

# 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

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## Summary

Listeriolysin O (LLO), a cholesterol-dependent cytolysin derived from *Listeria monocytogenes*, is a potent inducer of interleukin (IL)-12, IL-18 and interferon (IFN)- $\gamma$ . We have shown that LLO facilitates development of T cells mediating protective immunity against *L. monocytogenes* through the induction of IFN- $\gamma$  production at an early stage. Based on this finding, it is postulated that LLO inhibits differentiation of Th2 cells and the Th2 immune response. By using a murine model of ovalbumin (OVA)-induced allergic rhinitis, we investigated whether LLO has an ability to modulate the Th2-type immune disorder. In mice sensitized intraperitoneally with ovalbumin (OVA)/alum and challenged intranasally with OVA, a large number of eosinophils migrated into the nasal tissue, and high titres of anti-OVA IgE and IgG<sub>1</sub> antibodies were detected in sera. However, LLO treatment during sensitization markedly inhibited the eosinophil infiltration and production of these anti-OVA antibodies. A large number of T cells from mice sensitized and challenged with OVA produced high level of IL-4 and IL-5 but not IFN- $\gamma$  after stimulation with OVA. In contrast, OVA-specific IFN- $\gamma$ -producing T cells were preferentially induced in mice treated with LLO at the time of sensitization. In the absence of LLO administration, the expression level of GATA-3 and SOCS-3 in CD4<sup>+</sup> T cells was enhanced after sensitization with OVA. LLO treatment resulted in a reduction of GATA-3 and SOCS-3 expressions but induced the transcription of T-bet instead. Taken together, these data show clearly that LLO is capable of inhibiting Th2 immune response by skewing differentiation of antigen-specific T cells into Th1 cells.

**Keywords:** allergic rhinitis, GATA-3, listeriolysin O, SOCS-3, T-bet

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## Introduction

*Listeria monocytogenes* is a Gram-positive, facultative intracellular bacterium that causes sporadic and serious listeriosis in humans [1]. The major virulence genes (*plcA*, *hly*, *mpl*, *actA* and *plcB*) of *L. monocytogenes* have been identified [2] and the orchestrated action of these gene products is essential for evasion from the intracellular bactericidal mechanism and replication inside macrophages [3]. Among the virulence factors, listeriolysin O (LLO), a 58-kDa protein encoded by *hly*, is a member of a large family of cholesterol-dependent cytolysins (CDCs) [4], and contributes to intracellular multiplication by disruption of the phagosomal membrane and enabling this bacterium to escape into cytosol [5]. 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 (TNF)- $\alpha$ , IL-12, IL-18 and interferon (IFN)- $\gamma$  [6–8]. Administration of LLO promoted the development of protective immunity against a lethal dose of *L. monocytogenes* infection by inducing endogenous IFN- $\gamma$  production [9]. Because LLO-deficient *L. monocytogenes* never multiplied in macrophages or induced IFN- $\gamma$  production, it is certain that LLO exhibits critical roles for not only virulence expression in bacteria but also for the generation of protective immunity in an infected host [10].

Allergic rhinitis, including pollinosis, is a representative of Th2-type immune disorders, and is characterized by a high level of antigen-specific IgE production and eosinophil infiltration in nasal tissue where the antigen is trapped. Approx-

imately 15–20% of the population suffer from allergic rhinitis [11]. As the enhanced production of IgE and IgG<sub>1</sub> antibodies and inflammatory response are due to the predominant production of Th2 cytokines such as IL-4, IL-5 and IL-13, allergic symptoms can be alleviated by the inhibition of Th2 cytokine responses. As LLO is highly potent in the induction of Th1 cytokines *in vitro* and *in vivo*, we postulated that LLO is also capable of modulating Th2 immune response. Based on this assumption, we have examined the *in vivo* effect of recombinant LLO protein in mice on the course of allergic rhinitis induced by ovalbumin (OVA).

## 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.

### Generation and purification of recombinant LLO

Recombinant LLO was generated in *Escherichia coli* as His-tagged protein and purified as described previously [12]. The contaminating lipopolysaccharide (LPS) was removed to a level as low as 0.6 pg per µg protein by Detoxi-Gel endotoxin-removing gel column (Pierce Chemical Company, Rockford, IL, USA). Purified recombinant LLO exerted a high level of cytotoxic activity to mammalian cells, so we incubated LLO in phosphate buffered saline (PBS) (pH 8.0) for 2 h at 37°C according to a report showing the neutralization of cytotoxicity with this method [13]. Pretreated LLO at a concentration lower than 300 µg/ml did not exhibit cytotoxicity such as haemolytic activity, which was measured by haemoglobin release from sheep erythrocytes [8,12] (data not shown).

