

ORIGINAL PAPER

Inhibition of airway eosinophilia and pulmonary pathology in a mouse model of allergic asthma by the live vaccine strain of *Francisella tularensis*

R. KuoLee, H. Zhou, G. Harris, X. Zhao, H. Qiu, G. B. Patel and W. Chen

Institute for Biological Sciences, National Research Council Canada, Ottawa, ON, Canada

Clinical and Experimental Allergy

Summary

Background It has been suggested that exposure to certain microbes and their products, particularly during neonatal and early childhood periods, may shift the immune response towards a T-helper cell (Th) 1 phenotype and thereby prevent the development of and/or alleviate the clinical symptoms of allergic airway diseases.

Objective We evaluated the ability of the live vaccine strain (LVS) of *Francisella tularensis* to suppress airway eosinophilia and pulmonary pathology in a murine model of allergic airway disease.

Methods C57BL/6 mice were sensitized by intraperitoneal injection of ovalbumin (OVA) on days 1 and 14, and challenged intranasally (i.n.) with OVA on day 21 or thereafter. Some sensitized mice were i.n. treated with live LVS or its cell-free sonicate extract (CFSE) before i.n. OVA challenge. Bronchoalveolar lavage fluid, regional lymph node cells, lung tissues and serum samples were collected 3–7 days after the i.n. challenge.

Results Intranasal and, to a lesser degree, intradermal immunization of OVA-sensitized mice with LVS suppressed the development of airway eosinophilia and associated pulmonary pathology induced by i.n. OVA challenge. Moreover, CFSE prepared from LVS showed a similar inhibitory effect whereas neither LPS nor DNA purified from *F. tularensis* LVS had such an effect. The inhibition was associated with the reduction in mRNA expression and protein levels of Th2 cytokines IL-5 and IL-13 in the lungs and the enhanced production of OVA-induced IFN- γ by local draining lymph node cells, but not with the serum levels of OVA-specific IgG1 or IgE.

Conclusion *F. tularensis* LVS is capable of suppressing allergic airway inflammation probably through a Th1-mediated suppression of an ongoing Th2 response mechanism, and raises the possibility of exploring LVS and its components as potential therapeutic modalities for human allergic asthma.

Keywords asthma, cytokine, eosinophilia, *Francisella tularensis*, mouse

Submitted 3 May 2007; revised 12 December 2007; accepted 4 January 2008

Correspondence:

Wangxue Chen, Institute for Biological Sciences, National Research Council Canada, 100 Sussex Drive, Room 3100, Ottawa, ON, Canada K1A 0R6.
E-mail: wangxue.chen@nrc.gc.ca

Introduction

Allergic asthma is a chronic respiratory disease characterized by airway inflammation, excessive mucus production, reversible airway obstruction, and airway hyper-reactivity (AHR) [1]. The aetiopathogenesis of allergic asthma is complex and encompasses the interplay between genetic predisposition, environmental triggers, and dysregulated immune response [1, 2]. The precise mechanism of chronic airway inflammation that characterizes persistent asthma remains elusive, but appears to result from a strongly allergen-driven type 2-skewed

CD4⁺ T helper type 2 (Th2) lymphocyte infiltration of the airways with the induction of Th2 cytokines IL-4, IL-5, IL-13, and IL-25 [1]. These cytokines function in concert with CC chemokines (such as eotaxins and thymus and activation-regulated chemokines) and, to a lesser extent, CXC chemokines and result in the recruitment and activation of eosinophils and IgE production [1, 2]. Infiltration of eosinophils and other inflammatory cells in the lower respiratory tract is the hallmark of allergic asthma, and may be important in the pathogenesis of this disease [1, 2].

Asthma now affects approximately 8–10% of the US population, is the leading cause of hospitalization among

children <15 years of age, and costs the society billions of dollars annually [3]. Despite its public health and economic significance, there are relatively few novel therapies and strategies with proven efficacies available for clinical management of asthmatic patients [1, 4]. Although current standard therapies, inhaled bronchodilators (β_2 -receptor agonists) and anti-inflammatory drugs (corticosteroids), provide effective symptomatic control for the majority of asthma patients, they are sometimes accompanied by certain side-effects, particularly in children and in patients with severe asthma requiring high-dose treatments [1]. Moreover, these therapies require long-term daily administration, and they do not target the underlying immune mechanisms causing allergic asthma [1].

The rapid increase in asthma incidence in both the developed and developing countries over the past few decades suggests that certain environmental, in addition to genetic factors, may contribute to the development of allergic asthma [5]. In this regard, increasing epidemiological and clinical data have suggested that a lack of early childhood exposure to microbial stimulation could favour the development of allergic diseases in genetically predisposed individuals (hygiene hypothesis) [5]. As asthma and allergic airway diseases are disorders associated with a predominant Th2 immune response, suppression of the aberrant allergen-specific Th2 response through antagonism or the induction of regulatory T cells by certain microbes and their products, particularly during neonatal and early childhood periods, may shift the immune response towards a T-helper type 1 (Th1) phenotype and thereby prevent the development of and/or alleviate the clinical symptoms of allergic airway diseases [6–9]. Indeed, recent experimental and clinical studies have supported the notion that immunization with certain microbes and their products can reduce asthma-like responses [6–16]. However, not all microbe-elicited Th1-biased host responses can alleviate the asthma-like symptoms [17], and indeed some (e.g. *Bordetella pertussis*) may even exacerbate airway hyperresponsiveness under certain circumstances [18] although the relative contribution of infection-induced severe lung damage and immunological effects on airway hyperresponsiveness remains to be determined.

Francisella tularensis is a gram-negative, facultative intracellular bacterium and the causative agent of tularemia. The live vaccine strain (LVS) of *F. tularensis*, derived from a virulent type B strain of *F. tularensis* by passage on culture media [19], is attenuated for humans and had been used as an investigational new drug (IND) until very recently. LVS immunization induces strong local and systemic pro-inflammatory and Th1 cytokine responses in both human and murine vaccines [20, 21]. Because human allergic asthma and allergic airway diseases are Th2-mediated immune disorders, it was interesting to

investigate if LVS immunization would exert any influence on the development of allergic airway inflammation. In this study, we have examined the ability of LVS to suppress the development of airway eosinophilia and associated pulmonary pathology in a mouse model of allergic asthma. The results of this study raise the possibility of exploiting LVS and its cellular components as potential therapeutic modalities for human allergic asthma.

Materials and methods

Mice

Eight- to 10-week-old specific pathogen free (SPF) female C57BL/6 and Balb/c mice were purchased from Charles River Laboratories (St Constant, Quebec, Canada). B6.129S7-Ifng^{tm1Ts}/J (IFN- γ ^{-/-}) and C57BL/10SnJ (TLR-4^{+/+}) mice of a similar age and sex were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and age- and sex-matched C57BL/10ScNcr (TLR-4^{-/-}) mice were purchased from National Cancer Institute at Frederick (Frederick, MD, USA). Mice were housed under SPF conditions in the Animal Facility, Institute for Biological Sciences, National Research Council Canada (Ottawa) and given free access to sterile water and ovalbumin (OVA)-free diet. The animals were maintained and used in accordance with the recommendations of the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals.

Francisella tularensis live vaccine strain and preparation of cellular components from *Francisella tularensis* live vaccine strain

F. tularensis LVS (ATCC 29684) was grown in modified Mueller-Hinton broth, harvested and frozen at -70°C in the presence of 10% (w/v) sucrose [22]. The cell-free sonicate extract (CFSE) was prepared essentially in the same manner as that for *Helicobacter pylori* CFSE as described previously [23]. Briefly, *F. tularensis* LVS was grown on cysteine heart agar supplemented with 1% (w/v) haemoglobin for 72 h at 37°C . Bacterial cells were harvested into sterile saline and disrupted by 5 min of sonication (30 s pulses at 60%, at 1 min intervals) on ice using a Branson sonifier (model B15, Branson Sonic Power Company, Banbury, CT, USA). Cell debris and unbroken cells were removed by centrifugation at 14 000 g for 20 min at 4°C . The supernatant was aliquoted and stored at -80°C until use. The sterility of the CFSE was confirmed by plating on cysteine heart agar supplemented with 1% (w/v) haemoglobin, and its protein concentration was determined by the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin as the standard. In some experiments,

CFSE was heated in a water bath at 55 °C for 60 min or at 100 °C for 30 min before use.

Purified *F. tularensis* LVS lipopolysaccharide (LPS) was kindly provided by Dr Malcolm Perry [24]. *F. tularensis* LVS DNA was purified using QIAamp DNA Mini Kit (Qiagen Inc., Mississauga, ON, Canada).

