

Listeriolysin O derived from *Listeria monocytogenes* inhibits the effector phase of an experimental allergic rhinitis induced by ovalbumin in mice

K. Yamamoto,^{*,†} I. Kawamura,^{*}
T. Tominaga,^{*} T. Nomura,^{*} J. Ito[†] and
M. Mitsuyama^{*}

Departments of ^{*}Microbiology and

[†]Otolaryngology, Head and Neck Surgery, Kyoto
University Graduate School of Medicine, Kyoto,
Japan

Summary

Listeriolysin O (LLO) derived from *Listeria monocytogenes* is highly capable of inducing interleukin (IL)-12, IL-18 and interferon (IFN)- γ , and facilitates the generation of Th1 cells. We have recently shown that recombinant LLO (rLLO) inhibits generation of ovalbumin (OVA)-specific Th2 immune response by skewing maturation of antigen-specific T cells into Th1 cells. In the present study, we investigated the effect of rLLO on the effector phase of Th2-dependent allergic rhinitis in BALB/c mice sensitized with OVA. In mice sensitized intraperitoneally and challenged intranasally with OVA, nasal allergic symptoms such as sneezing and nose-scratching were observed at a high frequency. A high titre of anti-OVA IgE antibody was detected in sera and a large number of eosinophils migrated into the nasal tissue. However, rLLO treatment during the intranasal challenge inhibited the allergic symptoms, production of anti-OVA IgE antibody and eosinophil infiltration. Though rLLO did not affect antigen-specific cytokine production from splenic CD4⁺ T cells, rLLO significantly suppressed OVA-specific IL-4 and IL-5 production from nasal mononuclear cells. We further found that rLLO inhibited the recruitment of CD4⁺ T cells in nasal mucosa, and diminished the transcription and cell surface expression of CCR4 on splenic CD4⁺ T cells. Moreover, rLLO was able to inhibit the passive cutaneous anaphylaxis reaction mediated by anaphylactic antibodies (IgE and IgG₁) and mast cells. Taken together, these data showed that rLLO suppresses the effector phase of allergic rhinitis by inhibition of Th2 cell recruitment to nasal mucosa and type I allergic reaction.

Keywords: allergic rhinitis, CCR4, listeriolysin O, nasal mucosa, passive cutaneous anaphylaxis reaction

Accepted for publication 15 March 2006

Correspondence: Ikuo Kawamura, Department of Microbiology, Kyoto University Graduate School of Medicine, Yoshida konoecho, Sakyo-ku, Kyoto 606-8501, Japan.

E-mail: ikuo_kawamura@mb.med.kyoto-u.ac.jp

Introduction

Listeria monocytogenes is a gram-positive, facultative intracellular bacterium and the major virulence genes (*plcA*, *hly*, *mpl*, *actA* and *plcB*) of *L. monocytogenes* have been identified [1,2]. Among the virulence gene products, listeriolysin O (LLO), a 58-kD protein encoded by *hly*, is a member of a large family of cholesterol-dependent cytolysins and contributes to intracellular multiplication by disruption of the phagosomal membrane resulting in bacterial escape into the cytosol [3,4]. In addition to the membrane-lytic activity, we have found that LLO is highly capable of inducing various inflammatory cytokines, including interleukin (IL)-1, IL-6, tumour necrosis factor- α , IL-12, IL-18 and interferon (IFN)-

γ [5–7]. Furthermore, we have shown that IFN- γ is mainly produced by natural killer (NK) cells, which are activated by both IL-12 and IL-18 produced from macrophages in response to LLO [6,8]. Since LLO was recently reported to be a ligand of Toll-like receptor 4 (TLR4) [9], the proinflammatory cytokine-inducing property of LLO is thought to be a consequence of the activation of the TLR4 signal pathway. Due to such a high Th1 cytokine-inducing activity of LLO, the protective immunity to *L. monocytogenes* was induced when mice were immunized with killed or avirulent *L. monocytogenes* together with LLO, whereas the protection was not generated in mice immunized with killed or avirulent *L. monocytogenes* alone [10,11]. These results indicate that LLO serves as a strong Th1 adjuvant.

Allergic rhinitis including pollinosis is a typical Th2 immune disorder characterized by a high level of antigen-specific IgE production and eosinophil infiltration in a nasal tissue [12]. Approximately 15 to 20% of the population were estimated to suffer from allergic rhinitis [13]. Because the enhanced IgE production and inflammatory response in rhinitis are due to the predominant production of Th2 cytokines such as IL-4, IL-5 and IL-13, the allergic symptoms can be alleviated by inhibition of Th2 cytokine responses. Based on the idea of counterbalance between Th1 and Th2 cells [14], several adjuvants that preferentially induce Th1 response have been tested for their inhibitory activity to allergic response. There are many reports to show that Th1 adjuvants inhibited allergic response when they were administered during sensitization [15,16]. In addition, administration of CpG-oligodeoxynucleotides at the effector phase is reported to have exerted a suppressive effect as well [17]. These results suggest that some Th1 adjuvants may exert a therapeutic effect on Th2-dependent allergic disorders even if given at the effector phase. In those patients whose allergic state has been established, alleviation at the effector phase is of significant clinical importance.

We have recently shown that recombinant LLO (rLLO) is able to suppress allergic responses by the inhibition of the generation of antigen-specific Th2 cells when rLLO was administered during sensitization [8]. Thus, in the present study, we investigated whether rLLO is able to suppress the OVA-induced allergic rhinitis at the effector phase.

Materials and methods

Mice

Female BALB/c mice (Japan SLC, Shizuoka, Japan) raised and maintained under specific-pathogen-free conditions, were used at 7–8 weeks of age. All the experimental procedures performed on mice were approved by the Animal Ethics and Research Committee of Kyoto University Graduate School of Medicine.

Construction and purification of recombinant LLO

We have prepared rLLO as described previously [7]. The level of LPS was measured by a *Limulus* colour KY test (Wako Pure Chemical industries, Osaka, Japan), and was confirmed to be less than 50 pg of LPS per mg of protein. Since the purified rLLO exerted a high level of cytotoxic activity to mammalian cells, we incubated rLLO in PBS (pH 8.0) for 2 h at 37 °C to inhibit the membrane-lytic activity [8,18]. Pre-treated rLLO did not exhibit any haemolytic activity to sheep erythrocytes at the concentration (250 µg/ml) used for *in vivo* experiments [7]. The non-membrane-lytic rLLO was used for the experiments after confirming the presence of cytokine-inducing activity *in vitro* using murine spleen cells [8].

