

Therapeutic efficacy of an *E. coli* strain carrying an ovalbumin allergenic peptide as a fused protein to OMPC in a murine model of allergic airway inflammation

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Abstract

An *Escherichia coli* strain expressing the ovalbumin (OVA) 323–329 allergenic peptide on the bacterial surface was evaluated for its ability to reduce the lung inflammatory response in mice allergic to OVA. BALB/c mice were rendered allergic by means of two intraperitoneal injections of OVA suspended in alum 5 days apart, and one intratracheal boost 1 week later. The mice were then treated with two intranasal, 1 week apart, doses of 4×10^9 *E. coli*-UH302 transformed with plasmids pST13 or pST13-OVA_{323–329}, which bear the OmpC porin from *Salmonella enterica* serovar Typhi or the OmpC with the OVA allergenic 323–329 amino acid sequence inserted in the external loop 5. The allergic inflammatory reaction was evaluated on day 31, finding that mice treated with *E. coli*-UH302-pST13-OVA reduced four to seven times perivascular and peribronchial infiltrates, mucus production, goblet cell hyperplasia and eosinophils when compared with mice treated with *E. coli*-UH302-pST13 or saline solution. These results were consistent with a significant decrease of IL-5 mRNA and induction of IFN- γ mRNA in cells from bronchio-alveolar lavages (BAL). Specific serum IgE anti-OVA was also reduced, although the decrease did not reach statistical significance. These results demonstrate that the bacterial live vector bearing an allergenic peptide successfully moderated two important components of allergy, pulmonary inflammation and mucus overproduction.

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1. Introduction

Asthma is a clinical syndrome characterized by increased bronchial responsiveness to a variety of stimuli, which causes reversible airflow obstruction and inflammation. Extrinsic asthma, is the result of an allergic response to certain

environmental antigens, which in predisposed individuals triggers an immediate IgE-mediated hypersensitivity and late phase response caused by the release of proinflammatory substances by mast cells, eosinophils and basophils. The airflow is reduced due to smooth muscle contraction of bronchi and bronchioles, but without the involvement of alveoli, and mechanical obstruction with edema fluids, cell clusters and mucus plugs, combined with mucosal edema, viscid mucus secretion and goblet and mast cells hyperplasia. The basement membrane is thickened due to collagen deposition, and the *lamina propria* is infiltrated with CD4⁺ T cells, eosinophils, and neutrophils. The increase in airway

Abbreviations: OVA, ovalbumin; BAL, bronchioalveolar lavage; SS, saline solution; i.n., intranasal; i.p., intraperitoneal; i.t., intratracheal; LPS, lipopolysaccharide; H/E, hematoxylin/eosin; PAS, periodic acid Schiff

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resistance restrains inspired air to escape the lungs, reducing forced expiratory volumes and flow rates, and causing air trapping [1].

The pathogenesis of allergic asthma is associated to hyperactivity of CD4⁺ Th2 lymphocytes, which respond to allergens with the production of IL-4, IL-5, IL-9 and IL-13. These type 2 cytokines promote antibody switching to IgE, eosinophils expansion and activation, airway hyper-responsiveness and mucus overproduction by goblet cells [2]. In contrast, Th2 cells have an antagonist function to Th1 cells, because they release IL-2 and IFN- γ , which enhance cell-mediated immunity. Although the reasons for the excessive Th2 cell activity remain unknown, an attractive strategy for immunotherapy of allergies relies on restituting the Th1/Th2 balance by enhancing Th1 or inhibiting Th2 responses. The administration of Th1 cytokines, such as IL-12, IL-18 or IFN- γ [3–5], and Th2 antagonists, such as antibodies against IL-4 or IL-5, reduces the severity of asthma in allergic mice [6]. In humans, clinical trials for the treatment of asthma with antibodies against IL-5 reduced circulating eosinophils but had no effect on allergen induced bronchoconstriction. Nevertheless, the administration of an IL-4 soluble receptor in aerosol or monoclonal antibodies against IgE improved asthma symptoms, exacerbations and steroid requirements [7].

Other alternatives to modulate the immune response against allergens could be based on the use of attenuated bacterial strains to deliver antigens, since in animal models these vaccines have elicited cellular immune responses against viral, bacterial or parasitic passenger antigens. Furthermore, “bacterial vectors” may transport antigens directly to professional antigen presenting cells, such as dendritic cells or macrophages and induce an appropriate microenvironment for the differentiation of Th1 lymphocytes, some of their constituents, such as lipopolysaccharide (LPS), CpG DNA sequences or peptidoglycan, promote IL-12 production [8,9].

