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Attenuated *Salmonella typhimurium* reduces ovalbumin-induced airway inflammation and T-helper type 2 responses in mice

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Summary

Cytokines produced by Th2 cells are responsible for the pathogenesis of asthma. Th1-biased immune responses caused by attenuated salmonella have the potential to relieve asthmatic symptoms. We evaluated whether oral administration of attenuated salmonella could modulate allergic responses in a chicken ovalbumin (OVA)-induced asthmatic murine model. Mice were fed with attenuated salmonella SL7207 one dose before and three doses during the induction of an allergic response. Lung histology, percentages of eosinophil in bronchoalveolar lavage fluid, serum levels of OVA-specific antibodies and cytokine production by OVA-activated splenocytes were evaluated in mice with or without the administration of SL7207. A significant reduction in pulmonary eosinophilic infiltration was observed in mice receiving attenuated salmonella. Lower levels of OVA-specific IgG1 but higher titres of OVA-IgG2a in serum were also detected in this group. Splenocytes from salmonella-fed mice produced lower levels of Th2 cytokines upon OVA stimulation. The administration of attenuated salmonella significantly suppressed immunopathological symptoms in OVA-sensitized mice. Inhibition of Th2 responses might explain the potential mechanisms. This study provides some evidence for the feasibility of attenuated salmonella as an effective vaccine for allergic diseases.

Keywords: asthma, salmonella, vaccine, airway inflammation, Th2 cells, cytokines

Introduction

Bronchial asthma has emerged as a worldwide public health problem with increases in incidence, morbidity and mortality during the last two decades [1]. This disease is characterized as a chronic inflammatory disorder of the airways associated with enhanced Th2 responses to inhaled allergens, leading to bronchial eosinophil infiltration, airway-hypersensitivity and elevated serum IgE levels [2–4]. Th2-type cytokines such as interleukin (IL)-4 and IL-13 enhance IgE production [5,6]. IL-5 promotes the differentiation, maturation, activation and prolonged survival of eosinophils [7,8]. Novel effective strategies that have the potential to modulate Th2-type responses might therefore have beneficial effects towards allergic asthma.

Increasing evidence suggests that exposure to bacteria or its components, such as lipopolysaccharides (LPS), might influence the severity of asthma [9–12]. Furthermore, an inverse relationship between allergic disorder and microbial infection has been reported by an epidemiological survey [13]. The 'hygiene hypothesis' also proposes that reduced exposure to infectious organisms in childhood, due to cleaner environments, may have increased the prevalence of asthma and other atopic disorders [14]. The protective effect from infectious organisms may be mediated, at least in part, by microbe-induced Th1 cytokines, such as interferon (IFN)-γ, and consequently down-regulated allergic Th2 responses. Moreover, some experiments in mice support the idea that Th1 immune responses inhibit Th2-mediated diseases in a non-antigen-specific manner [15,16]. It may therefore be beneficial to use certain bacterial species as non-specific protective vaccines against allergic diseases such as asthma.

Attenuated *Salmonella* spp., with its well-understood genetics, known physiology and host Th1-bias immune responses, serves as a potential candidate for an effective vac-

cine against asthma [17]. Safe attenuated salmonella strains are available and are often applied to farm animals and humans for vaccination [18–20]. Several strains have been tested recently as vehicles of DNA delivery *in vivo* [21–23]. From those studies, T helper cell responses induced by genetic immunization seemed strongly biased toward the Th1-activity, as indicated by IFN-γ production from antigen-stimulated T cells and high serum antigen-specific IgG2a levels [24,25]. Thus, the goal of this study was to evaluate whether oral administration of the attenuated salmonella, SL7207, could modulate allergic responses in a chicken ovalbumin (OVA)-induced murine asthma model.

Materials and methods

Animals and bacteria

Female BALB/cByJ mice aged 6–8 weeks were purchased from the National Laboratory Animal Centre (Taipei, Taiwan, ROC). They were maintained and handled according to the guidelines of Animal Care Committee of Chang Gung University and NIH Guides for the Care and Use of Laboratory Animals. The auxotrophic *Salmonella typhimurium* aroA strain SL7207 [21] (*S. typhimurium* 2337–65 derivative hisG46, DEL407 (aroA::Tn10{Tc-s})) was kindly provided by Dr B. A. D. Stocker, Stanford University, CA, USA. Bacteria were grown in Luria-Bertani medium at 37 °C with vigorous shaking until they reached mid-log phase (regularly $0{\cdot}5–1 \times 10^8$ colony forming units (cfu)/ml).