Sensitization and challenge of mice with OVA

Mice were sensitized intraperitoneally with 10 µg of OVA (Nacalai Tesque, Kyoto, Japan) adsorbed onto 2 mg of alum (Pierce Chemical Company, Rockford, IL, USA) (OVA in alum) on day 1 and day 8. Non-immunized control mice received alum alone on the same schedule. Allergic rhinitis was induced by intranasal daily administration of 10 µl of PBS containing 100 µg of OVA from day 15 to day 21. To investigate the effect of rLLO on the effector phase of rhinitis, a group of mice were injected intraperitoneally with 100 µg of rLLO (250 µg/ml) on days 15, 18 and 20. A group of OVA-sensitized mice without rLLO administration were injected intraperitoneally with PBS. Non-immunized control mice were administered intranasally with OVA and intraperitoneally with PBS on the same schedule.

Observation of nasal allergic symptoms

The severity of allergic rhinitis was determined by measuring the frequency of sneezing and nose-scratching for 10 min after the last intranasal challenge with OVA as described previously [8,19]. In order to eliminate any biased measurement, mice in non-labelled cages were subjected to a single-blind observation by examiners without identifying the experimental group.

Detection of anti-OVA IgE antibody

One day after the last intranasal challenge, sera were collected. The titre of anti-OVA IgE antibody was determined by ELISA as described previously [13,20] and defined as the reciprocal of the highest serum dilution that gave a two-fold higher OD value at 450 nm than that of control group.

Histological examination

The nasal tissues were removed one day after the last intranasal challenge and fixed in 10% neutral formalin and decalcified. Coronal nasal sections were stained with haematoxylin and eosin, and the total number of eosinophils at the submucosal region of nasal septum was counted under a microscope with 10×40 magnification as described previously [8,12].

Cytokine production after stimulation with OVA *in vitro*

One day after the last intranasal challenge, splenic CD4⁺ T cells were enriched by using anti-CD4 Microbeads and the MACS system (Miltenyi Biotec, Gladbach, Germany) and suspended at 5 × 10⁶ cells/ml in the presence of peritoneal adherent cells (5 × 10⁴ cells/ml) as antigen-presenting cells in RPMI 1640 (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated FBS (Gibco-BRL), 50 µg/ml gentamicin (Wako Chemicals USA, Richmond, VA, USA)

and 5×10^{-5} M 2-mercaptoethanol (Nacalai Tesque). Then, the nasal cells were released from nasal tissues by treatment with collagenase (Liberase Blendzyme 3; Roche Diagnostics, Indianapolis, IN, USA), and mononuclear cells were separated using a stepwise Percoll gradient as described by Asanuma *et al.* [21]. The resultant nasal mononuclear cells were suspended at 2×10^6 cells/ml in culture medium. Cells were stimulated with 100 µg/ml of OVA for 72 h at 37 °C in 5% CO₂. In some experiments, spleen cells were collected from OVA-sensitized mice on day 15, and stimulated with 100 µg/ml of OVA alone or OVA plus 10 µg/ml of rLLO for 72 h. The level of IL-4 and IL-5 in the culture supernatant was determined by using OptEIA mouse ELISA set specific for IL-4 and IL-5 (BD Biosciences Pharmingen, San Diego, CA, USA). The level of IFN-γ production was determined by ELISA as described previously [6,7].

Detection of IL-5- or IFN-γ-producing cells by ELISPOT assay

One day after the last intranasal challenge, spleen cells were stimulated at 5×10^6 cells/ml with 100 µg/ml of OVA for 48 h. Cells were collected and antigen-specific IL-5- or IFN-γ-producing cells were enumerated by ELISPOT assay, as described previously [8].

Detection of CD4⁺ T cells in nasal mucosa by flow cytometric analysis

One day after the last intranasal challenge, cells in the nasal mucosa were isolated and stained with PE-Cy5-conjugated rat anti-mouse CD4 monoclonal antibody (Pharmingen). The population of CD4⁺ T cells was analysed using a FACSCalibur (BD Biosciences). Data were expressed as the percentage of positive cells in the nasal mononuclear cells and the total number of CD4⁺ T cells per nasal mucosa was also indicated.

Expression of TARC and MDC in nasal mucosa

One day after the last intranasal challenge, cells were collected from nasal tissues after collagenase treatment. Total RNA was extracted from the cells (1×10^6 cells) with NucleoSpin RNA II (Macherey Nagel, Duren, Germany) and cDNA was reverse transcribed using a random primer (Invitrogen, Carlsbad, CA, USA) as described previously [6]. PCR was performed by using KOD-plus DNA polymerase (TOYOBO, Osaka, Japan). The primers used in the present study are as follows [6,22,23]: 5'-CCTGGCTGCTCTGCTTCTGGGGAC-3' and 5'-GGCATCCCTGGAACACTCCACTG-3' for TARC, 5'-GCCCTCTGGTCATTAGACACCTG-3' and 5'-TCGTTGGCAAGGCTCTTGCTG-3' for MDC and 5'-TGAATCCTGTGGCATCCATGAAAC-3' and 5'-TAAACGCAGCTCAGTAACAGTCCG-3' for β-actin. Each PCR product (5 µl) was electrophoresed on a 2% agarose gel and

stained with ethidium bromide. The band of the PCR product was visualized on a UV transilluminator. The density of each band was compared using National Institutes of Health (NIH) image 1.63 software after normalization on the basis of the density of β-actin band. Data were expressed as a fold increase upon the intensity of corresponding PCR product of control group, which was arbitrarily assigned as 1.0.

CCR4 expression on splenic CD4⁺ T cells

To investigate the effect of *in vivo* administration of rLLO on CCR4 expression on CD4⁺ T cells, spleen cells were collected one day after the last intranasal challenge. Splenic CD4⁺ T cells were enriched by using anti-CD4 Microbeads and MACS system, and the transcriptional level of CCR4 was determined by RT-PCR using the following primer set: 5'-ATCGTGCACGCGGTATTCTCC-3' and 5'-GACGGGGTTAAGGCAGCAGTGA-3' for CCR4 [24]. We also determined the level of surface expression of CCR4 on CD4⁺ T cells. Spleen cells were stained with PE-Cy5-conjugated rat anti-mouse CD4 monoclonal antibody (Pharmingen) and rabbit anti-mouse CCR4 polyclonal antibody (Zymed Laboratories, South San Francisco, CA, USA), followed by FITC-conjugated goat anti-rabbit IgG (Zymed Laboratories). The level of surface expression of CCR4 on CD4⁺ T cells was analysed using a FACSCalibur. Data were expressed as the percentage of CCR4⁺ cells in CD4⁺ T cells and mean fluorescence intensity (MFI) of CCR4⁺ cells was also indicated.