Induction of airway eosinophilia and live vaccine strain immunization

Mice were sensitized by intraperitoneal (i.p.) injection of 2 µg OVA (Sigma Chemical, Co., St Louis, MO, USA) in 100 µL of Imject Alum (Pierce Laboratories, Rockford, IL, USA) on days 0 and 14 (hereafter referred to as sensitized mice). On day 21 or thereafter, mice were intranasally (i.n.) challenged with 100 µg OVA in 50 µL of phosphate-buffered saline (PBS). The live LVS immunization was given either i.n. (100 or 200 cfu in 50 µL of PBS) or intradermally (i.d.) (2×10^5 cfu in 50 µL of PBS) at least 3 weeks before the i.n. OVA challenge. This was to allow sufficient time for the mice to clear the infection since mice normally clear such infection by 15 days after administration [22]. In other experiments, sensitized mice were i.n. treated with heat-killed LVS (HK-LVS, equivalent to 10^7 cfu), 20 µg LVS LPS, 150 µg CFSE, 10 µg *F. tularensis* LVS DNA or PBS in 50 µL volume, 7 days before i.n. OVA challenge or at the time of OVA sensitization (day 0) as indicated in results. Mice were anesthetized by i.p. injection of xylazine and ketamine for all i.n. treatments and challenges.

Bronchoalveolar lavage and histopathology

Mice were killed by CO₂ asphyxiation 3 or 7 days after i.n. OVA challenge. Blood samples were collected by cardiac puncture, and sera separated and stored at -80 °C. The trachea was then exposed through a midline incision and cannulated with a plastic catheter. The lungs were lavaged by instillation of five 1.0 mL aliquots of PBS supplemented with 3 mM EDTA [25]. Total lavage cell numbers were counted on a haemocytometer, and differential cell counts were carried out on cytospin preparations stained with Hema3 Stain Set (Fisher Scientific, Middletown, VA, USA). BAL fluid (BALF) was centrifuged at 3000 g for 7 min, supernatants were removed and stored at -80 °C. In some experiments, the lungs were removed immediately after lavage, fixed by immersion in 10% neutral buffered formalin, and processed by standard paraffin embedding methods (Department of Pathology and Laboratory Medicine, University of Ottawa, Ottawa, Canada). Sections were cut 4 µm thick, stained with haematoxylin and eosin (H&E) and examined by light microscopy. In other experiments, the lungs from each mouse were minced into small pieces with scissors and homogenized in 2 mL of lysing buffer (300 mM NaCl₂, 15 mM Tris, 2 mM MgCl₂, and 2 mM

Triton X-100, pH 7.4) supplemented with Complete[®] protease inhibitors (Roche Diagnostics Canada, Laval, QC, Canada). The supernatant was collected following centrifugation at 14 000 g and used for cytokine assays.

Enzyme-linked immunosorbent assay for ovalbumin-specific immunoglobulin isotypes

Serum OVA-specific IgG1 and IgG2a were measured using OVA-coated microtitre plates (Immulon 2, Thermo Lab-systems, Franklin, MA, USA) and detected using alkaline phosphatase conjugated goat anti-mouse IgG1 or anti-mouse IgG2a, respectively (Caltag Laboratories, Burlingame, CA, USA), in conjunction with pNPP substrate (KPL Inc., Gaithersburg, MD, USA) [26]. Serum OVA-specific IgE was measured using anti-IgE mAb (4B-39, BD Biosciences, Mississauga, Ontario, Canada)-coated microtitre plates and detected using OVA-biotin/streptavidin-horse-radish peroxidase (HRP) in conjunction with TMB substrate (KPL Inc.) [6].

Total RNA isolation and real-time reverse transcriptase polymerase chain reaction for cytokine mRNA expression

For cytokine mRNA study, groups of five mice were killed at day 3 after i.n. OVA challenge and their lungs and tracheobronchial lymph nodes (TBLNs, the major draining lymph nodes of the lungs) were removed and immersed immediately in RNAlater[®] (Qiagen Inc.). Relative amounts of cytokine mRNA in the TBLN and lung were estimated using a real-time PCR-based method essentially as described elsewhere [27]. Briefly, total RNA was extracted from tissues, cDNA prepared, amplified and quantified using primers and probes designed with the Primer3 program available at http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi. Levels of PCR product were normalized to the housekeeping gene β -2-microglobulin (β 2m), and data presented as the average of relative expression values in CFSE-treated mice compared with those in the corresponding tissues of PBS-treated mice [27].

In vitro re-stimulation of local draining lymph node cells

Groups of OVA-sensitized C57BL/6 mice were i.n. treated with either CFSE or PBS 7 days before i.n. OVA challenge. Mice were killed 7 days after the challenge and their TBLN were collected and used for *in vitro* culture to determine cytokine production in response to OVA stimulation. Single-cell suspensions (4×10^6 cells/mL) from the TBLNs of the CFSE- or PBS-treated OVA-sensitized mice were prepared and cultured in 48-well tissue culture plates in complete RPMI-1640 (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% certified, heat-inactivated fetal bovine serum (Invitrogen, Grand Island, NY, USA), 50 µM

2-mercaptoethanol (Sigma-Aldrich), and 100 U/mL penicillin G and 100 µg/mL streptomycin (Invitrogen). The cells were either stimulated with 1 mg/mL OVA or medium alone. Culture supernatants were collected 72 h later and assayed for cytokine levels by Luminex.

Determination of cytokine levels in the bronchoalveolar lavage supernatant and lung homogenates

The levels of IFN- γ , IL-4, IL-5, IL-10, IL-12p70, and IL-13 in the BALF and lung homogenate supernatant were measured by the Beadlyte^R Mouse Cytokine Detection kit (Upstate Biotechnology, Lake Placid, NY, USA) on a Luminex^R 100IS system (Luminex Corp, Austin, TX, USA) [27]. The analysis was done in duplicate, and the cytokine concentrations were calculated against the standards using Beadview[®] software (ver. 1.03, Upstate). The lower limit of detection was < 2 pg/mL for IL-4, IL-10, and IL-13, 5.54 pg/mL for IL-12p70, 8.97 pg/mL for IFN- γ , and 30.9 pg/mL for IL-5, respectively.

Statistical analysis

All parametric data are presented as mean $\pm$ standard deviation (SD) for each group, and non-parametric data are presented as median (ranges). Differences between groups were analysed by Student's *t*-test or by one-way and two-way ANOVA followed by the Tukey-Kramer multiple comparison test, when appropriate. *P* < 0.05 was considered to be statistically significant. All statistical analyses were done using GraphPad Prism[®] version 4.0 (GraphPad Software, San Diego, CA, USA).

Results

Francisella tularensis live vaccine strain immunization inhibits airway eosinophilia and pulmonary pathology in ovalbumin-sensitized mice

Allergic asthma is a chronic pulmonary disease characterized by persistent airway and tissue infiltration of eosinophils and other inflammatory cell types. To examine the effect of *F. tularensis* LVS immunization on the development of airway eosinophilia and associated pulmonary pathology, groups of OVA-sensitized C57BL/6 mice were immunized with varying numbers of live LVS or PBS by either i.n. or i.d. route on day 0. The mice were i.n. challenged with OVA on day 21 and killed 7 days later. As anticipated, OVA challenge of PBS-treated sensitized mice induced the infiltration of large numbers of eosinophils and mononuclear cells, as well as some neutrophils into the lung, while the cohort group that was challenged with PBS contained only a few alveolar macrophages in their BALF (data not shown). In contrast, i.n. immunization of OVA-sensitized mice with either 100 or 200 cfu of

live *F. tularensis* LVS before the OVA challenge significantly inhibited eosinophil influx in the BALF (up to 95%) without affecting the recruitment of macrophages, as compared with PBS-treated mice (Fig. 1). Similarly, i.d. immunization of OVA-sensitized mice with 2×10^5 cfu of live *F. tularensis* LVS before OVA challenge resulted in a 60% inhibition of total eosinophil number in the BALF (Fig. 1). These results demonstrated that both i.n. and, to a lesser degree, i.d. LVS immunization prevented the development of allergic airway eosinophilia in mice that were prior sensitized to OVA.