Sensitization, treatment and challenge

Asthmatic model and salmonella-treated mice were sensitized by four intraperitoneal (i.p) injections of 50 μg OVA (grade V; Sigma, St. Louis, MO, USA) which had been emulsified in 0·8 mg aluminium hydroxide in 200 μl saline (OVA/alum) on days 7, 8, 9 and 20 (Fig. 1). Normal saline-control mice were injected with 0·8 mg aluminium hydroxide in 200 μl saline alone (NS/alum). Mice were challenged on days 20, 23, 27, 30 and 34 by inhalation of either normal saline (NS group) or OVA aerosols (OVA and OVA + SL7207 groups) in an exposure chamber for 20 min. Aerosols were generated by nebulizing 2% OVA solution in saline, or saline alone (NS-control group), with a Pulmo-Aide nebulizer (DeVilbiss, Sunrise Medical Corp., CA, USA). *S. typhimurium aroA* strain SL7207-treated mice were fed with 10^8 cfu bacteria in 100 μl PBS with 5% sodium bicarbonate on days 0, 7, 20 and 27 immediately before the i.p. injection of OVA/alum or receiving an OVA aerosol (Fig. 1). None of the mice exhibited any overt signs of illness during the experiment.

Bronchoalveolar lavage fluid and lung histology

Twenty-four hours after the final OVA challenge, lungs were lavaged three times with 1 ml of saline. To evaluate different cell types and numbers, cytospin preparations of bronchoalveolar lavage fluid (BALF) cells were stained with Liu stain and differential cell counts were performed on 500 cells, as based on the staining characteristics and morphology. The supernatant from the first lavage was collected and frozen at −70 °C until further analyses. Lungs were removed after BALF collection and embedded in OCT (Tissue-Tek, Sakura Finetek, Torrance, CA, USA) [26]. Multiple 8-μm sections were stained with haematoxylin and eosin (HE) for light microscopy.

Determination of OVA-specific IgG1, IgG2a and IgE

Serum samples were collected 24 h after the last challenge. OVA-specific IgG1, IgG2a and IgE antibody titres were determined by ELISA. Briefly, each microtitre plate well was coated with 10 μg/ml OVA, blocked with 3% bovine serum albumin (BSA, Sigma) in PBS, and incubated with 100 μl of diluted samples. Biotinylated antimouse IgG1, IgG2a or IgE (BD Pharmingen, San Diego, CA, USA) was then added to each well and followed by avidin-horseradish peroxidase (BD Pharmingen). The reaction was developed by the addition of 100 μl/well of substrate containing 0·2 mg/ml of

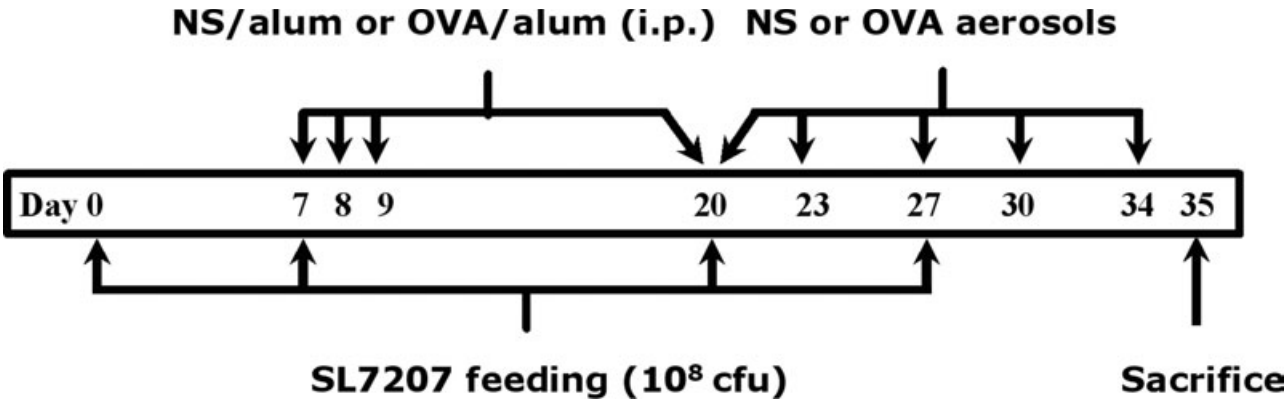

Fig. 1. Schematic diagram depicting protocols for SL7207-feeding and OVA-induced allergy protocol. Mice were fed with SL7207, then sensitized and challenged with OVA as described in Materials and methods. NS, normal saline; OVA, ovalbumin.