To investigate the effect of rLLO on CCR4 expression on CD4⁺ T cells *in vitro*, spleen cells were collected from OVA-sensitized mice on day 15 and stimulated for 72 h with 100 µg/ml of OVA alone or OVA plus 10 µg/ml of rLLO in the absence or presence of 2 µg/ml of goat anti-mouse IL-12p70 antibody (Genzyme/Teche, Minneapolis, MN, USA) or control antibody (normal goat IgG; Genzyme/Teche). Cells were collected for analysis of the level of transcription and surface expression of CCR4 on CD4⁺ T cells.

Passive cutaneous anaphylaxis (PCA) reaction at auricle

Mice were anaesthetized lightly with ether and injected intradermally into the auricle with 20 µl of appropriately diluted sera containing anti-OVA anaphylactic antibodies (IgE and IgG₁), which were obtained from mice immunized with OVA in alum. Twenty-four hours later, 250 µl of 0.25% Evans blue (Nacalai Tesque) in PBS containing OVA (1 mg/ml) was injected intravenously. To test the effect of rLLO on the PCA reaction, mice were injected intraperitoneally with 100 µg of rLLO 30 min before injection of the Evans blue solution. Auricles were collected 30 min after injection of the Evans blue solution and the amount of dye in the auricle was measured as described previously [25].

Statistical analysis

Mann–Whitney's *U*-test and Student's *t*-test were used to determine the statistical significance of the values obtained. $P < 0.05$ was considered statistically significant.

Results

Administration of rLLO at effector phase of rhinitis inhibits the allergic responses

We have recently shown that rLLO is capable of inhibiting generation of OVA-specific Th2 immune response by skewing maturation of antigen-specific T cells into Th1 cells [8]. The result prompted us to examine whether Th2-dependent allergic responses are suppressed when rLLO is administered at the effector phase. To test the possibility, we examined the effect of rLLO on a murine model of rhinitis induced by OVA. Upon intranasal challenge with OVA, nasal allergic symptoms such as sneezing and nose-scratching were observed at a high frequency in OVA-sensitized mice (OVA/OVA) but not in non-sensitized and OVA-challenged control mice (PBS/OVA) (Fig. 1a). In addition, both anti-OVA IgE titre in sera and eosinophil infiltration in nasal mucosa were increased in OVA/OVA mice (Fig. 1b,c), indicating that a typical allergic rhinitis was induced. On the other hand, the allergic symptoms were considerably alleviated in OVA-sensitized mice that were given rLLO during the intranasal challenge with OVA (OVA/OVA + rLLO) (Fig. 1a). Anti-OVA IgE titre in sera and eosinophil infiltration in nasal mucosa were significantly reduced (Fig. 1b,c). These results indicated that rLLO was capable of suppressing the effector phase of allergic rhinitis in OVA-sensitized mice.

Administration of rLLO at effector phase of rhinitis does not affect development of antigen-specific CD4⁺ T cells

To elucidate the mechanism by which rLLO suppressed the effector phase of allergic rhinitis in sensitized mice, we examined whether rLLO influences the generation of OVA-specific Th2 cells in spleen. One day after the last intranasal challenge with OVA, splenic CD4⁺ T cells were stimulated with the antigen and the production of IL-4, IL-5 and IFN- γ was measured. A high level of IL-4 and IL-5 production was observed in splenic CD4⁺ T cells of OVA/OVA mice (Fig. 2a,b), and IFN- γ production was also detected (Fig. 2c). A similar level of production of these cytokines was detected in splenic CD4⁺ T cells of OVA/OVA + rLLO mice (Fig. 2a–c). ELISPOT assays revealed that the number of IL-5- or IFN- γ -producing cells in the spleens of OVA/OVA mice was comparable to those of OVA/OVA + rLLO mice (Fig. 2d). These results indicated that rLLO administered at the effector phase did not affect the development of OVA-specific CD4⁺ T cells in spleen.

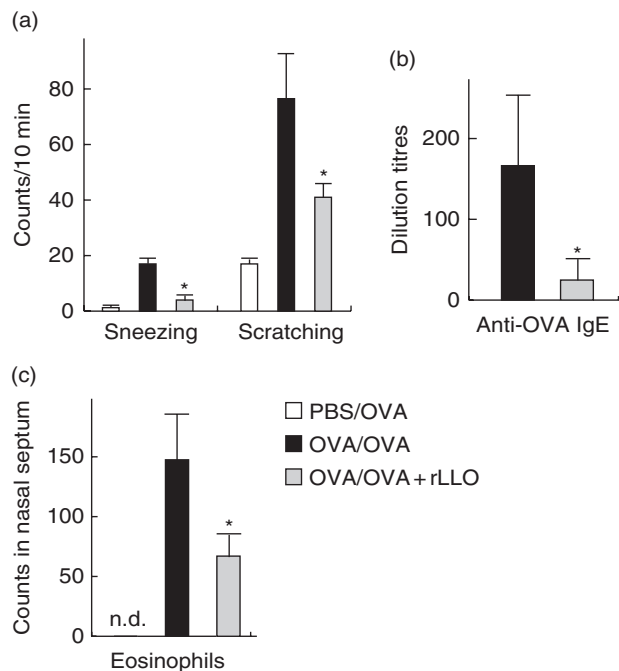


Fig. 1. The effect of rLLO administration on allergic rhinitis. BALB/c mice were sensitized and challenged with OVA, with or without rLLO. (a) The frequency of sneezing and nose-scratching was counted for 10 min after the last intranasal challenge ($n = 5$ or 6 mice for each group). (b) The titre of anti-OVA IgE in sera was determined by ELISA ($n = 5$ or 6 mice for each group). (c) The number of eosinophils infiltrating into the nasal septum was counted under a microscope with 10×40 magnification ($n = 5$ or 6 mice for each group). Data are the mean $\pm$ SD, and representative of two independent experiments. n.d., not detected. * $P < 0.05$ by Mann–Whitney's *U*-test, compared to OVA/OVA.