We next examined whether LVS infection of the host was essential for the above-observed suppression of allergic airway eosinophilia. In this series of experiments, groups of OVA-sensitized C57BL/6 mice were i.n. treated with either 150 µg CSFE or PBS 7 days before i.n. OVA challenge. As shown in Fig. 2, CFSE administration effectively inhibited the eosinophilia in the BALF at a magnitude comparable with that induced by live LVS inoculation (Fig. 1), resulting in approximately a 99% decrease in airway eosinophilia at day 3 and nearly 90% at day 7 following i.n. OVA challenge. Histological examination of lung sections showed that OVA challenge induced dramatic perivascular and peribronchial

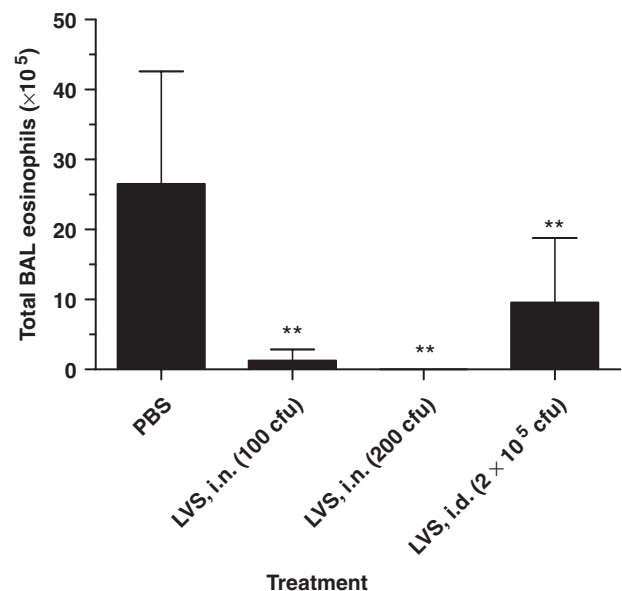


Fig. 1. Inhibition of airway eosinophilia in OVA sensitized mice by live *Francisella tularensis* LVS. Mice were sensitized i.p. with OVA/alum on days 0 and 14. Sensitized mice were treated with LVS or PBS by the indicated route on day 24, and intranasally (i.n.) challenged with OVA on day 45. Seven days after the i.n. OVA challenge, mice were euthanized, and their lungs were lavaged. Total and differential cell counts in the bronchoalveolar lavage (BAL) were enumerated on cytopsin preparations. Each bar represents the mean total number of eosinophils in the BAL fluid $\pm$ SD (*n* = 5). The data presented are representative of 1 of at least 2 separate experiments with similar results. ***P* < 0.01 compared with PBS-treated group. OVA, ovalbumin; LVS, live vaccine strain; PBS, phosphate-buffered saline; i.p., intraperitoneally; i.d., intradermally.

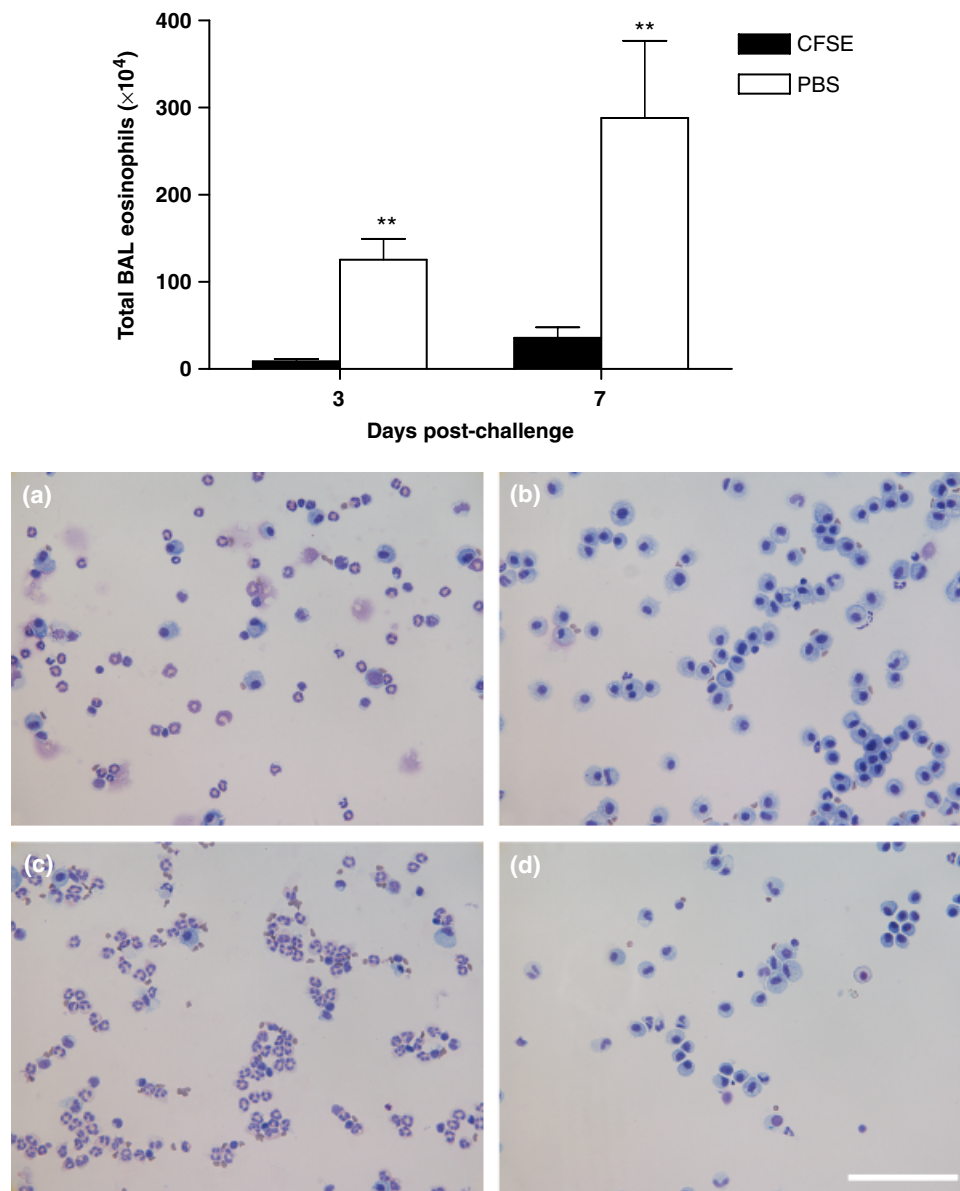


Fig. 2. Upper panel: Inhibition of airway eosinophilia in OVA-sensitized mice by intranasal treatment with *Francisella tularensis* LVS cell-free sonicate extract (CFSE). Mice were sensitized i.p. with OVA/alum on days 0 and 14. Sensitized mice were i.n. treated with either 150 μ g CFSE or PBS on day 29 and i.n. challenged with OVA on day 36. Cells in the bronchoalveolar lavage (BAL) were collected at 3 or 7 days after OVA challenge and differential cell types were enumerated on cytospin preparations. Each bar represents the mean total numbers of eosinophils in the BAL fluid $\pm$ SD ($n = 5$). The data presented are representative of one of at least two separate experiments with similar results. ** $P < 0.01$ compared with PBS-treated group. Lower panel: BAL cells from OVA-sensitized mice that were treated with PBS (a and c) or CFSE (b and d), and killed on day 3 (a and b) or day 7 (c and d) after i.n. challenge with OVA. The majority of BAL cells from CFSE-treated mice (b and d) are large alveolar macrophages with a foamy cytoplasm, whereas the BAL cells from PBS-treated mice (a and c) consist of mainly eosinophils with a donut- or horseshoe-shaped nucleus and a pink granular cytoplasm. HemaStat-3 staining, scale bar = 100 μ m. OVA, ovalbumin; LVS, live vaccine strain; PBS, phosphate-buffered saline; i.p., intraperitoneally; i.n., intranasally.

infiltration of various types of inflammatory cells in PBS-treated, OVA-sensitized mice, and CFSE treatment noticeably reduced the overall intensity of the cellular infiltration, pulmonary pathology (Fig. 3) and mucus production (Fig. 4). Similar inhibitory effects by CFSE on the airway eosinophilia and associated pulmonary pathology in response to OVA challenge were also observed in

Balb/c mice (data not shown) or when CFSE was administered at the time of OVA sensitization (day 0) (Fig. 5). In addition, i.n. administration of 10^7 cfu equivalent heat-killed LVS (HK-LVS) also resulted in inhibition of pulmonary eosinophilia (~60%) although the effect was not as strong and consistent as CFSE treatment (data not shown).