Foreign or passenger antigens may be delivered to the immune system from different bacterial compartments, such as the cytosol or the bacterial surface. Several strategies for antigen expression on the bacterial surface have been attempted, including expression in fimbria, flagella or outer membrane proteins (OMPs). These later proteins resemble a barrel embedded in the outer membrane, with loops oriented towards the bacterial outer surface [10], which may be permissive for the insertion of foreign sequences without disrupting the structure of the whole protein, and that such passenger antigens may induce humoral and cellular immune responses [11].

Herein, we report the therapeutic efficacy of an *Escherichia coli* strain carrying an allergenic peptide as a fused protein to OMPC in a murine model of allergic pulmonary inflammation to ovalbumin (OVA). Mice were sensitized with OVA, and then were treated with an *E. coli* strain expressing the OVA allergenic sequence in an external loop of the OmpC porin from *Salmonella enterica* serovar Typhi.

2. Materials and methods

2.1. Animals

Groups of 6–8 weeks old male BALB/c mice were obtained and maintained in a pathogen free environment in the facilities of the Instituto Nacional de Ciencias Médicas y de la Nutrición (México City).

2.2. Plasmids

- pST13: Contains the *S. enterica* serovar Typhi OmpC encoding gene (kindly donated by Dr. Felipe Cabello, New York Medical College Valhalla, NY, USA) [12].
- pST13-OVA_{323–339}: The OmpC loop 5 predicted strand was substituted by the OVA allergenic epitope, corresponding to amino acids 323–339 (as reported in this work).

2.3. Bacterial strains

- *E. coli*-UH302: Porinless strain (does not produce OmpA, OmpF and OmpC) [13].
- *E. coli*-UH302-pST13 (*E. coli*-OMP): Strain transformed with plasmid pST13, expresses OmpC from *S. enterica* serovar Typhi.
- *E. coli*-UH302-pST13-OVA_{323–339} (*E. coli*-OMPOVA): Strain transformed with plasmid pST13-OVA_{323–339}, expresses the OmpC–OVA_{323–339} fusion protein.

2.4. Bacterial culture

All bacterial strains were cultured in 5 ml of brain–heart infusion (BHI) (BIOXON) overnight at 37 °C in agitation. Then 1 ml was transferred to 100 ml of fresh medium and further cultured to late logarithmic growth phase (0.6 OD at 495 nm), 100 μ g/ml ampicillin were added when necessary. The cells were harvested and washed twice by centrifugation with sterile saline solution (SS) the bacterial concentration was estimated espectrophotometrically (540 nm) and was verified by viable counts.

2.5. Construction of plasmid pST13-OVA_{323–339}

The OVA 323–339 encoding strand was inserted in the OmpC loop 5 predicted region from *S. enterica* serovar Typhi by reverse PCR. Since the OmpC tertiary structure has not been reported, the external OmpC loop 5 strand was predicted by annealing with the *E. coli*-OmpF as described previously [14], and the following hybrid primers were designed: the sense OMPC-OVA1: 5'-ACATGCATGCAG-CTCACGCTGAAATTAATG AAGCTGGTAAAACGCT-CGTCTGTACGGT-3' and the antisense OMPC-OVA2: 5'TCCC GATATCTGCATGCAC AGCCT GGGAGAT-AGCGGTGTTGT TCTGGTC-3', containing the *ompC*

sequence (bold) (amino acids 227–232 in OMPC-OVA1 and 221–226 in OMPC-OVA2), the OVA 323–339 amino acids sequence (normal font) (amino acids 327–339 in OMPC-OVA1 and 323–326 in OMPC-OVA2), and *Sph*I (underlined) and *Eco*RV (underlined, italics) restriction sites.