o-orthophenylenediamine dihydrochloride (OPD) substrate (Sigma) and stopped with 3 M H_2SO_4. Arbitrary units of IgG1 and IgG2a were determined by the conversion of diluted serum with the comparison of a constructed standard curve with the use of pooled serum obtained from multiply OVA-sensitized mice. OD_{490nm} readings of IgE levels were obtained with the 5-fold diluted serum of all samples.

Cytokine production from OVA-stimulated splenocytes

Splenocytes were stimulated *in vitro* with 100 μg/ml OVA at a density of 5×10^6 cells/ml in RPMI 1640 medium (Gibco-BRL) with 10% fetal calf serum for 6 days. The concentrations of cytokines (IL-4, IL-5, IL-10, IL-13 and IFN-γ) in culture supernatants were evaluated with ELISA kits specific to each cytokine (IL-5 from R & D Systems, Minneapolis, MN, USA; others from BD Pharmingen). Cytokine concentrations were calculated based on standards run in parallel with recombinant cytokines. The limits of detection were: 15·6 pg/ml for IL-4 and IL-5; 31·3 pg/ml for IL-10 and IFN-γ; 39 pg/ml for IL-13.

Statistical analysis

All data are expressed as mean ± SD. All analyses were performed using Student's *t*-test. A probability value of $P < 0{\cdot}05$ was considered to be significant.

Results

Attenuated Salmonella reduces airway inflammation

To investigate whether the attenuated salmonella were able to modulate OVA-induced airway inflammation in asthmatic animals, we sensitized mice i.p. with OVA in alum, challenged them with repeated doses of OVA aerosol, and fed them with 10^8 cfu bacteria of log-phase SL7207 one dose before and three doses during the induction of allergic responses. OVA-induced eosinophilic pulmonary infiltration was greatly reduced in SL7207-fed mice after the final OVA challenge (Fig. 2). The percentage of eosinophils in BALF was reduced in the lungs of bacteria-treated mice (29·83 ± 17·86%) compared with that from controls (47·17 ± 18·10%, $P = 0{\cdot}0002$). The decrease of eosinophilia was also confirmed histologically with HE staining of lung tissue (Fig. 3). Lung tissue from sensitized control mice showed widespread inflammatory infiltrates mainly in the peribronchiolar and perivascular areas. Lung morphology in SL7207-fed mice, however, was similar to that observed in normal saline control mice.

Effects of SL7207 administration on cytokine production from splenocytes

To evaluate whether the administration of attenuated salmonella modulated the T-helper cell subpopulations, cytokine profiles were first analysed from the supernatants of spleen cells that were treated with heat-killed SL7207. As previously described [27], higher amount of IFN-γ, but not Th2-cytokines, such as IL-4, IL-5 and IL-13 were produced by splenocytes from SL7207-fed mice (data not shown). We next explored whether attenuated salmonella modulated OVA-specific T-helper cell responses and reduced pulmonary inflammation. Mice were sacrificed on day 35, and spleen cells were removed and stimulated with OVA *in vitro* for 6 days. Figure 4 shows that bacterial administration significantly down-regulated OVA-specific Th2-cytokine production (control *versus* SL7207-treated group: IL-4: 537·1 ± 281·8 pg/ml *versus* 332·7 ± 236·7 pg/ml, $P = 0{\cdot}0126$; IL-5: 14·2 ± 4·3 ng/ml *versus* 8·3 ± 6·3 ng/ml, $P = 0{\cdot}0007$; IL-13: 24·0 ± 8·8 ng/ml *versus* 15·6 ± 12·0 ng/ml, $P = 0{\cdot}0119$; IL-10: 4·1 ± 1·6 ng/ml *versus* 2·3 ± 1·5 ng/ml, $P = 0{\cdot}0003$). Conversely, no significant changes in IFN-γ production were detected in any experimental groups.