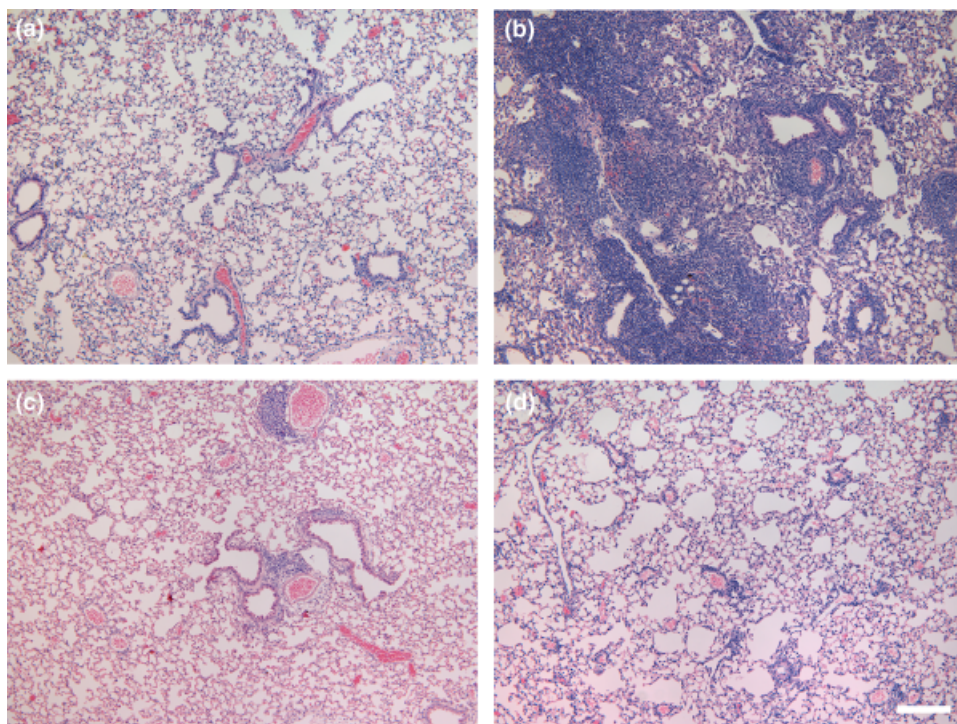


Fig. 3. Representative lung sections from a normal control mouse (a) and from OVA-sensitized mice treated with PBS (b), LVS (c) or CFSE (d) 7 days before i.n. OVA challenge. The mice were sensitized and treated as described in Fig. 2 and killed 7 days after the OVA challenge. The lungs were fixed and tissue sections were stained with haematoxylin and eosin (H&E). Compared with the control mouse (a), note the severe pulmonary inflammation in the areas adjacent to various-sized airways in PBS-treated, OVA-sensitized/challenged mouse (b) whereas the inflammation was relatively minor in the lungs of mice treated with LVS immunization (c) or with CFSE (d). Scale bar = 200 μ m. OVA, ovalbumin; LVS, live vaccine strain; PBS, phosphate-buffered saline; CFSE, cell-free sonicate extract; i.n., intranasally; BAL, bronchoalveolar lavage.

Although the above experiments convincingly showed that CFSE is effective in the inhibition of airway eosinophilia in OVA-sensitized mice, and that bacterial infection of the host is not essential for the inhibitory effect of LVS, it must be noted that the CFSE used in our study was nevertheless a crude preparation and could potentially contain other bacterial components (such as LPS and DNA) in addition to bacterial proteins. In this regard, recent studies have shown that earlier childhood exposure to bacterial LPS reduces the risk of developing allergic airway diseases [28] and treatment of mice with *Escherichia coli* LPS or bacterial DNA suppresses the development of airway hyper-reactivity in the mouse model of asthma [9, 14, 29]. For this reason, additional experiments were performed to determine the potential role of the LPS or bacterial DNA in *F. tularensis*-mediated inhibition of airway eosinophilia in OVA-sensitized mice. Groups of OVA-sensitized C57BL/6 mice were i.n. administered with either LPS or DNA purified from *F. tularensis* LVS, or with one of the two heat-treated CFSE preparations at 7 days before the i.n. OVA challenge. As shown in Fig. 6, neither LPS nor DNA purified from *F. tularensis* LVS at the indicated concentrations had any significant effect on the inhibition of airway eosinophilia in this model. On the other hand, treatment with CFSE significantly reduced

the number of eosinophils in the BAL. However, prior heating of CFSE at 100°C for 30 min, but not at 55°C for 60 min, significantly abolished the inhibitory effect of CFSE ($P < 0.05$). Moreover, the suppression of airway eosinophilia by CFSE was independent of the TLR4 pathway as the treatment remained effective in TLR4^{-/-} mice (Fig. 7). Collectively, these results suggest that a heat-sensitive component(s) in CFSE, other than LPS or DNA, is the likely active component in effecting the suppression of airway eosinophilia.

Inhibition of airway eosinophilia by live vaccine strain or cell-free sonicate extract does not significantly involve immunoglobulin isotype switch from immunoglobulin G1 and immunoglobulin E isotypes

As the first step toward understanding the potential mechanism(s) of LVS-mediated inhibition of airway eosinophilia, the effect of treatment of OVA-sensitized mice with LVS or CFSE on the serum levels of Th2-dependent production of antigen-specific IgG1 and IgE was examined and compared. In agreement with published reports [6, 10, 12, 13], OVA-sensitization alone induced only background levels of OVA-specific IgE and IgG1 (Fig. 8). i.n. OVA challenge induced a significant increase in serum

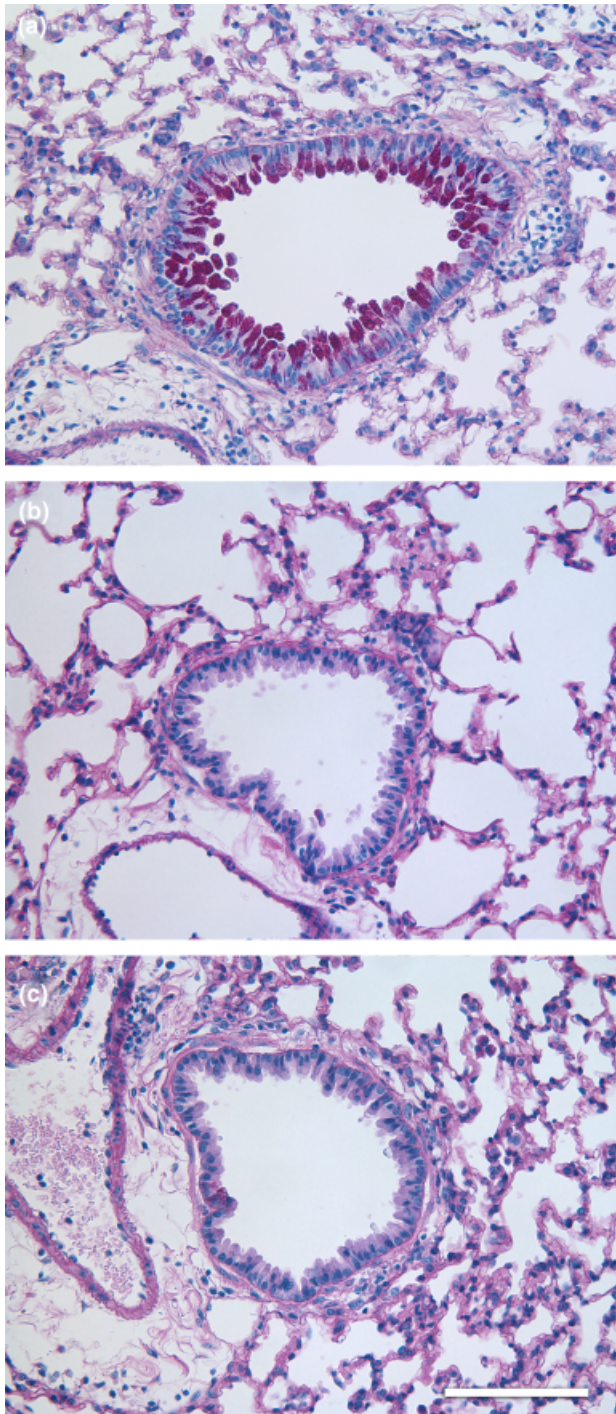


Fig. 4. Representative periodic acid-Schiff (PAS) stained lung sections from OVA-sensitized mice treated with PBS (a), LVS (b), or CFSE (c) 7 days before i.n. OVA challenge. The mice were sensitized and treated as described in Fig. 2 and killed 7 days after the OVA challenge. The lungs were fixed and tissue sections were stained with PAS to visualize the mucus-producing goblet cells (dark purple). Compared with the PBS-treated sensitized mouse (a), note nearly complete absence of goblet cell hyperplasia and mucus production in LVS (b) or CFSE (c) treated, OVA-sensitized/challenged mouse. Scale bar = 100 μ m. OVA, ovalbumin; LVS, live vaccine strain; CFSE, cell-free sonicate extract; PBS, phosphate-buffered saline; i.n., intranasally.

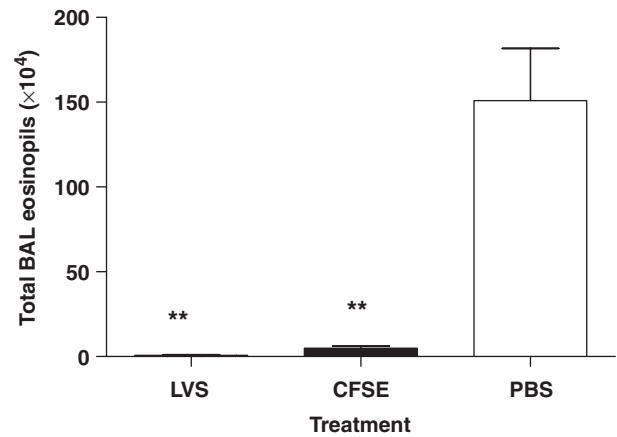


Fig. 5. Inhibition of airway eosinophilia in OVA-sensitized mice by live *Francisella tularensis* LVS and CFSE. Mice were treated i.n. with either live *F. tularensis* LVS (200 cfu), 150 μ g CFSE, or PBS at day 0 and sensitized i.p. with OVA/alum on days 0 and 14. The mice were then intranasally challenged with OVA on day 46. Seven days after the i.n. OVA challenge, mice were euthanized, and their lungs were lavaged. Total and differential cell counts in the bronchoalveolar lavage (BAL) were enumerated on cytospin preparations. Each bar represents the mean total number of eosinophils in the BAL fluid $\pm$ SD ($n = 5$). ** $P < 0.01$ compared with PBS-treated group. OVA, ovalbumin; LVS, live vaccine strain; CFSE, cell-free sonicate extract; PBS, phosphate-buffered saline; i.n., intranasally.