### IFN-γ production from whole spleen cells in response to LLO

Spleen was removed aseptically from naive BALB/c mice, and whole spleen cells were suspended at  $5 \times 10^6$  cells/ml in RPMI-1640 medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco-BRL), 50 µg/ml gentamicin (Wako Chemicals, USA, Richmond, VA, USA) and  $5 \times 10^{-5}$  M 2-mercaptoethanol (Nacalai Tesque, Kyoto, Japan), and stimulated with 10 µg/ml recombinant LLO in the absence or presence of 2 µg/ml of goat anti-mouse IL-12p70 antibody (Genzyme/Teche, Minneapolis, MN, USA), rat anti-mouse IL-18 antibody (Medical & Biological Laboratories, Nagoya, Japan) or control antibodies (normal goat IgG and normal rat IgG;

Genzyme/Teche) for 24 h at 37°C in 5% CO<sub>2</sub>. In some experiments, natural killer (NK) cells were depleted by using anti-NK (DX5) Microbeads and MACS system (Miltenyi Biotec, Gladbach, Germany) and the resultant cell population was stimulated with LLO. The level of IFN-γ in culture supernatants was determined by enzyme-linked immunosorbent assay (ELISA), as described previously [8].

### Induction of allergic rhinitis by sensitization and challenge of mice with OVA

BALB/c mice were sensitized by intraperitoneal injection with 400 µl PBS containing 10 µg of OVA (Nacalai Tesque) adsorbed to 2 mg of alum (Pierce Chemical Company) (OVA/alum) with or without 100 µg of LLO (250 µg/ml) on day 1 and day 8. Allergic rhinitis was elicited by intranasal challenge with 10 µl PBS containing 100 µg of OVA for 7 days from day 15. The control group mice were injected with alum alone, and PBS was administered intranasally in the same schedule. In some experiments, mice were administered intraperitoneally with 10 µg of rabbit anti-mouse IFN-γ polyclonal antibody (a gift from Daiichi Pharmaceutical Co., Tokyo, Japan) or normal rabbit IgG (Vector Laboratories, Burlingame, CA, USA) on days 1, 4, 8 and 11.

The severity of allergic rhinitis was determined by frequency of sneezing and nose-scratching for 10 min after the last intranasal challenge with OVA, as described previously [14].

### Detection of anti-OVA antibody

Sera were collected 1 day after the last intranasal challenge of OVA. Anti-OVA IgE and IgG<sub>1</sub> antibodies were measured by ELISA as described previously [15]. To determine the titre of anti-OVA antibodies, serially diluted serum samples were added to Nunc immunoplate wells (Nalge Nunc Int., Tokyo, Japan) that had been coated with OVA. The titre of anti-OVA antibodies was defined as the reciprocal of serum dilution that gave a twofold higher OD value than that of the control group.

### Histological examination

Nasal tissue was removed 1 day after the last challenge with OVA, and fixed in 10% formalin and decalcified. Coronal nasal section was stained with haematoxylin and eosin, and the number of eosinophils was counted under a microscope at four fields of submucosal region of nasal septum with  $10 \times 40$  magnification [16]. Eosinophils were defined morphologically by the existence of eosinophilic granules in the cytoplasm and two-lobed nucleus.

### Cytokine production after stimulation with OVA

Spleen was removed aseptically 1 day after the last intranasal challenge. CD4<sup>+</sup> T cells were enriched by using the anti-CD4

(L3T4) Microbeads and MACS system, suspended at  $5 \times 10^6$  cells/ml and stimulated with 100 µg/ml of OVA in the presence of peritoneal macrophages as antigen-presenting cells for 72 h at 37°C in 5% CO<sub>2</sub>. The level of IFN-γ in culture supernatants was determined by ELISA. The levels of IL-4 and IL-5 were determined by using OptEIA mouse ELISA set specifically for IL-4 and IL-5 (Pharmingen), according to the manufacturer's protocol.