Plasmid pST13, bearing the *OmpC* encoding gene was purified from *E. coli*-UH302-pST13 (CONCERTTM Rapid Plasmid Midiprep System, GIBCO BRL) and amplified by PCR using primers *OmpC*-Ova1 and OMPC-Ova2 (Vent_R DNA Polymerase; Biolabs, New England). The expected 5.2 kb product was purified using anion-exchange resin columns (GIBCO, BRL), digested with *Sph*I and autoligated (*T*₄ ligase, Roche-Boehringer). The resulting plas-

mid pST13-OVA_{323–339} (Fig. 1A) was transformed in *E. coli*-UH302. Appropriate clones were identified since the *Eco*RV site was lost after digestion with *Sph*I. Further restriction analysis with *Pvu*II showed that substitution was achieved when one *Pvu*II site was lost and a new *Sph*I site was created (not shown). Therefore, in the *OmpC*-OVA fusion protein amino acids 223–239 (predicted for loop 5) were substituted by the OVA 323–339 sequence. Expression of the fusion protein *OmpC*-OVA_{323–339} was verified by Western blot analysis, revealing with a cocktail of monoclonal antibodies anti-*OmpC* (kindly donated by Dr. Felipe Cabello) [15] and an anti-OVA 323–339 serum from a rabbit immunized with an OVA 323–339 synthetic peptide (ISQAVHAAHAEINEAGK) (Fig. 1B).