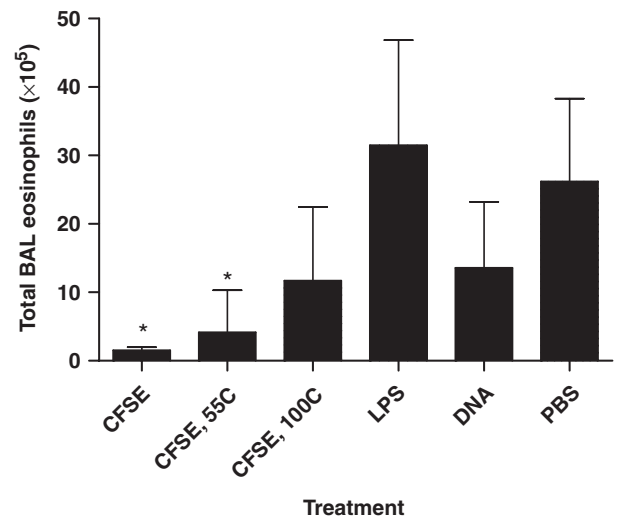


Fig. 6. Inhibition of airway eosinophilia in OVA-sensitized mice by different *Francisella tularensis* LVS components. Mice were sensitized i.p. with OVA/alum on days 0 and 14 and treated by i.n. with different LVS components in 50 μ L of PBS on day 24 as indicated: 150 μ g CFSE (CFSE), 150 μ g CFSE heated to 55 $^{\circ}$ C for 60 min (CFSE, 55C), 150 μ g CFSE heated to 100 $^{\circ}$ C for 30 min (CFSE, 100C), 20 μ g LPS (LPS), 10 μ g DNA (DNA) or PBS alone. Mice were i.n. challenged with OVA on day 31. Cells in the bronchoalveolar lavage (BAL) were collected 7 days after OVA challenge and different cell types were enumerated on cytospin preparations. Each bar represents the mean total eosinophils per mouse lung $\pm$ SD ($n = 5$). The data presented are representative of one of at least two separate experiments with similar results. * $P < 0.05$ compared with PBS-treated group. OVA, ovalbumin; LVS, live vaccine strain; CFSE, cell-free sonicate extract; PBS, phosphate-buffered saline; i.n., intranasally; i.p., intraperitoneally; LPS, lipopolysaccharide.

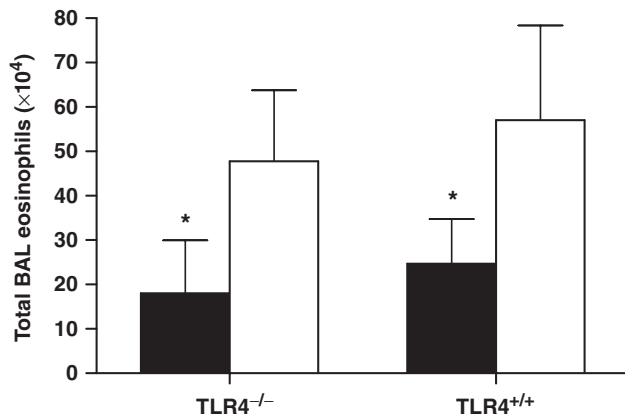


Fig. 7. Inhibition of airway eosinophilia in OVA by *Francisella tularensis* LVS cell-free sonicate extract (CFSE) in TLR4^{-/-} and TLR4^{+/+} mice. TLR4^{-/-} and TLR4^{+/+} mice were sensitized i.p. with OVA/alum on days 0 and 14 and treated by i.n. with 150 µg CFSE (solid bars) or PBS (open bars) on day 24. Mice were i.n. challenged with OVA on day 31. Cells in the bronchoalveolar lavage (BAL) were collected 7 days after OVA challenge and different cell types were enumerated on cytospin preparations. Each bar represents the mean total eosinophils per mouse lung ± SD ($n = 5$). The data presented are representative of one of at least two separate experiments with similar results. * $P < 0.05$ compared with PBS-treated group. OVA, ovalbumin; LVS, live vaccine strain; PBS, phosphate-buffered saline; i.n., intranasally; i.p., intraperitoneally.

OVA-specific IgG1 and IgE in sensitized mice ($P < 0.001$). However, treatment of sensitized mice with LVS or CFSE showed no significant effect on either the serum OVA-specific IgE or IgG1 levels, as compared with PBS treatment ($P > 0.05$) (Fig. 8) although a moderate reduction in the serum level of OVA-specific IgE was observed in LVS-treated sensitized mice (Fig. 8).

Inhibition of airway eosinophilia by cell-free sonicate extract is associated with local suppression of T helper type 2 immune responses

It is now well established that airway eosinophilia and inflammatory responses associated with allergic asthma are regulated by multiple pro-inflammatory mediators, and Th2 cytokines play crucial roles in the pathogenesis of allergic asthma [1, 2]. To examine the effect of CFSE treatment on the local cytokine profile, the mRNA expression of IFN- γ and IL-12p70 (Th1-associated), IL-4, IL-5, and IL-13 (Th2-associated), and IL-10 (regulatory/suppressor function associated) were determined in the lung and TBLNs, and their corresponding protein levels were assayed in the BAL and lung homogenate supernatants from mice killed at day 3 after i.n. OVA challenge. Compared with PBS treatment, CFSE treatment moderately reduced the IL-5 and IL-13 mRNA expression and slightly increased the IFN- γ and IL-12p40 mRNA expression in the TBLNs (Table 1). Similarly, CFSE treatment showed substantial inhibitory effect on the expression of

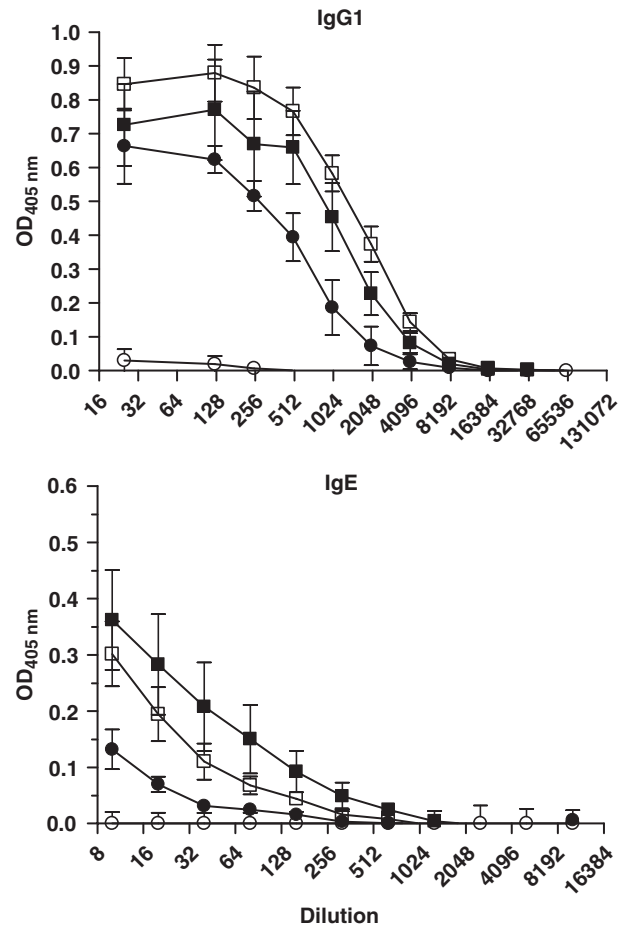


Fig. 8. OVA-specific serum IgG1 (top panel) and IgE (bottom panel) levels in mice sensitized with OVA only (○) and in OVA-sensitized mice treated with LVS (●), CFSE (■) or PBS (□). Groups of five mice were euthanized 7 days after i.n. OVA challenge, and serum was collected. The OVA-specific IgE, and IgG1 levels were measured using ELISA. Each data point represents the mean OD value ± SD of five mice in each group. Data are representative of one of at least two independent experiments. OVA, ovalbumin; LVS, live vaccine strain; CFSE, cell-free sonicate extract; PBS, phosphate-buffered saline; i.n., intranasally.

IL-5 and IL-13 mRNA in the lungs but the effect on mRNA expression of other cytokines was less discernable (Table 1). Consistent with the mRNA expression pattern, CFSE treatment also significantly reduced the levels of IL-4, IL-5, and IL-13 in the BAL and, to a lesser degree, in the lung homogenates in response to the i.n. OVA challenge (Fig. 9).