#### Detection of IFN-γ and IL-5-producing cells by enzyme-linked immunospot (ELISPOT) assay

Whole spleen cells were stimulated with 100 µg/ml of OVA for 48 h. To enumerate the antigen-specific IFN-γ-producing cells, graded numbers of collected viable cells were plated in wells of Multiscreen Immobilon-P 96-well Filtration Plates (Millipore, Bedford, MA, USA) that had been coated with rat anti-mouse IFN-γ monoclonal antibody (mAb) (XMG1.2; Endogen, Woburn, MA, USA), and cultured for 24 h. Wells were washed thoroughly with PBS containing 0.05% Tween 20, and incubated with rabbit anti-mouse IFN-γ polyclonal antibody (a gift from Daiichi Pharmaceutical Co., Tokyo, Japan) for 90 min. After several washes, horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Zymed Laboratories Inc., San Francisco, CA, USA) was added and plates were incubated for 90 min. The spots representing IFN-γ-producing cells were developed by the addition of 0.27 mg/ml 3-amino-9-ethylcarbazole (Sigma, St Louis, MO, USA) solution containing 0.03% H<sub>2</sub>O<sub>2</sub>. Detection of the antigen-specific IL-5-producing cells was performed with reagents of OptEIA mouse IL-5 ELISA.

#### RNA isolation and real-time reverse transcription polymerase chain reaction (RT-PCR) analysis

Mice were injected intraperitoneally with 10 µg of OVA/alum, 10 µg of OVA/alum plus 100 µg of LLO, 100 µg of LLO or PBS. Six hours after the injection, total RNA was extracted from whole spleen cells or CD4<sup>+</sup> T cells by using NucleoSpin RNA II (Macherey Nagel, Duren, Germany) and cDNA was reverse-transcribed using a random primer (Invitrogen, Carlsbad, CA, USA). The levels of IFN-γ, T-bet (T-box expressed in T cells), GATA-3 (GATA binding protein 3), SOCS-3 (suppressor of cytokine signalling-3) and β-actin mRNA expressions were determined by real-time PCR using an ABI PRISM 7000 KS-11 Thermal Cycler (Applied Biosystems) and qPCR Core Kit for SYBR Green I (Eurogentec, Seraing, Belgium). The levels of these mRNA expressions were analysed by ABI Prism 7000 Sequence Detection System (version 1.0). Data were normalized against β-actin and expressed as an index of each transcription factor mRNA/β-actin mRNA. The primers used in this study are as follows [8,17,18]: 5'-AGCGGCTGACTGAAGTCAGATTGTAG-3' and 5'-GTCACAGTTTTCAGCTGTATAGGG-3' for IFN-γ, 5'-CAACAACCCCTTTGCCAAAG-3' and 5'-TCCCCCAA

GCAGTTGACAGT-3' for T-bet, 5'-AGAACCGGCCCCCTATCAA-3' and 5'-AGTTCGCGCAGGATGTCC-3' for GATA-3, 5'-GAACCTACGCATCCAGTGTGAG-3' and 5'-CGTTGACAGTCTTCCGACAAAG-3' for SOCS-3, and 5'-TGGAATCCTGTGGCATCCATGAAAC-3' and 5'-TAAAA CGCAGCTCAGTAACAGTCCG-3' for β-actin.

#### Statistical analysis

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

## Results

### Induction of IFN-γ by LLO

We have reported previously that native LLO had an ability to induce IFN-γ production from NK cells via the induction of both IL-12 and IL-18 from macrophages in spleens of C3H/HeN and C57BL/6 mice [8]. In this experiment, we first determined whether recombinant LLO induces IFN-γ production from spleen cells of BALB/c mice. A high level of IFN-γ production was observed when whole spleen cells were stimulated with recombinant LLO (Fig. 1a). The IFN-γ production was inhibited completely by the addition of anti-IL-12 and anti-IL-18 antibodies, while control antibodies did not affect cytokine production. LLO-induced IFN-γ production was also inhibited by the depletion of NK cells. The result was consistent with our previous study [8]. Moreover,

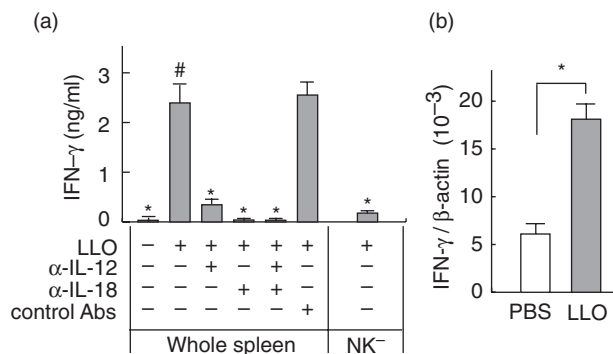


**Fig. 1.** Interferon (IFN)-γ-inducing activity of listeriolysin O (LLO). (a) Whole or natural killer (NK)-depleted spleen cells were stimulated with 10 µg/ml LLO in the absence or presence of an antibody specific for interleukin (IL)-12 or IL-18. The level of IFN-γ in culture supernatants was determined by enzyme-linked immunosorbent assay (ELISA). Statistical analysis was performed compared to whole spleen cells stimulated with LLO alone (#). (b) BALB/c mice were injected intraperitoneally with phosphate buffered saline (PBS) or LLO. Six hours after injection, total RNA was extracted from whole spleen cells, and quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed. Data represent the mean ± s.d. of triplicate assays, and are representative of three independent experiments. \**P* < 0.05, by Student's *t*-test.

we found that LLO promoted the expression of IFN- $\gamma$  mRNA in spleen after intraperitoneal injection (Fig. 1b).