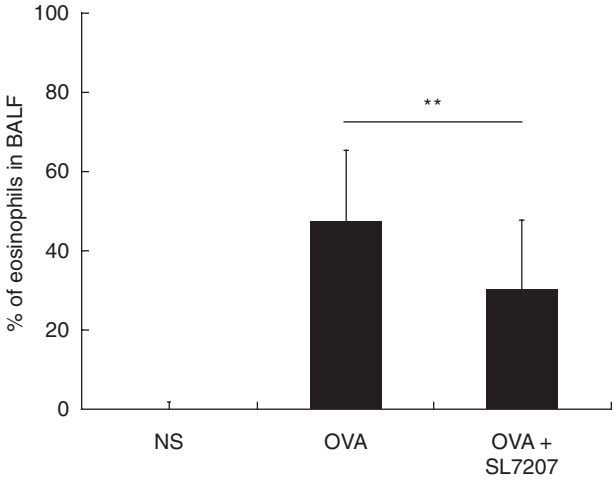

Fig. 2. Reduced eosinophil infiltration in the lungs of mice fed with SL7207. Following the final OVA challenge, percentages of eosinophils in BALF cells were counted with the Liu staining. Data are represented as mean percentage ± SD, NS, normal saline control group ($n = 22$); OVA, OVA-sensitized and challenged group ($n = 33$) and OVA + SL7207, SL7207-treated group ($n = 33$). $^{**}P < 0{\cdot}01$ when compared with the OVA-sensitized control group.

The administration of SL7207 modulates serum levels of OVA-specific IgG1 and IgG2

OVA sensitization and challenge evoked higher OVA-specific Th2 responses and serum IgG1 and IgE levels. We therefore investigated whether treatment with attenuated salmonella affected serum anti-OVA immunologlobulin levels. When compared with controls, treatment with SL7207 during immunization with OVA notably reduced the level of OVA-specific IgG1 (208·24 ± 70·74 KU/ml in treated mice *versus* 299·39 ± 85·68 KU/ml in control mice, $P = 0{\cdot}0004$), but significantly increased IgG2a production (32·68 ± 34·53 KU/ml in treated mice *versus* 16·07 ± 14·78 KU/ml in control mice,