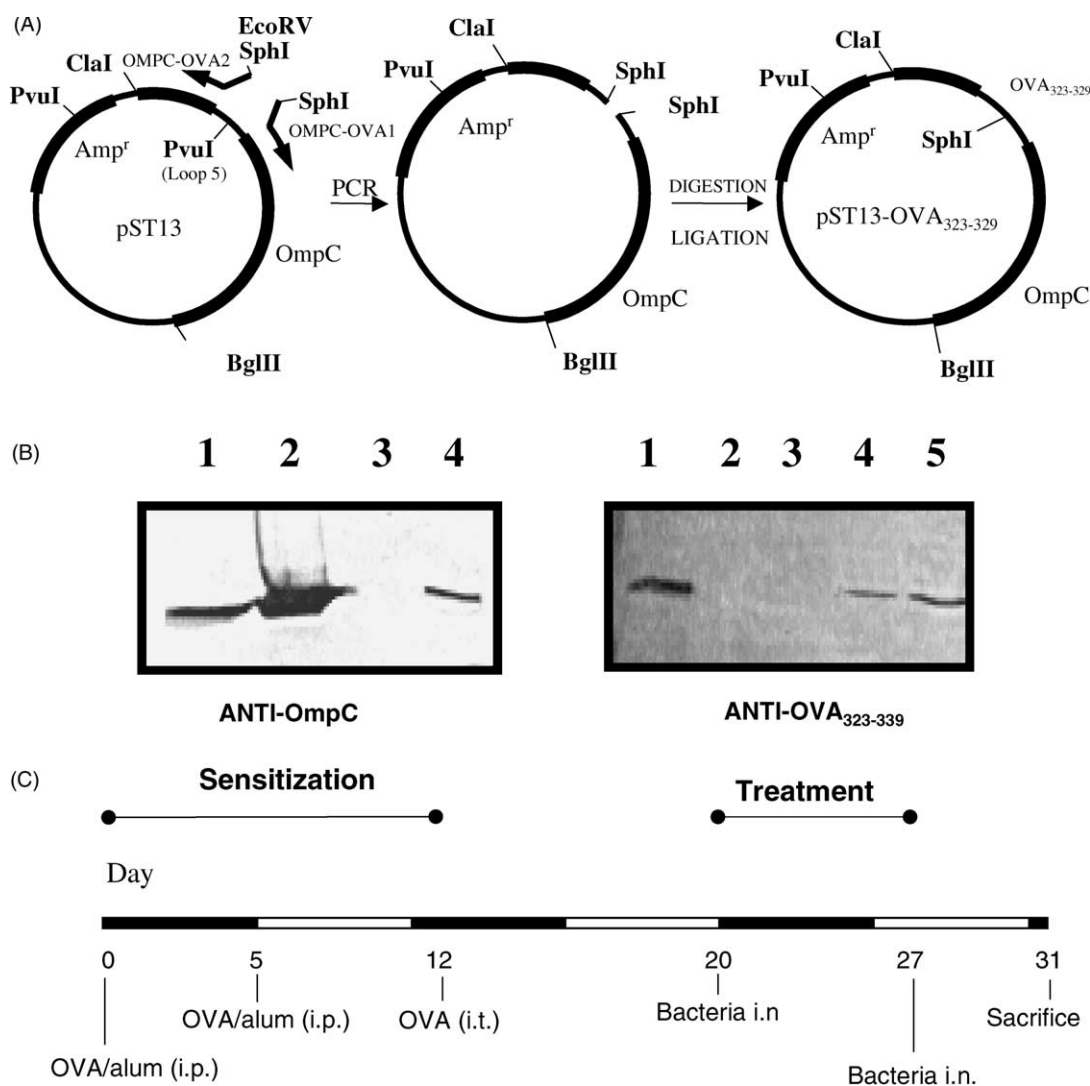


Fig. 1. (A) Insertion of the OVA_{323–339} peptide in *OmpC* from *S. enterica* serovar *Typhi*. The OVA_{323–339} amino acid encoding strand was inserted in plasmid pST13 by PCR in the loop 5 predicted region of *S. enterica* serovar *Typhi* *OmpC*. (B) *OmpC*-OVA_{323–339} fusion protein expression. Left panel: Western blot revealed with monoclonal anti-*OmpC* antibodies: lane 1, purified recombinant *OmpC*; lane 2, *E. coli*-OMP; lane 3, *E. coli*-UH302; lane 4, *E. coli*-OMPOVA. Right panel: Western blot revealed with a rabbit serum anti-OVA_{323–339} peptide: lanes 1, purified OVA; lane 2, *E. coli*-UH302; lane 3, *E. coli*-OMP; lanes 4 and 5, *E. coli*-OMPOVA. (Electrophoresis was performed with 5×10^8 bacteria in 40 μ l of loading buffer.) (C) Sensitization and treatment schedules.

2.6. Sensitization and treatment schedules

The sensitization and treatment schedules are depicted in Fig. 1C. Briefly, groups of 10 mice received 100 μ l of 10 μ g OVA suspension in 1 mg alum by i.p. injection on days 0 and 5, and they were boosted with 100 μ l of 0.75% OVA-SS by intratracheal (i.t.) route on day 12. Control groups received only 100 μ l of SS on the same days (sham-ST). After sensitization, the mice were treated with *E. coli*-OMP or *E. coli*-OMPOVA, two doses of 40 μ l of a 1×10^{11} ml⁻¹ bacterial suspension in SS were administered by intranasal (i.n.) route in days 20 and 27, and finally the mice were sacrificed on day 31. Control groups received only SS.

2.7. Bronchoalveolar lavages

After the mice were sacrificed by exsanguination a tracheal cannula was introduced and the lungs were washed six times with 1 ml of SS. The cells were harvested by centrifugation ($500 \times g$ for 10 min at 4 °C) from the wash fluid, were counted in a hemacytometer and adjusted to 1×10^5 cells/ml. Differential counts from four to six H/E stained cytospin preparations for each experimental group were determined by microscopy, 500 cells were counted by standard procedures at 400 $\times$ magnification. Results are presented as cells/ml averages from all the slides per group.

2.8. Processing of lung sections and morphometric analysis

After the mice were sacrificed by exsanguination, the lungs were fixed by intratracheal instillation of 1 ml of ethanol. After 24 h fixation, the lungs were embedded in paraffin, and 5 μ m wide sections were stained with H/E or periodic acid Schiff (PAS) for the assessment of cell infiltrate or mucus production, respectively. The morphometric analysis was performed using an image analyzer (Leica Q5001W) under light microscopy (40 $\times$) [16]. The cell infiltrate area (μ m²) was calculated from H/E stained slides selecting venules with diameter of 100–200 μ m and bronchi with 150–300 μ m of diameter, five blood vessels or bronchi from five to eight lung sections were analyzed for each experimental group, and results were expressed as average and standard deviations of infiltrate area (μ m²). Mucus production was assessed from lung sections stained with PAS. The PAS-positive material was measured in 200 μ m squares (40,000 μ m²), five squares from five to eight lung sections were analyzed for each experimental group and results are expressed as average and standard deviations of PAS-positive area (μ m²).

2.9. RT-PCR of BAL cytokine mRNA

The cells were harvested from BAL by centrifugation and total RNA was purified using the TRIzol (GIBCO, BRL) reagent according to the technique described previously [17].