To further determine the potential mechanism underlying the suppression of airway eosinophilia and associated pathology induced by CFSE, groups of OVA-sensitized C57BL/6 mice were i.n. treated with either CFSE or PBS 7 days before i.n. OVA challenge. Mice were killed 7 days after the challenge and the lung draining lymph nodes, TBLN, were collected and used for *in vitro* culture to determine cytokine responses to OVA restimulation. As it can be seen in Fig. 10, compared with TBLN cells from PBS-treated, OVA-sensitized mice, TBLN cells from

Table 1. Relative cytokine mRNA expression in lung and tracheobronchial lymph nodes (TBLNs) in OVA-sensitized and CFSE-treated mice after i.n. OVA challenge

Cytokine	TBLNs	Lung
IL-4	0.96 (0.62–1.48)	0.36 (0.27–0.50)
IL-5	0.57 (0.45–0.72)	0.19 (0.12–0.30)
IL-13	0.59 (0.37–0.96)	0.17 (0.11–0.24)
IL-10	0.70 (0.56–0.88)	1.53 (2.58–2.58)
IFN- γ	1.27 (0.71–2.28)	0.57 (0.41–0.81)
IL-12p35	0.72 (0.49–1.07)	0.37 (0.18–0.76)
IL-12p40	1.60 (0.80–3.23)	2.51 (1.48–4.26)

Mice were sensitized and treated as described in Fig. 2 and were euthanized 3 days after i.n. OVA challenge. The lungs and TBLNs were collected for RNA extraction. Relative levels of cytokine mRNA expression were determined by real-time RT-PCR analysis as described in 'Materials and methods'. Mouse β 2 microglobulin RNA was measured and used to calculate relative expression using the formula $\text{Rel Exp} = 2 - (\Delta\Delta\text{CT})$. Results shown are the average of relative expression values determined using cDNA from 5 CFSE-treated mice with ranges in parentheses in relation to the corresponding expression levels in 5 PBS-treated mice.

OVA, ovalbumin; CFSE, cell-free sonicate extract; i.n., intranasally; TBLNs, tracheobronchial lymph nodes; PBS, phosphate buffered saline.

CFSE-treated, OVA-sensitized mice produced significantly higher amounts of IFN- γ ($P < 0.05$) and substantially lower, although not statistically significant, amounts of IL-10, IL-5, and IL-13 in response to OVA re-stimulation. The amount of IL-12p70 and IL-4 was generally at the limit of detection (data not shown). On the other hand, medium stimulation induced only minimal amounts of cytokine production by TBLN cells in both groups of mice (Fig. 10). Furthermore, the suppression of airway eosinophilia by CFSE treatment was significantly, although not completely, impaired in OVA-sensitized IFN- $\gamma^{-/-}$ mice as compared with WT mice (Fig. 11).

Discussion

The hygiene hypothesis proposes that a lack of early childhood exposure to microbial stimulation could favour the development of allergic diseases in genetically predisposed individuals [5]. It predicts that certain infections prevent the induction of aberrant allergen-specific Th2 immune responses through antagonism or the induction of regulatory T cells. Indeed, several recent clinical and experimental studies have shown the potential of using bacteria-based vaccines (particularly those from *Mycobacterium* spp.) as new immunotherapeutics for the prevention and treatment of asthma and other allergic disorders [8]. However, not all infections that elicit a predominantly Th1 immune response in the host alleviate effects of aberrant allergen-specific Th2 immune responses, and some such as *B. pertussis* infection even contradictorily exacerbate the allergy symptoms [17, 18]

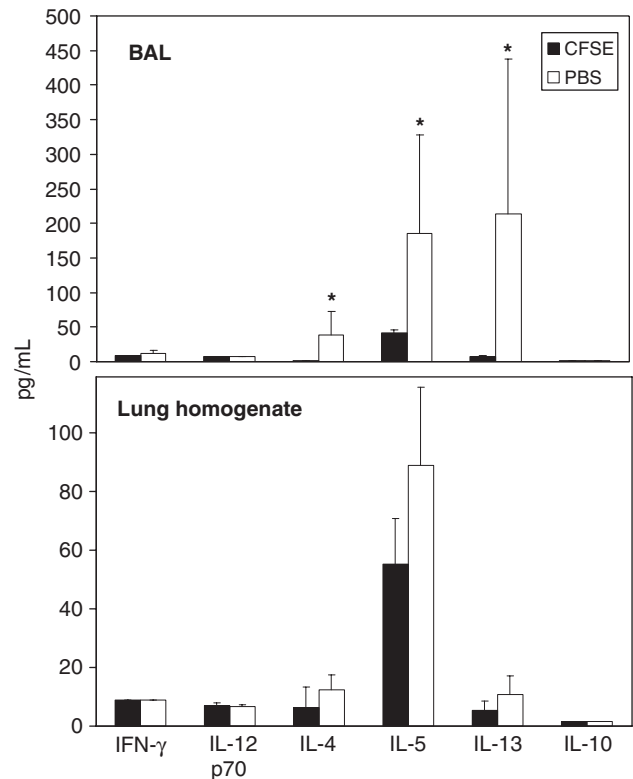


Fig. 9. Effect of CFSE treatment on cytokine levels in the BAL (top panel) and lung homogenates (bottom panel) in OVA-sensitized mice following i.n. OVA challenge. Mice were sensitized and treated as described in Figure 2 and were euthanized 3 days after i.n. OVA challenge. The levels of indicated cytokines in BAL and in the lung homogenate supernatants were measured on a Luminex 100IS system using the Beadlyte mouse cytokine detection kit. Each bar represents the mean pg cytokine/mL $\pm$ SD ($n = 4$). The data are representative of two to three independent experiments. * $P < 0.05$ compared with the PBS-treated group. OVA, ovalbumin; BAL, bronchoalveolar lavage; PBS, phosphate-buffered saline; i.n., intranasally; CFSE, cell-free sonicate extract.

although the relative contribution of infection-induced severe lung damage and immunological effects on airway hyperresponsiveness remains to be determined. On the other hand, systemic immunization with heat-killed whole-cell *B. pertussis* inhibits allergic airway reactions (airway eosinophilia, lung inflammation, and airway hyperresponsiveness) [16], despite the fact that both *B. pertussis* infection and immunization with heat-killed *B. pertussis* cells induce Th1 immune responses [16, 18]. Hence, it is impossible to rationally predict the potential effect of different Th1 response-eliciting microbes and their components on the allergic airway disease. In this report, using a mouse model of allergic airway disease, we found that both live *F. tularensis* LVS and its CFSE, but not LPS or DNA purified from *F. tularensis* LVS, significantly inhibited airway eosinophilia and its associated pulmonary pathology as well as the pulmonary Th2 cytokine (IL-5 and IL-13) response. Although previous studies have demonstrated the ability of a number of microbes and

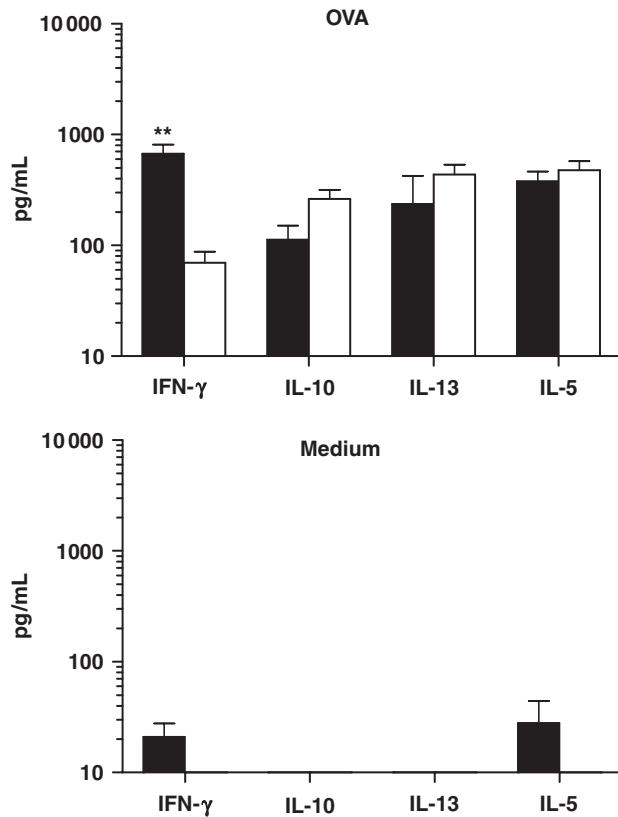


Fig. 10. Cytokine responses to *in vitro* restimulation with OVA (top panel) or medium (bottom panel) by tracheobronchial lymph node (TBLN) cells from CFSE (closed bars)- or PBS (open bars)-treated OVA-sensitized mice. Groups of OVA-sensitized C57BL/6 mice were i.n. treated with either CFSE or PBS 7 days before i.n. OVA challenge. Mice were killed 7 days after the challenge and their TBLNs were collected and used for *in vitro* culture to determine cytokine production in response to OVA stimulation. Single cell suspensions (2×10^6 cells/mL) were re-stimulated *in vitro* for 48 h with either OVA (1 mg/mL) or culture medium only. The level of cytokines in the supernatants was determined by Luminex. Data are presented as mean $\pm$ SD ($n = 5$) and are representative of two independent experiments. ** $P < 0.01$ compared to cells from PBS-treated group. OVA, ovalbumin; PBS, phosphate-buffered saline; i.n., intranasally.

their products to modulate the course of allergic asthma in the mouse model, many of these treatments were only successful if initiated before OVA sensitization or immediately before airway challenge [6, 16, 30]. It is therefore interesting to note that LVS or CFSE immunization/treatment in the current study was capable of inhibiting airway eosinophils and its associated pulmonary pathology in mice that had already been sensitized to OVA. In this regard, our results further support a broad role for microbes and their products in different aspects of the pathogenesis of allergic asthma, and raise the possibility of exploring LVS and its cellular components as potential immunotherapeutics for the treatment of human allergic asthma.