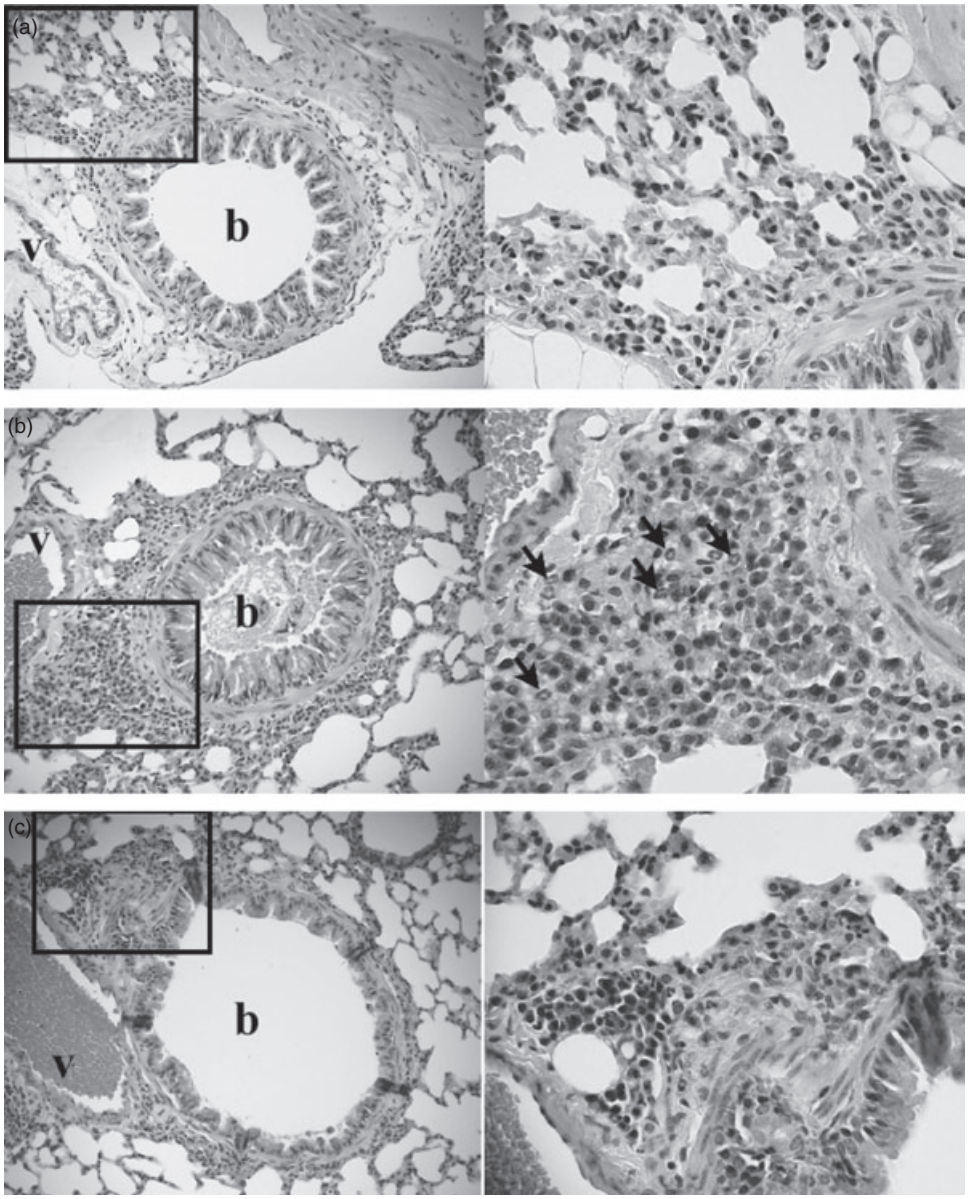

Fig. 3. SL7207 inhibits OVA-induced airway inflammation. Lungs were obtained from (a) normal saline control, (b) OVA-sensitized and challenged and (c) SL7207-fed mice groups after BALF collection, and embedded in OCT. Representative HE-stained sections at 200× are shown for each experimental group. Sections at 400× view are also shown for the indicated areas. Eosinophils are indicated by arrows in the perivascular (v) and peribronchial (b) spaces.

$P = 0{\cdot}0442$) (Fig. 5). However, no significant difference in OVA-specific IgE production was detected following treatment with SL7207 (OD_{490nm}: $0{\cdot}053 \pm 0{\cdot}003$ in NS control mice; $0{\cdot}612 \pm 0{\cdot}258$ in OVA-sensitized control mice; and $0{\cdot}499 \pm 0{\cdot}257$ in SL7207-treated mice; $P = 0{\cdot}185$ between the latter two groups).

Discussion

The results demonstrate that oral feeding with attenuated *Salmonella typhimurium* strain SL7207 reduced important manifestations of atopic asthma in a mouse model. Four doses of salmonella bacteria significantly suppressed eosinophilia in OVA-sensitized BALB/c mice. Production of Th2-type cytokines from OVA-activated splenocytes was also greatly reduced.

Interactions between the host immune system and bacterial pathogens involve specific and non-specific humoral and cellular responses. Th1 responses are induced during most bacterial and viral infections, and have the potential to down-regulate allergic Th2 responses [28]. It was therefore postulated that an approved bacterial vaccine might have the potential to improve allergic asthma. Safe attenuated strains are already available as vaccines against pathogenic salmo-