Briefly, the cellular pellet was resuspended in 1 ml of TRIzol, then 200 μ l of chloroform were added and mixed gently for 3 min. The suspension was centrifuged at $12,000 \times g$ for 20 min at 4 °C. The aqueous phase was transferred to a clean tube and 500 μ l of isopropanol were added, the solution was mixed and incubated at room temperature for 15 min. After the incubation period, the suspension was centrifuged at $12,000 \times g$ for 10 min at 4 °C, the supernatant was discharged and the pellet was washed with 1 ml of 75% ethanol. The obtained RNA was resuspended in 30 μ l water previously treated with DEPC and was incubated at 65 °C for 20 min. Finally, the RNA was quantified spectrophotometrically at 260 nm.

The first cDNA chain was generated from 1 μ g of RNA through reverse transcription (RT) using RT-AMV (Avian Myeloblastosis Virus Reverse Transcriptase, Roche Molecular Biochemical, La Jolla, CA, USA) and hexanucleotide mix (Roche) in 20 μ l of reaction. The RT product was employed as template of PCR amplification in a thermocycler (AMPLITOR II) using specific oligonucleotides for IL-5, IFN- γ and 3-phosphate dehydrogenase (GAPDH). Amplification was achieved after 40 cycles utilizing 94 °C for 30 seg for denaturation; 57 °C for 1 min for annealing; and 72 °C by 1.5 min for extension as previously described [18,19]. A total of 15 μ l of the PCR products were observed by 1.5% agarose gel electrophoresis stained with ethidium bromide. Samples from amplification without previously using RT-AMV were included as controls. The GAPDH enzyme expression was used as internal control for RNA quantity and integrity. Band densitometry analysis from electrophoresis gels was performed using the Sigma Gel software for Windows[®].

2.10. Determination of serum OVA-specific IgE

Serum levels of specific anti-OVA IgE were determined by ELISA. Briefly, 96 flat-bottom well microtiter plates (COSTAR) were coated with 20 μ g of OVA and stored overnight at 4 °C. The wells were then blocked with 100 μ l of 5% skim milk in PBS pH 7.4 for 1 h at 37 °C. A total of 100 μ l of mice sera diluted 1:10 in 5% PBS-milk were added and incubated for 1 h at 37 °C. Then, a goat anti-mouse IgE conjugated with biotin (Zymed) was added and incubated for 1 h at 37 °C. Then a streptavidine–peroxidase conjugate (Zymed) was added diluted 1:5000 and incubated for 1 h at 37 °C. The plates were washed eight times with 0.05% PBS-Tween 20 between incubations. Color was developed adding OPD in substrate buffer (Sanofi Diagnostic Pasteur). The reaction was arrested with 50 μ l H₂SO₄ and the OD determined at 495 nm.

2.11. Statistical analysis

All experiments were performed blindly and an unrelated researcher broke the code. The results were expressed as average and standard deviations. Multiple comparisons

between groups were performed by ANOVA (Kruskal–Wallis), further analysis using the Mann–Whitney *U*-test was performed for the comparison between two individual groups. All data were analyzed using SPSS for Windows[®] and a *P*-value ≤ 0.05 was considered as significant.

3. Results

3.1. Expression of the OmpC–OVA fusion protein

The OVA 323–339 amino acid sequence, which is a known allergenic epitope for BALB/c mice was fused to the OmpC porin from *S. enterica* serovar *Typhi* by genetic engineering as described in material and methods. In summary, the encoding sequence for the OmpC external loop 5 was substituted by the OVA 323–339 sequence though a single round of reverse PCR amplification using plasmid pST13 as template [12] and OmpC–OVA hybrid primers, the amplification product contained the OVA sequence an suitable restriction sites at each end which were digested and ligated. The parental pST13 and the resulting pST13-OVA_{323–339} plasmids were transformed in the porinless *E. coli*-UH302 (OmpC[−], OmpF[−], OmpA[−]) [13] and the strains were designed as *E. coli*-OMP and *E. coli*-OMPOVA, respectively.