### Effect of LLO on allergic rhinitis

We have shown that LLO promotes generation of Th1 cells that are able to control *L. monocytogenes* infection [9] and that induction of Th1 cells is dependent on the activity of LLO to induce endogenous IFN- $\gamma$  production [7,10]. Because IFN- $\gamma$  is known to promote differentiation of Th1 cells but inhibit generation of Th2 cells, it is conceivable that LLO may affect the Th2-mediated inflammatory response. Therefore, we investigated in this study whether LLO inhibits the generation of OVA-induced allergic rhinitis. Mice were sensitized and challenged with OVA, and monitored for frequency of sneezing and nose-scratching to score the degree of allergic rhinitis. The counts of sneezing and nose-scratching were higher in mice sensitized and challenged with OVA compared to mice from the control group (Fig. 2a). We further found that a significant number of eosinophils migrated

into the nasal tissue of mice sensitized and challenged with OVA (Fig. 2b). On the other hand, no histological changes were seen in the lungs (data not shown). In addition, high titres of anti-OVA IgE antibody were detected in the sera of mice sensitized and challenged with OVA (Fig. 2c). Similarly, the serum level of IgG<sub>1</sub>, which is known to be a Th2-associated subclass of IgG, increased markedly (Fig. 2d). These results indicated that an experimental allergic rhinitis was elicited in mice sensitized and challenged with OVA in our procedure. Conversely, local symptoms were markedly reduced in mice given LLO during sensitization with OVA (Fig. 2a), and the infiltration of cells was also decreased significantly in mice with LLO treatment (Fig. 2b). In addition, the level of anti-OVA IgE and IgG<sub>1</sub> antibodies remained at a low level in mice with LLO treatment (Figs 2c,d). These results indicated that LLO is highly capable of inhibiting the development of allergic status induced by OVA in mice.

### Effect of LLO on differentiation of Th cells in mice sensitized and challenged with OVA

Based on the above results, it was likely that LLO inhibited the generation of Th2 cells in mice sensitized and challenged with OVA. We next examined OVA-specific cytokine production from splenic CD4<sup>+</sup> T cells to elucidate whether LLO affected the development of OVA-specific Th2 cells. A high level of OVA-specific IL-4 (Fig. 3a) and IL-5 (Fig. 3b) production was observed in splenic CD4<sup>+</sup> T cells of mice sensitized and challenged with OVA, whereas the level of OVA-specific IFN- $\gamma$  production from the cells remained at a low level (Fig. 3c). In contrast, splenic CD4<sup>+</sup> T cells of mice sensitized with OVA along with LLO produced a large amount of IFN- $\gamma$  in response to OVA, but the level of OVA-specific IL-4 and IL-5 production was decreased considerably (Fig. 3a–c). OVA-specific cytokine production from the splenic CD4 negative cell population was at the level of the control group, and it was also shown by FACS analysis that LLO treatment did not influence the proportions of CD4<sup>+</sup> or CD8<sup>+</sup> cells in spleen (data not shown). Moreover, the effect of LLO was completely inhibited by the administration of anti-IFN- $\gamma$  antibody (Fig. 3a–c).

To confirm that the distinct profile of the cytokine-producing pattern was due to the difference in the proportional development of OVA-specific Th1 or Th2 cells, we performed an ELISPOT assay to enumerate the OVA-specific IFN- $\gamma$ - and IL-5-producing T cells (Fig. 3d). A small number of IFN- $\gamma$ -producing T cells and a large number of IL-5-producing T cells were detected in spleen cells of mice sensitized and challenged with OVA. In contrast, significantly higher numbers of IFN- $\gamma$ -producing T cells were induced in mice treated with LLO, while the number of IL-5-producing T cells was markedly reduced. These results indicated that administration of LLO during sensitization promotes differentiation of OVA-specific T cells into Th1 cells and inhibits generation of Th2 cells.