Administration of rLLO inhibits antigen-specific Th2 cytokine production from nasal mononuclear cells

Since administration of rLLO did not affect the development of OVA-specific Th1 or Th2 cells in spleen, we next investigated cytokine production from cells of the nasal mucosa. One day after the last intranasal challenge, the nasal tissues were treated with collagenase and the average number ($\times 10^6 \pm$ SD) of cells per nasal mucosa was 1.89 ± 0.11 in PBS/OVA mice, 4.26 ± 0.24 in OVA/OVA mice and 2.1 ± 0.30 in OVA/OVA + rLLO mice. Then, nasal mononuclear cells were separated and stimulated with OVA and the production of IL-4, IL-5 and IFN- γ was measured. A high level of IL-4 and IL-5 production was detected in cells of OVA/OVA mice (Fig. 3a,b). However, administration of rLLO significantly reduced the production of these Th2 cytokines (Fig. 3a,b). On the other hand, we detected the control level of IFN- γ production from cells of both OVA/OVA mice and OVA/OVA + rLLO mice (Fig. 3c). Production of IL-4, IL-5 and IFN- γ was not detected in cells of control mice (PBS/OVA). In addition, we found that though the percentage and the number of CD4⁺ T cells increased significantly in the nasal

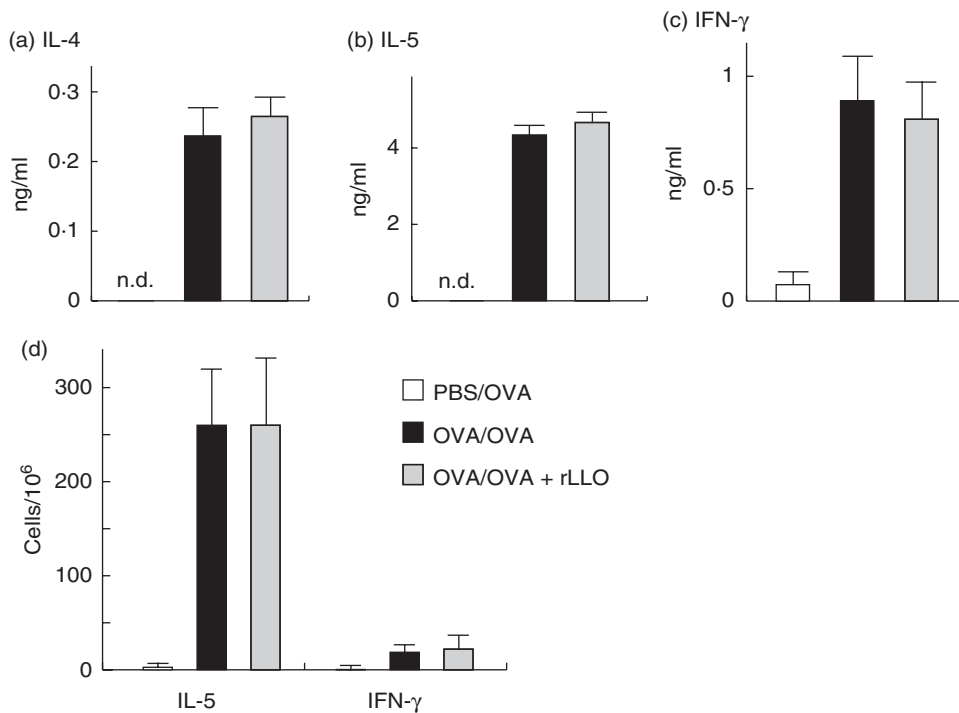


Fig. 2. The effect of rLLO administration on OVA-specific cytokine production in spleen. BALB/c mice were sensitized and challenged with OVA, with or without rLLO. Splenic CD4⁺ T cells were stimulated with OVA for 72 h. The level of (a) IL-4, (b) IL-5 and (c) IFN-γ in culture supernatants was determined by ELISA. (d) Spleen cells were stimulated with OVA for 48 h. Cells were collected and IL-5- and IFN-γ-producing cells were enumerated by ELISPOT assay. Data are the mean $\pm$ SD, and representative of two independent experiments. n.d., not detected.

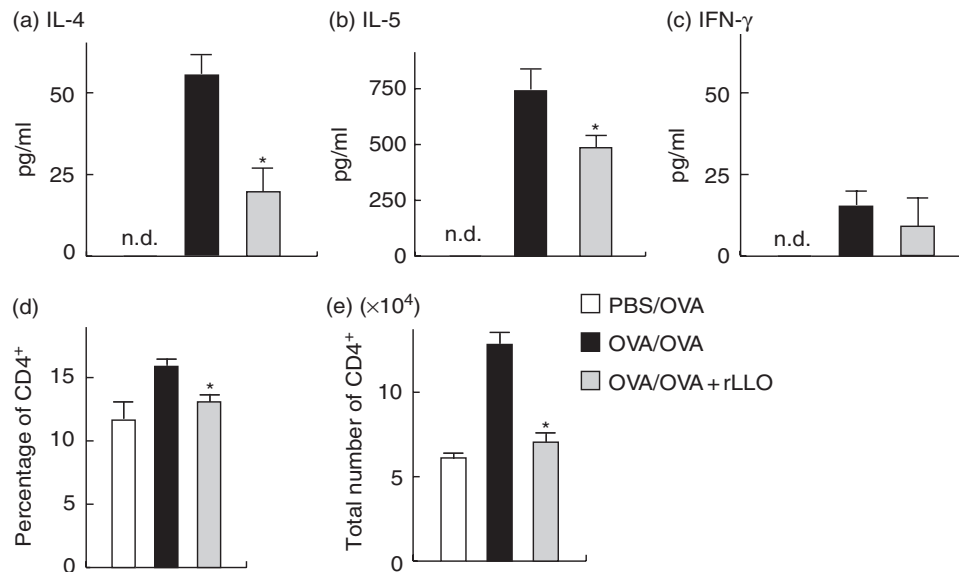


Fig. 3. The effect of rLLO administration on OVA-specific cytokine production and the number of CD4⁺ T cells in nasal mucosa. BALB/c mice were sensitized and challenged with OVA, with or without rLLO. Nasal mononuclear cells were stimulated with OVA for 72 h. The level of (a) IL-4, (b) IL-5 and (c) IFN-γ in culture supernatants was determined by ELISA. (d,e) Cells obtained from nasal mucosa were counted and stained with PE-Cy5-conjugated anti-mouse CD4 monoclonal antibody. Flow cytometric analysis was performed. Data are expressed as (d) the percentage of positive cells in nasal mononuclear cells and (e) the total number of CD4⁺ T cells per nasal mucosa was also indicated. Data are the mean $\pm$ SD, and representative of two independent experiments. n.d., not detected. * $P < 0.05$ by Student's *t*-test, compared to OVA/OVA.

mucosa of OVA/OVA mice, they did not increase in OVA/OVA + rLLO mice (Fig. 3d,e). These results suggest that rLLO inhibits the recruitment of CD4⁺ T cells to nasal mucosa, resulting in the suppression of Th2 immune responses and allergic symptoms.