Expression of the OmpC–OVA_{323–339} fusion protein was assessed by western blot analysis of crude protein extracts. A cocktail of monoclonal antibodies against OmpC revealed the 36 kDa protein in *E. coli*-OMP and *E. coli*-OMPOVA, but not in *E. coli*-UH302, whereas and a rabbit polyclonal anti-OVA_{323–339} serum revealed the protein only in *E. coli*-OPOVA (Fig. 1B). These results demonstrate that *E. coli*-OMPOVA expressed the passenger OVA_{323–339} peptide in the context of OmpC and that the fusion protein has similar molecular weight to the parental porin. This observation may be explained because the encoding loop 5 was substituted by the OVA_{323–339} sequence, thereby not affecting the amino acid number and resulting in a slight molecular weight change not detectable by electrophoresis. Surface expression of the OVA_{323–339} passenger peptide was further demonstrated by flow cytometry using antibodies against OmpC or OVA_{323–339}. Interestingly, OmpC–OVA expression was around 50% lower than the native OmpC which may be related to codon usage (data not shown).

3.2. *E. coli*-OMPOVA reduced pulmonary inflammation and goblet cells hyperplasia

Groups of BALB/c mice were rendered allergic to OVA with two i.p. injections of OVA-alum suspension 5 days apart and one intratracheal boost 1 week later. Four days after sensitization, the mice were treated with two doses of *E. coli*-OMP or *E. coli*-OMPOVA administered by intranasal route 1 week apart. The intensity of the allergic reaction was evaluated on day 31, assessing the extent of the pulmonary

inflammation, mucus production, IL-5 and IFN- γ transcription, and specific anti-OVA IgE antibodies. Control groups included mice “sensitized” only with SS (sham-ST) in order to discriminate the non-specific inflammatory response due to the sensitization procedure, and mice sensitized with OVA but treated only with SS. Pulmonary inflammation was estimated in H&E stained lung sections by morphological analysis in periarteriole, perivascular and peribronchial areas and was expressed in mm² of infiltrate.

Allergic mice treated with *E. coli*-OMPOVA showed significant reduction of perivascular cell infiltrates (Fig. 2A) in comparison to mice treated with *E. coli*-OMP ($\bar{x}$ = 2036 μ m² versus 5380 μ m², *P* < 0.05) or SS ($\bar{x}$ = 2036 μ m² versus 5419 μ m², *P* < 0.05); but more importantly, the inflammation level was similar to the sham-ST mice ($\bar{x}$ = 2036 μ m² versus 1439 μ m², *P* not significant). Similar results were obtained in peribronchial areas (Fig. 2B) and were consistent with the mucus production (Fig. 3). Mice treated with *E. coli*-OMPOVA presented 4.2 times less mucus than those treated with *E. coli*-OMP ($\bar{x}$ = 879 μ m² versus 3669 μ m² of PAS-positive material/40,000 mm²) and 3.3 times as compared with SS ($\bar{x}$ = 879 μ m² versus 2860 μ m², *P* < 0.05). Nevertheless, despite this decrease, the mucus production was still higher when compared with the sham-ST controls (2.4 times higher, $\bar{x}$ = 879 μ m² versus 362 μ m²).

Fig. 4 shows lung sections stained with PAS from the different experimental groups. Allergic mice treated with SS developed an intense inflammatory infiltrate and mucus production, affecting both perivascular and periarteriole regions with marked goblet cells hyperplasia and hypertrophy, and altered alveolar architecture (Fig. 4B). This overall picture was similar in mice treated with *E. coli*-OMP (Fig. 4C); but in contrast, mice treated with *E. coli*-OMPOVA (Fig. 4D) presented slight inflammatory infiltrate similar to the sham-ST mice (Fig. 4A). Taken together, these results demonstrate that treatment with *E. coli*-OMPOVA reduced the inflammation and the associated mucus production in mice sensitized with OVA.

3.3. *E. coli*-OMPOVA reduced BAL eosinophils

Leukocytes were measured in bronchio-alveolar lavages (BAL) from allergic mice and differential counts were determined by microscopy in H/E stained cytospin preparations. While in sham-ST mice monocytes predominated in BAL, mice treated only with SS developed important leukocytosis characterized mainly by monocytes and lymphocytes. Although treatment with *E. coli*-OMP or *E. coli*-OMPOVA tended to diminish the total BAL cell yield no statistical differences were found, and the proportion of macrophages, lymphocytes and neutrophils were also similar (Fig. 5A).