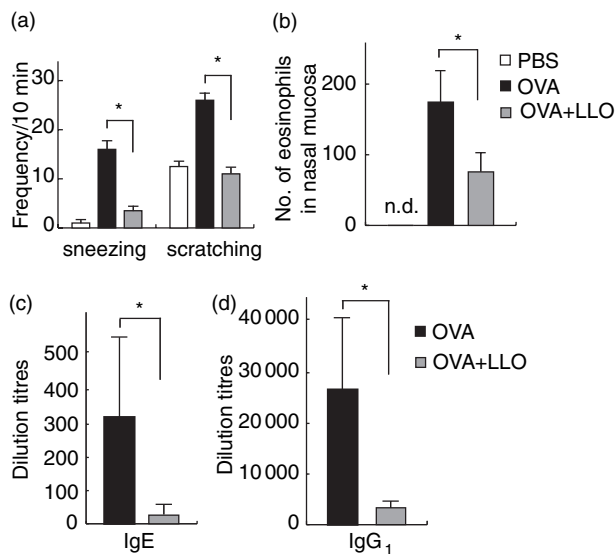


**Fig. 2.** Listeriolysin O (LLO) markedly inhibits allergic rhinitis. Mice were sensitized with ovalbumin (OVA)/alum with or without LLO, and challenged intranasally with OVA. (a) Frequency of sneezing and nose-scratching was monitored for 10 min after the last intranasal challenge. (b) One day after the last challenge, coronal nasal section was stained with haematoxylin and eosin, and the number of eosinophils in nasal septum was counted. (c) One day after the last challenge, sera were collected. The sera were added to enzyme-linked immunosorbent assay (ELISA) plates that had been coated with OVA, and the titre of anti-OVA IgE antibody was detected by using antimouse IgE antibody. (d) The titre of anti-OVA IgG<sub>1</sub> antibody in the sera was measured by using OVA-coated ELISA plate and antimouse IgG<sub>1</sub> antibody. These experiments were performed at  $n = 4$ –6 for each group. Data represent the mean  $\pm$  s.d., and are representative of three independent experiments. n.d., not detected. \* $P < 0.05$ , by Mann–Whitney's  $U$ -test.

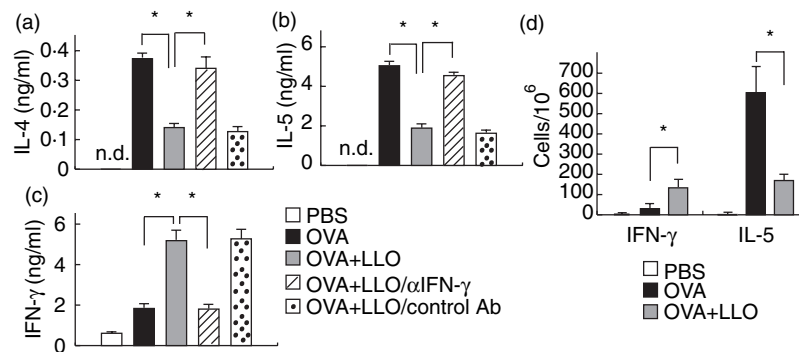


**Fig. 3.** Listeriolysin O (LLO) enhances antigen-specific Th1 response but reduces Th2 response. Mice were sensitized with ovalbumin (OVA)/alum with or without LLO and challenged intranasally with OVA. In one group of mice, anti-interferon (IFN)- $\gamma$  antibody or control antibody was administered during sensitization and mice were challenged with OVA. Splenic CD4<sup>+</sup> T cells were incubated with OVA 100  $\mu$ g/ml for 72 h, and concentrations of interleukin (IL)-4 (a), IL-5 (b) and IFN- $\gamma$  (c) in culture supernatants was determined by enzyme-linked immunosorbent assay (ELISA). (d) Whole spleen cells were incubated with OVA 100  $\mu$ g/ml for 48 h, and IFN- $\gamma$ - and IL-5-producing cells were enumerated by enzyme-linked immunospot (ELISPOT) assay. Data represent the mean  $\pm$  s.d. of triplicate assays and are representative of three independent experiments. n.d., not detected. \* $P$  < 0.05, by Student's *t*-test.

#### Effect of LLO on the expression of T-bet, GATA-3 and SOCS-3 mRNAs in CD4<sup>+</sup> T cells during sensitization with OVA

It has been shown that the transcription factors T-bet and GATA-3 play a critical role for differentiation of T cells into Th1 and Th2, respectively [19,20]. Also, SOCS-3 is known to contribute to the development of Th2 cells [21]. Because LLO has been shown to promote differentiation of Th1 cells by induction of IFN- $\gamma$  production [9] and IFN- $\gamma$  is known to enhance T-bet expression [22], there was a possibility that LLO modulates expression of these transcription factors during sensitization with OVA and skews the differentiation of Th cells into Th1 cells.