Administration of rLLO down-regulates CCR4 expression on CD4⁺ T cells

It has been shown that CCR4 is a chemokine receptor that is exclusively expressed on Th2 cells and responds to CCL17 (TARC: thymus and activation-regulated chemokine) and CCL22 (MDC: macrophage-derived chemokine) that are produced at the site of allergic inflammation [24,26,27]. Since our results suggest that rLLO modulates the recruitment of Th2 cells to nasal mucosa, we extracted total RNA one day after the last intranasal challenge and analysed the transcriptional level of TARC and MDC in nasal mucosa and CCR4 transcription in splenic CD4⁺ T cells of OVA/OVA mice and OVA/OVA + rLLO mice. The transcription of these chemokines was increased in both experimental groups of mice and there was no significant difference in the transcriptional level (Fig. 4a). However, the administration of rLLO

might have reduced the production of these chemokines, since a reduction of the cells infiltrating into the nasal mucosa was noted. On the other hand, we detected enhanced transcription of CCR4 in CD4⁺ T cells of OVA/OVA mice (Fig. 4b) but the transcriptional level was profoundly suppressed in CD4⁺ T cells of OVA/OVA + rLLO mice (Fig. 4b). In addition, the percentage of CCR4⁺ cells in CD4⁺ T cells was markedly increased in the spleen of OVA/OVA mice, but was significantly reduced by rLLO administration (Fig. 4c). We also observed that the cell surface intensity of CCR4 was decreased in OVA/OVA + rLLO mice as compared to OVA/OVA mice (Fig. 4d). These results suggested that administration of rLLO down-regulated the expression of CCR4 on Th2 cells *in vivo*, resulting in an inhibition of Th2 cell recruitment to nasal mucosa. Moreover, rLLO administration decreased the expression level of CCR2 (chemoattractant receptor-homologous molecule expressed on Th2 cells), another Th2-type chemoattractant receptor for PGD₂ [28] (data not shown).

Recombinant LLO down-regulates CCR4 expression via induction of IL-12 production

In order to elucidate the mechanism by which rLLO administration down-regulates the expression of CCR4 *in vivo*, we stimulated spleen cells from OVA-sensitized mice *in vitro*, with OVA alone or OVA plus rLLO, and determined the transcriptional level of CCR4 in CD4⁺ T cells. Consistent with the *in vivo* result, RT-PCR analysis revealed that stimulation with OVA induced the transcription of CCR4 but rLLO inhibited the transcription (Fig. 5a). It has been reported that IL-12 has the ability to down-regulate the expression of Th2-type chemoattractant receptors [29–31] and LLO is able to induce the production of IL-12 [6,8]. Based on these findings, we speculated that rLLO might inhibit the transcription of CCR4 via induction of IL-12 production. To test this possibility, we added anti-IL-12 antibody to investigate its effect on CCR4 transcription. As expected, the antibody almost restored the transcriptional level that existed prior to reduction by rLLO, while control antibody did not (Fig. 5a). Moreover, flow cytometric analysis indicated that the percentage of CCR4⁺ cells in CD4⁺ T cells was dramatically increased after stimulation with OVA, but was significantly inhibited by rLLO (Fig. 5b). Recombinant LLO also reduced the cell surface intensity of CCR4 (Fig. 5c). The inhibitory effect of rLLO was cancelled by anti-IL-12 antibody (Fig. 5b,c). These results indicate that rLLO down-regulates the expression of CCR4 via the induction of endogenous IL-12 production. We also observed a high level of IL-4 and IL-5 production in spleen cells from OVA-sensitized mice on stimulation with OVA *in vitro* (Fig. 5d). However, rLLO suppressed the production of Th2 cytokines and increased the production of IFN- γ (Fig. 5d). Moreover, we found that rLLO did not affect the production of these cytokines from the resultant CD4⁺ T cells after re-stimulation with OVA

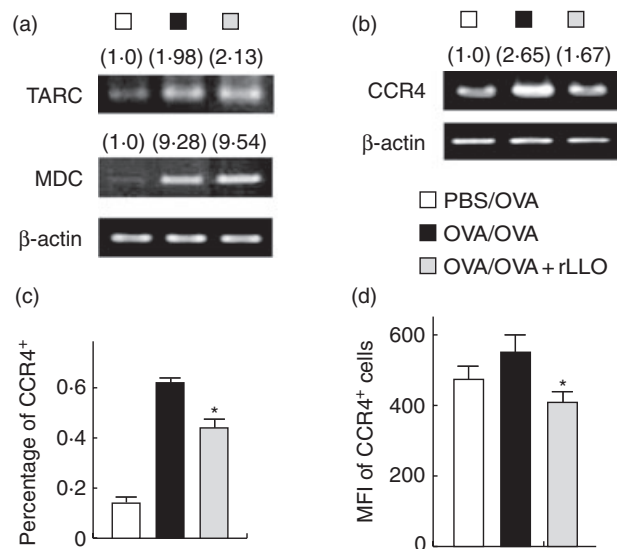


Fig. 4. The effect of rLLO administration on the expression of TARC, MDC and CCR4. BALB/c mice were sensitized and challenged with OVA, with or without rLLO. (a) The nasal cells were collected and the transcriptional level of TARC and MDC was examined by RT-PCR. (b) The transcriptional level of CCR4 in splenic CD4⁺ T cells was examined by RT-PCR. The number in parentheses represents a fold increase, which was determined by densitometric analysis as described in Materials and methods. (c, d) The level of surface expression of CCR4 on splenic CD4⁺ T cells was examined by flow cytometric analysis and data were expressed as (c) the percentage of CCR4⁺ cells in CD4⁺ T cells and (d) mean fluorescence intensity (MFI) of CCR4⁺ cells was also indicated. Data are expressed as the mean $\pm$ SD and are representative of two independent experiments. **P* < 0.05 by Student's *t*-test, compared to OVA/OVA.