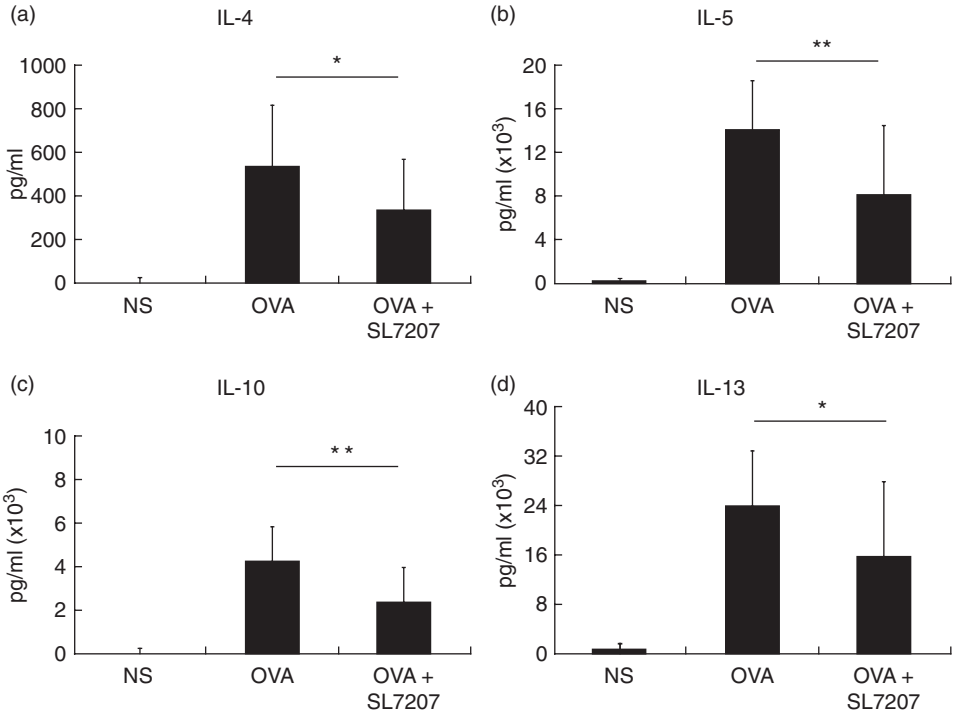

Fig. 4. Effects of SL7207 administration on cytokine production. Splenocytes were cultured *in vitro* with 100 μg/ml OVA in complete RPMI 1640 medium for 6 days. The concentrations of (a) IL-4, (b) IL-5, (c) IL-10 and (d) IL-13 in supernatants were determined with ELISA. Data are presented as mean ± SD, $n = 22$ for each group. $^*P < 0{\cdot}05$ and $^{**}P < 0{\cdot}01$ when compared to the OVA-sensitized control group.

nella infection and are also commonly used for vaccination in farm animals [18,19]. Several attenuated *Salmonella enterica serovar Typhi* strains have been developed, and their safety and efficacy as candidate typhoid fever vaccines have been evaluated in clinical trials [20]. In addition, Darji *et al.* [21] studied the potential use of attenuated *S. typhimurium* strain SL7207 carrying protective antigen against *L. momocytogenes* infection. Strong Th1 responses were demonstrated in mice treated with SL7207. Furthermore, other reports of the application of Salmonella demonstrated similar conclusions [29,30]. Although it is important to elicit antigen-specific Th1 responses to allergens in asthmatic individuals, many patients suffer from unknown allergen or more than one allergen [31,32]. Enhanced non-specific Th1 responses may therefore offer therapeutic benefits.

Other bacteria, including *Mycobacterium,* have been studied for allergy suppressive activity. An inverse association between tuberculin responses and atopic disorder was observed by Shirakawa *et al.* [17]. In addition, mycobacterial infection suppressed the development of asthma in mice [9,33]. Although strong Th1-biased immune responses do develop with *Mycobacterium* or BCG administration, intraperitoneal and subcutaneous injections are not particularly favourable clinically. However, oral administration of attenuated Salmonella might enhance the expression of cytokines or other immuno-modulatory genes that might function as adjuvant and enhance the Th1 activity in allergic individuals.

The biased Th1 response induced with attenuated salmonella might provide a potential mechanism for the reduction of Th2 responses in SL7207-fed mice. Following oral administration, Salmonella egress from the gut lumen via the M cells of Peyer's Patches [34,35], migrate into lymph nodes and spleen, and then induce systemic immune responses with increased Th1 activity. Toll-like receptors (TLR) have recently been described as molecules that link innate and adaptive immunity [36]. TLR-4 appears to be the signalling receptor for Salmonella lipopolysaccharides (LPS) [37] and mediates increases in the expression of pro-inflammatory cytokines, such as TNF-α and IL-12 [38,39]. Therefore, the increased production of IFN-γ might be caused by the interaction between LPS of Salmonella and TLR-4. Indeed, splenocytes of SL7207-fed mice secreted high amounts of IFN-γ upon heat-killed salmonella stimulation, in agreement with previous studies [27]. This provides a possible mechanism for the reduction of Th2 responses in SL7207-fed mice. Whether the administration of Salmonella will modulate the expression of airway remodeling genes requires further investigation.