We determined the effect of LLO on the expression of T-bet, GATA-3 and SOCS-3 mRNAs in mice injected with OVA/alum. After injection of OVA/alum, the level of T-bet expression decreased below the control level (Fig. 4a) but the level of GATA-3 and SOCS-3 expressions was increased significantly (Fig. 4b,c). In contrast, administration of LLO resulted in the induction of T-bet expression (Fig. 4a) and inhibited the OVA/alum-induced GATA-3 and SOCS-3 expression (Fig. 4b,c). It appears that the alteration in the expression of some essential transcription factors promoted the development of OVA-specific Th1 cells and inhibited generation of Th2 cells, resulting in inhibition of allergic rhinitis.

#### Discussion

It has been demonstrated that LLO is the major virulence factor and is essential for the escape of *L. monocytogenes* from phagosome to cytosol in host macrophages [5]. In a series of recent studies, we have found that LLO is highly capable of inducing various cytokines, including IFN- $\gamma$ , IL-

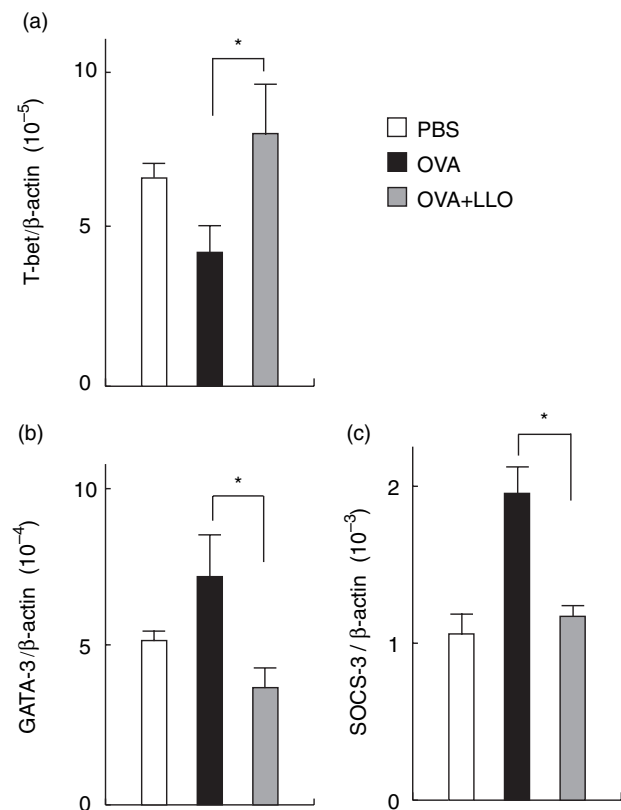


**Fig. 4.** Listeriolysin O (LLO) induces expressions of T-bet but reduces GATA-3 and SOCS-3 expressions. BALB/c mice were injected intraperitoneally with phosphate buffered saline (PBS), ovalbumin (OVA)/alum, or OVA/alum + LLO. Six hours after injection, total RNA was extracted from splenic CD4<sup>+</sup> T cells, and transcriptional levels of T-bet (a), GATA-3 (b) and SOCS-3 (c) were analysed by quantitative real-time transcription-polymerase chain reaction (RT-PCR). Data represent the mean  $\pm$  s.d. of triplicate assays, and are representative of three independent experiments. \* $P$  < 0.05, by Student's *t*-test.

12 and IL-18, and this novel activity is independent of the well-known cytotoxic activity of LLO protein [6–8,12]. IFN- $\gamma$  and IL-12 promote the differentiation of Th1 cells but inhibit the generation of Th2 cells. In our previous study using purified LLO, we found that LLO exhibited a very potent adjuvant activity in the induction of protective Th1 cells against *L. monocytogenes* [9]. These observations prompted us to investigate the beneficial effect of LLO on Th2-mediated immune disorder. In this study, we have shown that LLO is able to affect the OVA-specific Th2 cell generation by skewing differentiation of Th cells into Th1 cells, resulting in the inhibition of allergic rhinitis. In addition, OVA-specific IL-4 and IL-5 production was notably detected in nasal mononuclear cells in mice sensitized and challenged with OVA, whereas Th2 cytokine production was decreased significantly in the cells of mice treated with LLO (data not shown). On the other hand, no significant cytokine production was detected in bronchoalveolar lavage fluids (data not shown). Intranasal administration with a low volume of OVA may not influence the lungs in our murine model, as reported previously [14].