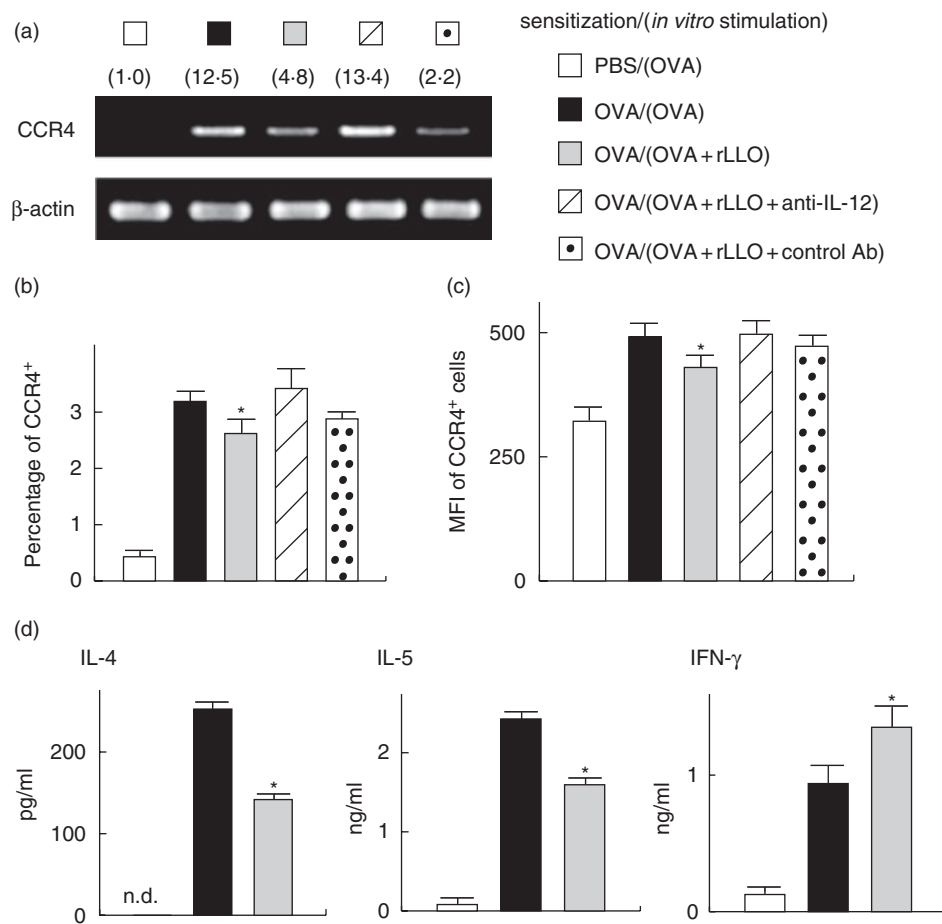


Fig. 5. *In vitro* effect of rLLO on CCR4 expression and OVA-specific cytokine production. Spleen cells from OVA-sensitized mice were stimulated with OVA alone or OVA plus rLLO in the absence or presence of anti-IL-12 antibody or control antibody for 72 h. (a) The transcriptional level of CCR4 in splenic CD4⁺ T cells was examined by RT-PCR. The number in parentheses represents a fold increase, which was determined by densitometric analysis as described in Materials and methods. (b,c) The level of surface expression of CCR4 on splenic CD4⁺ T cells was examined by flow cytometric analysis and data were expressed as (b) the percentage of CCR4⁺ cells in CD4⁺ T cells and (c) mean fluorescence intensity (MFI) of CCR4⁺ cells was also indicated. (d) The level of IL-4, IL-5 and IFN-γ in the culture supernatants was determined by ELISA. Data are expressed as the mean ± SD and are representative of three independent experiments. n.d., not detected. **P* < 0.05 by Student's *t*-test, compared to OVA/(OVA).

alone (data not shown). These results suggest that rLLO is able to inhibit the production of IL-4 and IL-5 from Th2 cells without affecting the development of CD4⁺ T cells *in vitro* as well as rLLO administration *in vivo*. Taken together, it appears that rLLO is capable of modulating several functions of Th2 cells such as CCR4 expression and the production of Th2 cytokines.

Recombinant LLO inhibits PCA reaction

In the present study, we showed that nasal allergic symptoms were remarkably alleviated in mice injected three times with rLLO. However, a similar alleviation was observed in mice given a single injection of rLLO before the last intranasal challenge (data not shown). The result raised a possibility that rLLO was capable of directly inhibiting type I allergic reaction. To test this possibility, we determined the effect of

rLLO on the PCA reaction. The sera containing OVA-specific anaphylactic antibodies were injected into the auricle of naïve mice, and the magnitude of the PCA reaction was measured 30 min after injection of the Evans blue solution containing OVA. We observed a massive extravasation of dye at the auricle (Fig. 6). However, rLLO treatment inhibited the response dramatically (Fig. 6). In addition, although the inhibitory effect on the PCA reaction was induced when rLLO was administered 30 min before injection of Evans blue solution, such an effect was not detected when it was administered 3 h before the injection. Based on these findings, it seems that the inhibitory property of rLLO to type I allergic reaction may be involved in inhibition of the effector phase of allergic rhinitis, independent of IL-12 production and inhibition of Th2 cell recruitment. The precise mechanism of the effect of rLLO on PCA reaction is to be determined in a future study.

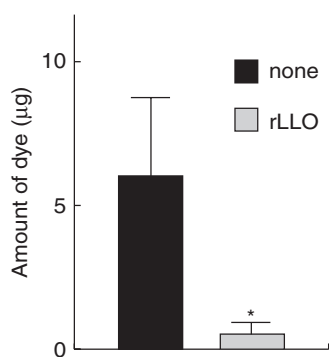


Fig. 6. The effect of rLLO on the PCA reaction at auricle. Twenty microliter of sera pooled from mice immunized with OVA in alum was injected intradermally into the auricle. Twenty-four hours later, 250 µl of Evans blue solution containing OVA was injected intravenously. To test the effect of rLLO on the PCA reaction, mice were injected intraperitoneally with 100 µg of rLLO 30 min before injection of the Evans blue solution. The magnitude of the PCA reaction was determined by measurement of the amount of dye in the auricle as described in Materials and methods ($n = 3$ mice for each group). Data are the mean $\pm$ SD, and are representative of three independent experiments. * $P < 0.05$ by Student's t -test, compared to none (no treatment).

Discussion

Allergic rhinitis is an immune disorder characterized by a high level of antigen-specific IgE production and eosinophil infiltration in a nasal tissue, and is caused by the predominant production of Th2 cytokines such as IL-4, IL-5 and IL-13 [12,14]. Because LLO is able to serve as a Th1 adjuvant by induction of IL-12 and IFN- γ , which interfere with the activity of Th2 cytokines [6,10,11], and suppress allergic response when rLLO is administered during sensitization [8], in the present study, we tested whether rLLO exerts its inhibitory effect on the effector phase of allergic rhinitis. The results clearly showed that rLLO remarkably suppressed nasal allergic symptoms induced upon intranasal challenge with OVA. The level of anti-OVA IgE titre in sera and eosinophil infiltration in nasal mucosa were decreased by administration of rLLO. We further found that administration of rLLO suppressed antigen-specific Th2 cytokine production from nasal mononuclear cells of OVA/OVA mice and decreased the number of CD4⁺ T cells in nasal mucosa. The inhibitory action of rLLO appears to be attributed to inhibition of Th2 cell recruitment to the site of allergic responses.