The current understanding of the immunopathogenesis of allergic asthma suggests that CFSE may suppress OVA-

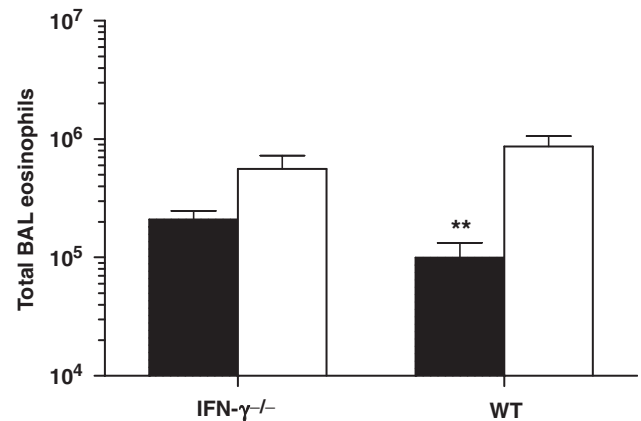


Fig. 11. Inhibition of airway eosinophilia in OVA by *Francisella tularensis* LVS cell-free sonicate extract (CFSE) in IFN- $\gamma^{-/-}$ and wild-type (WT) C57BL/6 mice. IFN- $\gamma^{-/-}$ and WT mice were sensitized i.p. with OVA/alum on days 0 and 14 and treated by i.n. with 150 μ g CFSE (solid bars) or PBS (open bars) on day 24. Mice were i.n. challenged with OVA on day 31. Cells in the bronchoalveolar lavage (BAL) were collected 7 days after OVA challenge and different cell types were enumerated on cytospin preparations. Each bar represents the mean total eosinophils per mouse lung $\pm$ SD ($n = 5$). ** $P < 0.01$ compared with the PBS-treated group. OVA, ovalbumin; LVS, live vaccine strain; PBS, phosphate-buffered saline; i.n., intranasally.

induced allergic airway eosinophilia through a number of mechanisms such as the induction of Th1 immune responses and suppression of Th2 immune responses and the induction of IL-10 and TGF- β -secreting regulatory T cells (Th3 cells, CD4⁺CD25⁺ cells, T_R cells, and NKT cells) [31, 32]. In this study, we have shown (Table 1 and Figs 9 and 10) that CFSE treatment increased IFN- γ production in response to OVA by local draining lymph node cells and reduced the local expression and production of several Th2 cytokines (IL-5 and IL-13) which are known to play crucial roles in the pathogenesis of human allergic asthma [2, 33]. On the other hand, LVS immunization appears to have no substantial effect on local mRNA expression and production of IL-10. These data imply that LVS suppresses allergic airway eosinophilia in this model through a Th1 (IFN- γ)-mediated suppression of the ongoing Th2 response. Indeed, it is well established that LVS immunization has diverse local and systemic immunomodulatory functions including the induction of a strong Th1 (IFN- γ and IL-12) immune response [34, 35]. Our data have also shown that the suppression of airway eosinophilia by CFSE treatment was significantly reduced, although not completely abolished, in OVA-sensitized IFN- $\gamma^{-/-}$ mice, as compared with WT mice (Fig. 11). The observation of only a partial reduction of the CFSE treatment effect in IFN- $\gamma^{-/-}$ mice was not entirely surprising since it is possible that additional, probably redundant, mechanisms are also involved in CFSE-mediated suppression.

One of the consistent findings in this study was the inhibition of local mRNA expression and production of

IL-13 by LVS and CFSE treatment. In this regard, IL-13 contributes to asthma associated pulmonary inflammation probably through multiple mechanisms. IL-13 is the major Th2 cytokine that induces the chemoattractants for eosinophils (eotaxin) and Th2 cells [thymus and activation regulated chemokine (TARC), and eotaxin] [36]. IL-13 enhances IgE production [37], which may augment early-phase inflammation. In the chronic asthma model, anti-IL-13 mAb dramatically reduced levels of eotaxin as well as KC and MIP-1 α , further confirming its important role in regulating chemokine production [38]. However, neutralization of endogenous IL-13 alone only partially suppressed airway eosinophilia and pulmonary inflammation [38], indicating the pathological redundancy among multiple pro-inflammatory cytokines/chemokines in the perpetuation of asthmatic airway inflammation.

The similar levels of serum OVA-specific IgE and IgG1 between PBS- and CFSE-treated groups (Fig. 8) suggests that inhibition of airway eosinophilia did not involve the suppression of these Th2 response-associated parameters in this study. This finding corroborates studies examining the suppression of allergic airway diseases in mice using live or heat-killed BCG [6, 30], heat-killed *Mycobacterium vaccae* [10], and mycobacterial PIM [12], but differs from others where a reduction in OVA-specific IgE level was observed in the treatment group [9, 13, 39]. It is somewhat surprising that there was no change in the serum levels of OVA-specific IgE and IgG1 in the CFSE-treated mice in the current study, whereas these mice demonstrated a substantial reduction in the mRNA expression and production of Th2 cytokines IL-5 and IL-13. The most likely explanation for this is that cytokines other than IL-5 and IL-13 are sufficient to maintain serum levels of these antibodies. In this regard, CFSE did not significantly alter the local mRNA expression and production of IL-4, another key Th2 cytokine that mediates important pro-inflammatory functions in asthma [40]. IL-4 induces Ig isotype switching from IgM to IgE in B cells and induces Th2 cell differentiation and up-regulates low-affinity IgE receptors (FcRII, CD 23) [40]. Although most of these Th2 cytokines (IL-4, IL-5, and IL-13) share many of their biological functions, there are subtle differences in the regulation of host immune responses among these cytokines [33, 40]. For example, IL-13 is the most widely produced cytokine in allergic inflammatory tissues and IL-5 is the most important eosinophilopoietic cytokine whereas IL-4 plays the most significant role in the induction of IgE production [40]. IL-4^{-/-} mice fail to develop detectable IgG1 or IgE in the serum following anti-IgD treatment or infection with the nematode *Nippostrongylus brasiliensis*, both potent inducers of IgG1 and IgE responses [41]. On the other hand, IL-5^{-/-} mice do not develop blood and tissue eosinophilia in response to helminth infection but their serum levels of specific IgG1 and IgE are not significantly affected [42].

The immune response to whole live bacterium is complex and multifaceted. This is likely to lead to some side-effects and impede its potential use as a therapeutic agent for the treatment of allergic asthma. Continuous efforts have been made to identify and evaluate the potential active bacterial component(s) responsible for the protective effect. In this regard, a number of bacterial components, including LPS, unmethylated DNA (CpG), heat-shock proteins and *Mycobacterium bovis* lipoarabinomannan (LAM) and phosphatidylinositol mannan (PIM), have been demonstrated to be capable of inhibiting airway eosinophilia in mouse models of allergic asthma and in human clinical trials [8, 9, 12, 14, 29, 43, 44]. In this study, we have shown that bacterial protein(s), rather than LPS or DNA, from LVS is the likely candidate for the inhibition of airway eosinophilia and its associated pulmonary pathology. It should be noted that *F. tularensis* LPS does not share many biological characteristics with other gram-negative bacteria [45] and LAM and PIM are not the constituents of *F. tularensis* cell walls [46]. Moreover, CFSE remains effective in the inhibition of airway eosinophilia in TLR4^{-/-} mice, suggesting that TLR4, the major receptor for LPS function, does not play an important role in the inhibition. Thus, further identification and characterization of the active immunomodulatory components of *F. tularensis* LVS responsible for the protective effects in allergic airway diseases may lead to the generation of synthetic compounds or agonists devoid of the unwanted characteristics of whole bacteria for evaluation in human clinical trials.

Acknowledgements

We thank Dr Malcolm Perry for the LPS from *F. tularensis* LVS used in this study and Mr Tom Devcseri for his assistance in the preparation of the photomicrography. This work was supported by the National Research Council Canada (A-base).