Nevertheless, eosinophils presented a different behavior. Allergic mice treated with SS developed important eosinophilia in comparison to sham-ST mice (30.8 times

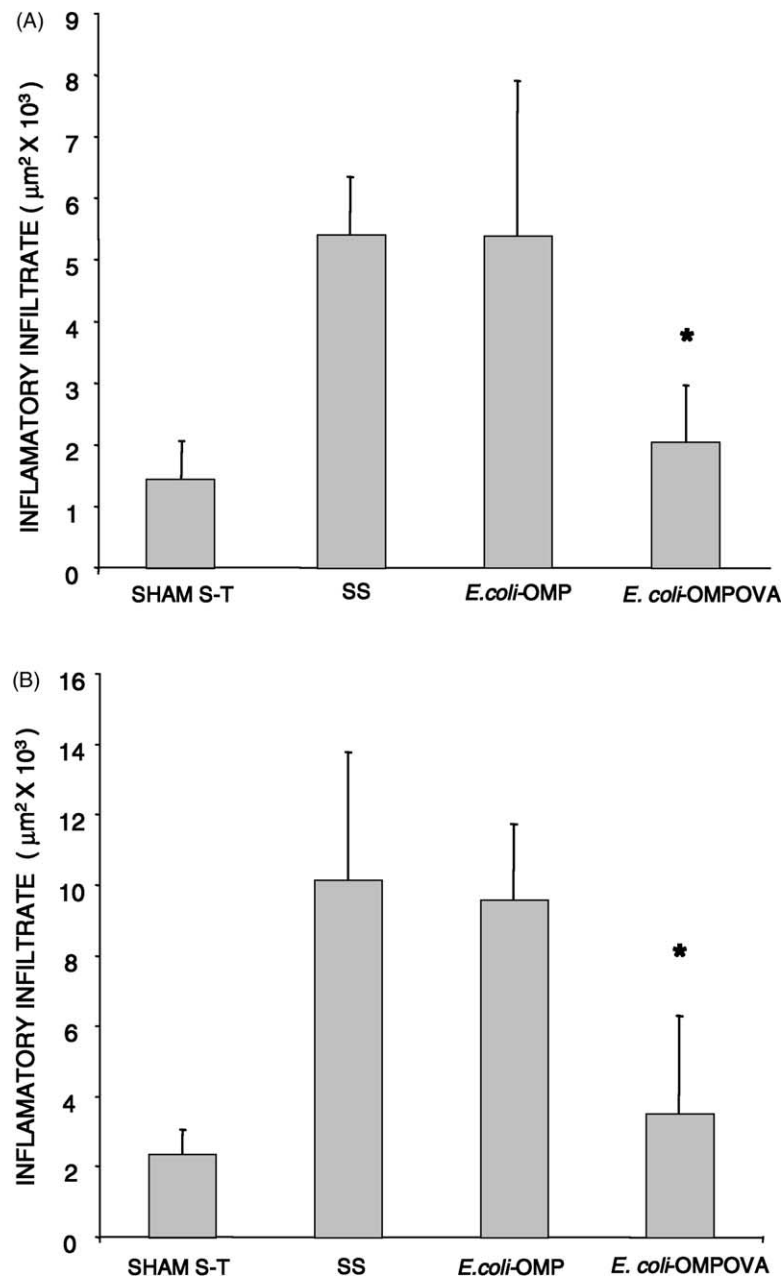


Fig. 2. *E. coli*-OMPOVA reduced the lung inflammatory infiltrate in allergic mice to OVA. Lung sections from five mice from each experimental group were stained with H/E and analyzed under light microscopy (40×). The inflammatory infiltrate was measured in venules and middle size veins with 100–200 μm of diameter, and 150–300 μm diameter bronchi using an image analyzer (five venules or bronchi from five to eight lung sections were analyzed for each experimental group). Results are expressed as averages and standard deviations of infiltrate area. (A) Perivascular infiltrate. (B) Peribronchial infiltrate. * $P < 0.05$ comparisons between groups (ANOVA).

higher, $\bar{x} = 39,771$ cells/ml versus 1295 cells/ml). Mice treated with *E. coli*-OMP presented a slight eosinophils reduction when compared with SS controls (21,178 cells/ml versus 39,771 cells/ml), but this difference did not reach statistical significance. In contrast, mice treated with *E. coli*-OMPOVA showed important reduction of BAL eosinophilia, being 6.8 times lower than in mice treated with SS ($\bar{x} = 5857$ cells/ml versus 39,771 cells/ml, $P < 0.05$) (Fig. 5B).