CD4⁺ T cells are divided into two subsets based on their profiles of cytokine production [32]. In the last decade, some surface molecules including chemokine receptors have been reported to be preferentially expressed on Th1 or Th2 cells [33]. CXCR3 and CCR5 are expressed on Th1 cells, while CCR3, CCR4 and CCR8 are expressed on Th2 cells [24,26,27,33]. Among these chemokine receptors, CCR4 appears to act as a predominant receptor for the recruitment of Th2 cells when antigen was administered repeatedly in a murine model of allergic inflammation [24]. Our results

clearly indicate that administration of rLLO decreased the transcription and cell surface expression of CCR4 on splenic CD4⁺ T cells. It is probable that down-regulation of the receptor by rLLO modulates Th2 cell recruitment to nasal mucosa and is one of mechanisms by which rLLO suppresses allergic responses at the local sites.

In the present study, we showed that rLLO is capable of suppressing the production of Th2 cytokines *in vitro* (Fig. 5d). We believe that administration of rLLO at the effector phase of allergic rhinitis might suppress the production of Th2 cytokines *in vivo* as well. This may be one of the reasons why administration of rLLO suppressed the production of anti-OVA IgE antibody without affecting the development of CD4⁺ T cells in spleen, since IL-4 and IL-13 are the only cytokines known to induce Ig class switching to IgE [34].

IL-4 is known to serve not only as a mast cell growth factor but also as the major mast cell chemoattractant [35,36]. Therefore, it is possible that the decrease of Th2 responses in nasal mucosa modulated the mast cell (IgE)-mediated nasal symptoms, in concert with the decreased production of anti-OVA IgE antibody.

It has been shown that eosinophil recruitment to the site of allergic inflammation involves Th2 cytokines, chemokines (RANTES, eotaxin) and other molecules, and the eotaxin/CCR3 signalling pathway is a fundamental regulator of allergen-induced eosinophilia [37]. As IL-4 and IL-5 are known to cooperate with eotaxin in eosinophil recruitment [37], it is possible that the decreased local production of these Th2 cytokines inhibited eosinophil infiltration in nasal mucosa.

It was reported that IL-12 has the ability to down-regulate the expression level of Th2-type chemoattractant receptors *in vitro* [29–31]. We also found that the down-regulation of CCR4 expression by rLLO was cancelled by anti-IL-12 antibody in *in vitro* experiments. It is likely that administration of rLLO decreased *in vivo* expression of CCR4 via induction of IL-12 production, since LLO, one of the TLR4 ligands is capable of inducing IL-12 from APCs [6]. On the other hand, it was reported that LPS, a representative TLR4 ligand suppressed allergic responses, including the PCA reaction, via induction of nitric oxide synthase 2 (NOS2) and this inhibitory effect was independent of IL-12 because suppression of allergic rhinitis by LPS was detected in IL-12^{-/-} mice [38]. This finding raises the alternative possibility that rLLO may be able to suppress allergic responses independent of IL-12. Actually, we found that rLLO was capable of inhibiting the PCA reaction. The inhibitory effect was observed when rLLO was administered 30 min before injection of the Evans blue solution but not 3 h before the injection. In addition, our preliminary data showed that rLLO is also capable of inhibiting histamine-induced skin reaction, suggesting that rLLO directly or indirectly inhibits the histamine/H1 receptor signalling, and affects vascular endothelial cells to cause a decrease in vascular permeability. In the case of rhinitis induced by OVA, we found that the allergic symptoms were

alleviated by a single injection of rLLO before the last intranasal challenge. These results suggest that rLLO has an additional alternative ability to inhibit the allergic responses independently of the inhibition of Th2 cell recruitment. The precise inhibitory mechanism of rLLO on the PCA reaction is currently under investigation.

In summary, we demonstrated that rLLO was capable of inhibiting Th2 cell response at the effector phase of allergic rhinitis in OVA-sensitized mice, and the inhibitory mechanism was possibly attributed to suppression of Th2 cell recruitment to the nasal mucosa and type I allergic reaction. We believe that the further study from such a viewpoint may provide important informations into the therapy against allergic disorders such as allergic rhinitis, bronchial asthma and atopic dermatitis.

Acknowledgements

This work was supported by Grants from The Ministry of Education, Science, Culture and Sports of Japan and from The Japan Society for the Promotion of Science.