It has been shown clearly that the development of Th1 and Th2 cells is regulated primarily by expression of transcription factors, T-bet and GATA-3, respectively [23], and the expression of T-bet in naive CD4<sup>+</sup> T cells is induced by IFN- $\gamma$  [22]. It was also reported that T-bet expression was induced in monocytes, macrophages, dendritic cells and B cells, in addition to T cells and NK cells [22,24], and T-bet expression in B cells resulted in the inhibition of Ig class-switching to IgG<sub>1</sub> and IgG<sub>2</sub> [24].

Our previous studies have shown that not only LLO used in this study but also seeligeriolysin O (LSO), a protein toxin allied to LLO, possess a profound IFN- $\gamma$ -inducing activity [25]. These toxins induced IFN- $\gamma$  production from NK cells via the induction of both IL-12 and IL-18 from macrophages [8,25]. Our preliminary studies have shown that LSO as well as LLO have an ability to induce T-bet expression in CD4<sup>+</sup> T cells and the expression was inhibited substantially by anti-IFN- $\gamma$  antibodies. The result implied that endogenous IFN- $\gamma$  induced by LSO had a major role in the induction of T-bet transcription in CD4<sup>+</sup> T cells. Because the administration of LLO also induced IFN- $\gamma$  expression during the early period after injection and anti-IFN- $\gamma$  antibody cancelled the inhibitory activity of LLO, it seems that the expression of T-bet in CD4<sup>+</sup> T cells was enhanced by endogenous IFN- $\gamma$  induced after injection with LLO.

GATA-3 is a transcription factor specific for Th2 cells and mediates chromatin remodelling of the IL-4/IL-13 locus [26], an essential step in Th2 differentiation. It is reported that GATA-3 suppresses Th1 development by down-regulation of STAT4, which is an important signal transducer for IL-12 receptor [17]. SOCS-3 belongs to a family of molecules that act as negative regulators for cytokine signalling and its expression is also known to result in a reduction of Th1 differentiation as a consequence of inhibition of IL-

12R/STAT4 signalling [21]. Our results showed that LLO was capable of reducing GATA-3 and SOCS-3 expressions in CD4<sup>+</sup> T cells at the early stage after immunization with OVA/alum. Because T-bet is capable of repressing both IL-4 and IL-5 expression [19] and transcription of GATA-3 and SOCS-3 is induced by IL-4 [21,26], it is probable that the early expression of T-bet induced by LLO inhibited IL-4 expression, resulting in a simultaneous decrease in expression of GATA-3 and SOCS-3.

It has been reported that intracellular bacteria, including *Mycobacterium bovis* BCG [27], *M. vaccae* [28] and *L. monocytogenes* [29], preferentially induce Th1 immune response after infection and suppress generation of Th2-type immune response. Although some bacterial components such as CpG-oligodeoxynucleotides are reported to modulate the immune response via the Toll-like receptor (TLR)-mediated pathway, particularly into Th1 dominance [12,13], some TLR ligands such as peptidoglycan (PGN) are reported to promote a Th2 response [30]. These bacterial components, CpG-ODN and PGN, are ligands for TLR9 and TLR2, respectively. Furthermore, lipopolysaccharide (LPS), a representative TLR4 ligand, also has the ability to modulate the host immune response [31]. Recently, LLO has been shown to be a TLR4 ligand [32]. Because TLR signals are shown to play an important role in the innate immune response, it is likely that interaction of TLR with the ligand gives a critical signal to polarize differentiation of Th cells. The molecular basis for the preferential induction of Th1 response by *L. monocytogenes* has not yet been elucidated; however, it can be argued that LLO, which stimulates the cytokine production via the TLR molecule, plays an essential role in the induction of Th1-type immune response.

In conclusion, we have shown that LLO inhibited the generation of OVA-specific Th2 cells by skewing differentiation of Th cells into Th1 cells, and IFN- $\gamma$  induced by LLO enhanced T-bet expression and reduced GATA-3 and SOCS-3 expression, resulting in the inhibition of allergic rhinitis. It is possible that the ability of LLO is due mainly to its IFN- $\gamma$ -inducing activity. In addition, our preliminary study showed that LLO alleviated the symptoms of allergic rhinitis by administration to OVA-sensitized mice, suggesting that LLO has an ability to inhibit the expression phase of allergic response. We believe that LLO is a potent inducer of Th1 cytokines and may be a candidate for immunomodulator on Th2-type immune disorder.

