodes via modulation of the response to chemokines (24). Thus, it will be interesting to determine whether cysLT-pulsed DCs are also differentially responsive to chemokines.

References