We observed significant reductions in Th2-type cytokines produced from the OVA-stimulated splenocyte cultures of SL7207-fed mice. Due to the pivotal role of Th2 activity in the pathogenesis of allergic asthma [2–4], higher levels of IL-4, IL-5, IL-10 and IL-13 as well as more severe asthmatic symptoms were demonstrated after sensitization. Some con-

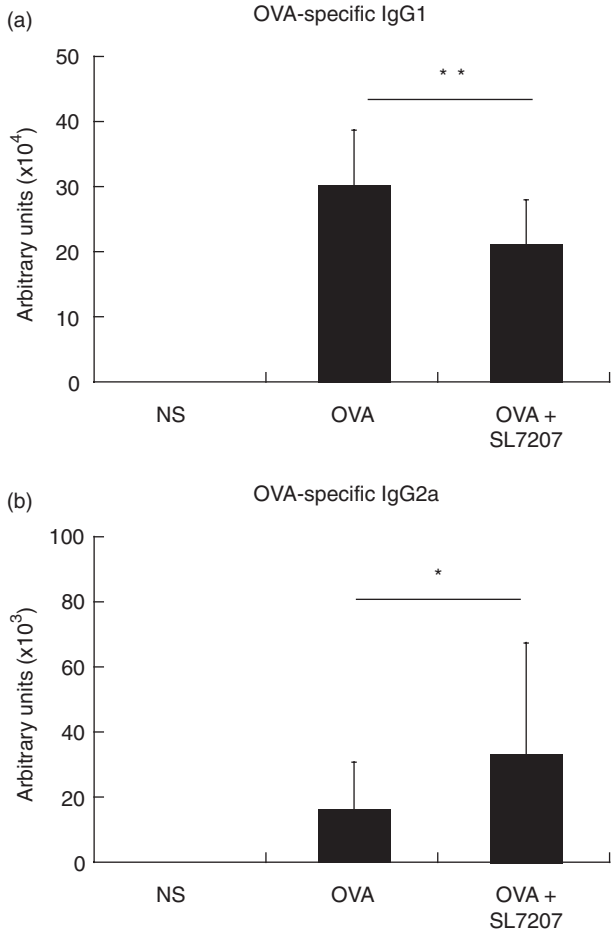

Fig. 5. Serum OVA-specific IgG1 (a) and IgG2a (b) levels. OVA-specific antibodies were determined from three groups by ELISA. n.d. not detectable. Data are represented as mean ± SD, $n = 22$ for each group. $^{*}P < 0{\cdot}05$ and $^{**}P < 0{\cdot}01$ when compared to the OVA-sensitized and challenged control group.

troversy exists about the role of IL-10 as a Th2-cytokine. IL-10 has been considered as one of the major mediators for T regulatory cells, however, high levels of IL-10 were produced from splenocytes of OVA-sensitized mice upon antigen stimulation in our animal model. Furthermore, treatment with SL7207 reduced IL-10 production from OVA-activated splenocytes. Both anti- and pro-inflammatory effects of IL-10 have been demonstrated in asthma [40,41]. The dual role of IL-10 during immune responses has been reviewed by Mocellin *et al.* [42]. Moreover, the transcription factor Stat6, which is activated by IL-4 signalling and is vital for the development of a Th2 response, is critical for the expression of IL-5, IL-10, and IL-13 [43]. In a similar murine model, the expression of IL-10 as well as IL-4, IL-5, and IL-13 was detected in the Th2 cell population [44]. Thus, IL-10 can be considered as a Th2-cytokine in our model rather than a regulatory cytokine.

In conclusion, the oral administration of attenuated *S. typhimurium*, strain SL7207, down-regulated allergen-induced airway inflammation and Th2 cytokine secretion. The development of an oral Salmonella vaccine might have the dual benefit of controlling Salmonella infection and allergic asthma in humans.

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