References

- Portnoy DA, Chakraborty T, Goebel W, Cossart P. Molecular determinants of *Listeria monocytogenes* pathogenesis. *Infect Immun* 1992; **60**:1263–7.
- Vazquez-Boland JA, Dominguez-Bernal G, Gonzalez-Zorn B, Kreft J, Goebel W. Pathogenicity islands and virulence evolution in *Listeria*. *Microb Infect* 2001; **3**:571–84.
- Geoffroy C, Gaillard JL, Alouf JE, Berche P. Purification, characterization, and toxicity of the sulfhydryl-activated hemolysin listeriolysin O from *Listeria monocytogenes*. *Infect Immun* 1987; **55**:1641–6.
- Lety MA, Frehel C, Dubail I, Beretti JL, Kayal S, Berche P, Charbit A. Identification of a PEST-like motif in listeriolysin O required for phagosomal escape and for virulence in *Listeria monocytogenes*. *Mol Microbiol* 2001; **39**:1124–39.
- Nishibori T, Xiong H, Kawamura I, Arakawa M, Mitsuyama M. Induction of cytokine gene expression by listeriolysin O and roles of macrophages and NK cells. *Infect Immun* 1996; **64**:3188–95.
- Nomura T, Kawamura I, Tsuchiya K *et al*. Essential role of interleukin-12 (IL-12) and IL-18 for gamma interferon production induced by listeriolysin O in mouse spleen cells. *Infect Immun* 2002; **70**:1049–55.
- Kohda C, Kawamura I, Baba H, Nomura T, Ito Y, Kimoto T, Watanabe I, Mitsuyama M. Dissociated linkage of cytokine-inducing activity and cytotoxicity to different domains of listeriolysin O from *Listeria monocytogenes*. *Infect Immun* 2002; **70**:1334–41.
- Yamamoto K, Kawamura I, Tominaga T, Nomura T, Kohda C, Ito J, Mitsuyama M. Listeriolysin O, a cytolysin derived from *Listeria monocytogenes* inhibits generation of ovalbumin-specific Th2 immune response by skewing maturation of antigen-specific T cells into Th1 cells. *Clin Exp Immunol* 2005; **142**:268–74.
- Park JM, Ng VH, Maeda S, Rest RF, Karin M. Anthrolysin O and other Gram-positive cytolysins are Toll-like receptor 4 agonists. *J Exp Med* 2004; **200**:1647–55.
- Tanabe Y, Xiong H, Nomura T, Arakawa M, Mitsuyama M. Induction of protective T cells against *Listeria monocytogenes* in mice by immunization with a listeriolysin O-negative avirulent strain of bacteria and liposome-encapsulated listeriolysin O. *Infect Immun* 1999; **67**:568–75.
- Xiong H, Tanabe Y, Ohya S, Mitsuyama M. Administration of killed bacteria together with listeriolysin O induces protective immunity against *Listeria monocytogenes* in mice. *Immunology* 1998; **94**:14–21.
- Okano M, Azuma M, Yoshino T *et al*. Differential role of CD80 and CD86 molecules in the induction and the effector phases of allergic rhinitis in mice. *Am J Respir Crit Care Med* 2001; **164**:1501–7.
- McCusker C, Chicoine M, Hamid Q, Mazer B. Site-specific sensitization in a murine model of allergic rhinitis: role of the upper airway disease in lower airway disease. *J Allergy Clin Immunol* 2002; **110**:891–8.
- Romagnani S. Immunologic influences on allergy and the T_H1/T_H2 balance. *J Allergy Clin Immunol* 2004; **113**:395–400.
- Major Jr T, Wohlleben G, Reibetanz B, Erb KJ. Application of heat killed *Mycobacterium bovis*-BCG into the lung inhibits the development of allergen-induced Th2 responses. *Vaccine* 2002; **20**:1532–40.
- Hattori H, Okano M, Yamamoto T *et al*. Intranasal application of purified protein derivative suppresses the initiation but not the exacerbation of allergic rhinitis in mice. *Clin Exp Allergy* 2002; **32**:951–9.
- Jain VV, Kitagaki K, Kline JN. CpG DNA and immunotherapy of allergic airway diseases. *Clin Exp Allergy* 2003; **33**:1330–5.
- Schuerch DW, Wilson-Kubalek EM, Tweten RK. Molecular basis of listeriolysin O pH dependence. *Proc Natl Acad Sci USA* 2005; **102**:12537–42.
- Saito H, Howie K, Wattie J, Denburg A, Ellis R. Allergen-induced murine upper airway inflammation: local and systemic changes in murine experimental allergic rhinitis. *Immunology* 2001; **104**:226–34.
- Kline JN, Kitagaki K, Businga TR, Jain VV. Treatment of established asthma in a murine model using CpG oligodeoxynucleotides. *Am J Physiol Lung Cell Mol Physiol* 2002; **283**:L170–9.
- Asanuma H, Inaba Y, Aizawa C, Kurata T, Tamura S. Characterization of mouse nasal lymphocytes isolated by enzymatic extraction with collagenase. *J Immunol Methods* 1995; **187**:41–51.
- Lieberman I, Forster I. The murine beta-chemokine TARC is expressed by subsets of dendritic cells and attracts primed CD4⁺ T cells. *Eur J Immunol* 1999; **29**:2684–94.
- Schanuel C, Pardali E, Sallusto F *et al*. Activated murine B lymphocytes and dendritic cells produce a novel CC chemokine which acts selectively on activated T cells. *J Exp Med* 1998; **188**:451–63.
- Lloyd CM, Delaney T, Nguyen T, Tian J, Martinez-A C, Coyle AJ, Gutierrez-Ramos JC. CC chemokine receptor (CCR) 3/eotaxin is followed by CCR4/monocyte-derived chemokine in mediating pulmonary T helper lymphocyte type 2 recruitment after serial antigen challenge in vivo. *J Exp Med* 2000; **191**:265–73.
- Katayama S, Shionoya H, Ohtake S. A new method for extraction of extravasated dye in the skin and the influence of fasting stress on passive cutaneous anaphylaxis in guinea pigs and rats. *Microbiol Immunol* 1978; **22**:89–101.

- 26 D'Ambrosio D, Iellem A, Bonecchi R, Mazzeo D, Sozzani S, Mantovani A, Sinigaglia F. Cutting edge. selective up-regulation of chemokine receptors CCR4 and CCR8 upon activation of polarized human type 2 Th cells. *J Immunol* 1998; **161**:5111–5.
- 27 Imai T, Nagira M, Takagi S *et al.* Selective recruitment of CCR4-bearing Th₂ cells toward antigen-presenting cells by the CC chemokines thymus and activation-regulated chemokine and macrophage-derived chemokine. *Int Immunol* 1999; **11**:81–8.
- 28 Hirai H, Tanaka K, Yoshie O *et al.* Prostaglandin D₂ selectively induces chemotaxis in T helper type 2 cells, eosinophils, and basophils via seven-transmembrane receptor CRTH2. *J Exp Med* 2001; **193**:255–61.
- 29 Nagata K, Tanaka K, Ogawa K *et al.* Selective expression of a novel surface molecule by human Th2 cells in vivo. *J Immunol* 1999; **162**:1278–86.
- 30 Kim CH, Nagata K, Butcher EC. Dendritic cells support sequential reprogramming of chemoattractant receptor profiles during naïve to effector T cell differentiation. *J Immunol* 2003; **171**:152–8.
- 31 Annunziato F, Cosmi L, Manetti R, Brugnolo F, Parronchi P, Maggi E, Nagata K, Romagnani S. Reversal of human allergen-specific CRTH2⁺Th₂ cells by IL-12 or the PS-DSP30 oligodeoxynucleotide. *J Allergy Clin Immunol* 2001; **108**:815–21.
- 32 Mosmann TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol Today* 1996; **17**:138–46.
- 33 Bonecchi R, Bianchi G, Bordignon PP *et al.* Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. *J Exp Med* 1998; **187**:129–34.
- 34 Fish SC, Donaldson DD, Goldman SJ, Williams CM, Kasaian MT. IgE generation and mast cell effector function in mice deficient in IL-4 and IL-13. *J Immunol* 2005; **174**:7716–24.
- 35 Ikeda RK, Miller M, Nayar J *et al.* Accumulation of peribronchial mast cells in a mouse model of ovalbumin allergen induced chronic airway inflammation: modulation by immunostimulatory DNA sequences. *J Immunol* 2003; **171**:4860–7.
- 36 Olsson N, Taub DD, Nilsson G. Regulation of mast cell migration by T and T cytokines: identification of tumor necrosis factor- α and interleukin-4 as mast cell chemotaxis. *Scand J Immunol* 2004; **59**:267–72.
- 37 Pope SM, Zimmermann N, Stringer KF, Karow ML, Rothenberg ME. The eotaxin chemokines and CCR3 are fundamental regulators of allergen-induced pulmonary eosinophilia. *J Immunol* 2005; **175**:5341–50.
- 38 Rodriguez D, Keller AC, Faquim-Mauro EL, de Macedo MS, Cunha FQ, Lefort J, Vargaftig BB, Russo M. Bacterial lipopolysaccharide signaling through Toll-like receptor 4 suppresses asthma-like responses via nitric oxide synthase 2 activity. *J Immunol* 2003; **171**:1001–8.