References

- 1 Lazaar AL, Panettieri RA. Pathogenesis and treatment of asthma: recent advances. *Drug Discov Today:Dis Mech* 2004; 1:111–6.
- 2 Lukacs NW. Role of chemokines in the pathogenesis of asthma. *Nat Rev Immunol* 2001; 1:108–16.
- 3 Elias JA, Lee CG, Zheng T, Ma B, Homer RJ, Zhu Z. New insights into the pathogenesis of asthma. *J Clin Invest* 2003; 111:291–7.
- 4 Weinberger M. Respiratory infections and asthma: current treatment strategies. *Drug Discov Today* 2004; 9:831–7.
- 5 Strachan DP. Hay fever, hygiene, and household size. *BMJ* 1989; 299:1259–60.
- 6 Erb KJ, Holloway JW, Sobeck A, Moll H, Le Gros G. Infection of mice with *Mycobacterium bovis*-Bacillus Calmette-Guerin (BCG) suppresses allergen-induced airway eosinophilia. *J Exp Med* 1998; 187:561–9.

- 7 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+T cells and IL-18. *J Immunol* 2000; **164**: 223–30.
- 8 Matricardi PM, Bjorksten B, Bonini S *et al*. Microbial products in allergy prevention and therapy. *Allergy* 2003; **58**:461–71.
- 9 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.
- 10 Smit JJ, Van Loveren H, Hoekstra MO, Van der Kant PA, Folkerts G, Nijkamp FP. Therapeutic treatment with heat-killed *Mycobacterium vaccae* (SRL172) in a mild and severe mouse model for allergic asthma. *Eur J Pharmacol* 2003; **470**: 193–9.
- 11 Jain VV, Businga TR, Kitagaki K, George CL, O'Shaughnessy PT, Kline JN. Mucosal immunotherapy with CpG oligodeoxynucleotides reverses a murine model of chronic asthma induced by repeated antigen exposure. *Am J Physiol Lung Cell Mol Physiol* 2003; **285**:L1137–46.
- 12 Sayers I, Severn W, Scanga CB, Hudson J, Le Gros G, Harper JL. Suppression of allergic airway disease using mycobacterial lipoglycans. *J Allergy Clin Immunol* 2004; **114**:302–9.
- 13 Ennis DP, Cassidy JP, Mahon BP. Whole-cell pertussis vaccine protects against *Bordetella pertussis* exacerbation of allergic asthma. *Immunol Lett* 2005; **97**:91–100.
- 14 Rodriguez D, Keller AC, Faquim-Mauro EL *et al*. Bacterial lipopolysaccharide signaling through Toll-like receptor 4 suppresses asthma-like responses via nitric oxide synthase 2 activity. *J Immunol* 2003; **171**:1001–8.
- 15 Wohlleben G, Muller J, Tatsch U *et al*. Influenza A virus infection inhibits the efficient recruitment of Th2 cells into the airways and the development of airway eosinophilia. *J Immunol* 2003; **170**:4601–11.
- 16 Kim YS, Kwon KS, Kim DK, Choi IW, Lee HK. Inhibition of murine allergic airway disease by *Bordetella pertussis*. *Immunology* 2004; **112**:624–30.
- 17 Schwarze J, Hamelmann E, Bradley KL, Takeda K, Gelfand EW. Respiratory syncytial virus infection results in airway hyperresponsiveness and enhanced airway sensitization to allergen. *J Clin Invest* 1997; **100**:226–33.
- 18 Ennis DP, Cassidy JP, Mahon BP. Prior *Bordetella pertussis* infection modulates allergen priming and the severity of airway pathology in a murine model of allergic asthma. *Clin Exp Allergy* 2004; **34**:1488–97.
- 19 Eigelsbach HT, Downs CM. Prophylactic effectiveness of live and killed tularemia vaccines. I. Production of vaccine and evaluation in the white mouse and guinea pig. *J Immunol* 1961; **87**:415–25.
- 20 Elkins KL, Cowley SC, Bosio CM. Innate and adaptive immune responses to an intracellular bacterium, *Francisella tularensis* live vaccine strain. *Microbes Infect* 2003; **5**:135–42.
- 21 Tarnvik A. Nature of protective immunity to *Francisella tularensis*. *Rev Infect Dis* 1989; **11**:440–51.
- 22 Conlan JW, Chen W, Shen H, Webb A, KuoLee R. Experimental tularemia in mice challenged by aerosol or intradermally with virulent strains of *Francisella tularensis*: bacteriologic and histopathologic studies. *Microb Pathog* 2003; **34**:239–48.
- 23 Chen W, Shu D, Chadwick VS. Inhibition of mitogen-induced murine lymphocyte proliferation by *Helicobacter pylori* cell-free extract. *J Gastroenterol Hepatol* 2000; **15**: 1000–6.
- 24 Vinogradov E, Perry MB, Conlan JW. Structural analysis of *Francisella tularensis* lipopolysaccharide. *Eur J Biochem* 2002; **269**:6112–8.
- 25 Chen W, Havell EA, Moldawer LL, McIntyre KW, Chizzonite RA, Harmsen AG. Interleukin 1: an important mediator of host resistance against *Pneumocystis carinii*. *J Exp Med* 1992; **176**: 713–8.
- 26 Patel GB, Zhou H, KuoLee R, Chen W. Archaeosomes as adjuvants for combination vaccines. *J Liposome Res* 2004; **14**: 191–202.
- 27 KuoLee R, Zhao X, Austin J, Harris G, Conlan JW, Chen W. Mouse model of oral infection with virulent type A *Francisella tularensis*. *Infect Immun* 2007; **75**:1651–60.
- 28 Douwes J, Le Gros G, Gibson P, Pearce N. Can bacterial endotoxin exposure reverse atopy and atopic disease? *J Allergy Clin Immunol* 2004; **114**:1051–4.
- 29 Kline JN, Waldschmidt TJ, Businga TR *et al*. Modulation of airway inflammation by CpG oligodeoxynucleotides in a murine model of asthma. *J Immunol* 1998; **160**:2555–9.
- 30 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.
- 31 Akbari O, Stock P, DeKruyff RH, Umetsu DT. Role of regulatory T cells in allergy and asthma. *Curr Opin Immunol* 2003; **15**: 627–33.
- 32 Umetsu DT. Revising the immunological theories of asthma and allergy. *Lancet* 2005; **365**:98–100.
- 33 Taube C, Dakhama A, Gelfand EW. Insights into the pathogenesis of asthma utilizing murine models. *Int Arch Allergy Immunol* 2004; **135**:173–86.
- 34 Golovliov I, Sandstrom G, Ericsson M, Sjostedt A, Tarnvik A. Cytokine expression in the liver during the early phase of murine tularemia. *Infect Immun* 1995; **63**:534–8.
- 35 Stenmark S, Sunnemark D, Bucht A, Sjostedt A. Rapid local expression of interleukin-12, tumor necrosis factor alpha, and gamma interferon after cutaneous *Francisella tularensis* infection in tularemia-immune mice. *Infect Immun* 1999; **67**: 1789–97.
- 36 Li L, Xia Y, Nguyen A *et al*. Effects of Th2 cytokines on chemokine expression in the lung: IL-13 potently induces eotaxin expression by airway epithelial cells. *J Immunol* 1999; **162**:2477–87.
- 37 Defrance T, Carayon P, Billian G *et al*. Interleukin 13 is a B cell stimulating factor. *J Exp Med* 1994; **179**:135–43.
- 38 Yang G, Volk A, Petley T *et al*. Anti-IL-13 monoclonal antibody inhibits airway hyperresponsiveness, inflammation and airway remodeling. *Cytokine* 2004; **28**:224–32.
- 39 Zuany-Amorim C, Manlius C, Trifilieff A *et al*. Long-term protective and antigen-specific effect of heat-killed *Mycobacterium vaccae* in a murine model of allergic pulmonary inflammation. *J Immunol* 2002; **169**:1492–9.
- 40 Haitchi HM, Krishna MT, Holloway JH, Dent G, Buckley MG, Holgate ST. Asthma: clinical aspects and mucosal immunology. In: Mestecky J, Lamm ME, Strober W, Bienenstock J, McGhee JR,

- Mayer L, eds. *Mucosal Immunology*. Amsterdam: Elsevier Academic Press, 2005; 1415–32.
- 41 Kopf M, Le Gros G, Bachmann M, Lamers MC, Bluethmann H, Kohler G. Disruption of the murine IL-4 gene blocks Th2 cytokine responses. *Nature* 1993; 362:245–8.
- 42 Kopf M, Brombacher F, Hodgkin PD *et al.* IL-5-deficient mice have a developmental defect in CD5+B-1 cells and lack eosinophilia but have normal antibody and cytotoxic T cell responses. *Immunity* 1996; 4:15–24.
- 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.
- 44 Horner AA, Takabaysahi K, Zubeldia JM, Raz E. Immunostimulatory DNA-based therapeutics for experimental and clinical allergy. *Allergy* 2002; 57 (Suppl. 72):24–9.
- 45 Sandstrom G, Sjostedt A, Johansson T, Kuoppa K, Williams JC. Immunogenicity and toxicity of lipopolysaccharide from *Francisella tularensis* LVS. *FEMS Microbiol Immunol* 1992; 5:201–10.
- 46 Larsson P, Oyston PC, Chain P *et al.* The complete genome sequence of *Francisella tularensis*, the causative agent of tularemia. *Nat Genet* 2005; 37:153–9.