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## References

- Low JC, Donachie W. A review of *Listeria monocytogenes* and listeriosis. *Vet J* 1997; **153**:9–29.
- 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.
- Alouf JE. Introduction of the family of the structurally-related cholesterol-binding cytolysins (sulphydryl-activated toxins). In: Alouf JE, Freer JH, eds. *The comprehensive sourcebook of bacterial toxins*, 2nd edn. London: Academic Press, 1999:443–56.
- Lety M-A, Frehel C, Dubail I, Beretti J-L, 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.
- Yoshikawa H, Kawamura I, Fujita M, Tsukada H, Arakawa M, Mitsuyama M. Membrane damage and interleukin-1 production in murine macrophages exposed to listeriolysin O. *Infect Immun* 1993; **61**:1334–9.
- 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.
- 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, Kawamura I, Nishibori T, Mitsuyama M. Cytokine gene expression in mice at an early stage of infection with various strains of *Listeria* spp. differing in virulence. *Infect Immun* 1994; **62**:3649–54.
- Okano M, Nishizaki K, Nakada M *et al.* Prevalence and prediction of allergic rhinitis using questionnaire and nasal smear examination in schoolchildren. *Acta Otolaryngol (Suppl)* 1999; **540**:58–63.
- Kohda C, Kawamura I, Baba H *et al.* Dissociated linkage of cytokine-inducing activity and cytotoxicity to different domains of listeriolysin O from *Listeria monocytogenes*. *Infect Immun* 2002; **70**:1334–41.
- Beauregard KE, Lee K-D, Collier RJ, Swanson JA. pH-dependent perforation of macrophage phagosomes by listeriolysin O from *Listeria monocytogenes*. *J Exp Med* 1997; **186**:1159–63.
- 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.
- Okano M, Nishizaki K, Abe M *et al.* Strain-dependent induction of allergic rhinitis without adjuvant in mice. *Allergy* 1999; **54**:593–601.
- Usui T, Nishikomori R, Kitani A, Strober W. GATA-3 suppresses Th1 development by downregulation of Stat4 and not through effects on IL-12R $\beta$ 2 chain or T-bet. *Immunity* 2003; **18**:415–28.
- Fox SW, Haque SJ, Lovibond AC, Chambers TJ. The possible role of TGF- $\beta$ -induced suppressors of cytokine signaling expression in osteoclast/macrophage lineage commitment *in vitro*. *J Immunol* 2003; **170**:3679–87.
- Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 2000; **100**:655–69.
- Zheng W, Flavell RA. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell* 1997; **89**:587–96.
- Seki Y, Inoue H, Nagata N *et al.* SOCS-3 regulates onset and maintenance of T<sub>H</sub>2-mediated allergic responses. *Nat Med* 2003; **9**:1047–54.
- Lighvani AA, Frucht DM, Jankovic D *et al.* T-bet is rapidly induced by interferon- $\gamma$  in lymphoid and myeloid cells. *PNAS* 2001; **98**:15137–42.
- Yates A, Callard R, Stark J. Combining cytokine signalling with T-bet and GATA-3 regulation in Th1 and Th2 differentiation: a model for cellular decision-making. *J Theor Biol* 2004; **231**:181–96.
- Liu N, Ohnishi N, Ni L, Akira S, Bacon KB. CpG directly induces T-bet expression and inhibits IgG1 and IgE switching in B cells. *Nat Immunol* 2003; **4**:687–93.
- Ito Y, Kawamura I, Kohda C *et al.* Seeligeriolysin O, a cholesterol-dependent cytolysin of *Listeria seeligeri*, induces gamma interferon from spleen cells of mice. *Infect Immun* 2003; **71**:234–41.
- Seki N, Miyazaki M, Suzuki W *et al.* IL-4-induced GATA-3 expression is a time-restricted instruction switch for Th2 cell differentiation. *J Immunol* 2004; **172**:6158–66.
- Major 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.
- Zuany-Amorim C, Sawicka E, Manlius C *et al.* Suppression of airway eosinophilia by killed *Mycobacterium vaccae*-induced allergen-specific regulatory T-cells. *Nat Med* 2002; **8**:625–9.
- Hansen G, Yeung VP, Berry G, Umetsu DT, DeKruyff RH. Vaccination with heat-killed *Listeria* as adjuvant reverses established allergen-induced airway hyperreactivity and inflammation. role of CD8<sup>+</sup> T cells and IL-18. *J Immunol* 2000; **164**:223–30.
- Redecke V, Hacker H, Datta SK *et al.* Cutting edge. activation of Toll-like receptor 2 induces a Th2 immune response and promotes experimental asthma. *J Immunol* 2004; **172**:2739–43.
- Eisenbarth SC, Piggott DA, Huleatt JW, Visintin I, Herrick CA, Bottomly K. Lipopolysaccharide-enhanced, Toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *J Exp Med* 2002; **196**:1645–51.
- 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.