3.4. *E. coli*-OMPOVA decreased IL-5 and induced IFN-γ expression

IL-5 and IFN-γ mRNA transcription was examined in cells harvested from BAL by RT-PCR and estimated by densitometry analysis considering the GAPDH amplification as reference. As expected, sham ST mice did not express IL-5 nor IFN-γ and mice sensitized with OVA and treated with SS expressed IL-5 but not IFN-γ. Mice treated

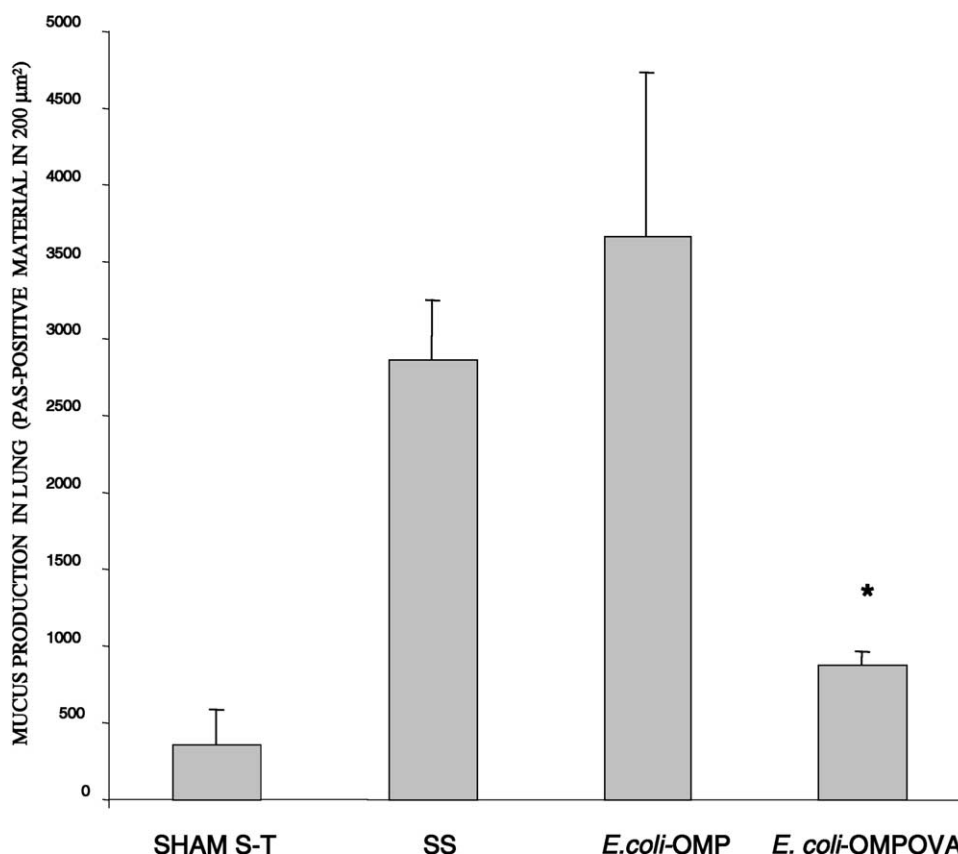


Fig. 3. *E. coli*-OMPOVA reduced mucus production in lungs from allergic mice to OVA. Lung sections from five mice from each experimental group were stained with PAS and analyzed under light microscopy (40 $\times$). The PAS-positive material was measured in 200 μm^2 squares (40,000 μm^2) using an image analyzer (five squares from five to eight lung sections were analyzed for each experimental group). Results are expressed as averages and standard deviations of PAS-positive area (μm^2). * $P < 0.05$ comparisons between groups (ANOVA).

with *E. coli*-OMPOVA showed important and significant decrease in IL-5 mRNA expression and induction of IFN- γ . Noteworthy, *E. coli*-OMP also induced IFN- γ transcription; observation which may be explained by the fact that both strains contain LPS which promotes Th1 cytokines. Nevertheless, the densitometry analysis demonstrated that treatment with *E. coli*-OMPOVA induced more IFN- γ transcription (IFN- γ /GAPDH ratio $\bar{x} = 0.6$ versus 0.3), and inhibited IL-5 expression (IL-5/GAPDH ratio $\bar{x} = 0.6$ versus 2.0). Moreover, no differences were observed in IL-5 mRNA between mice treated with *E. coli*-OMP and SS (IL-5/GAPDH ratio $\bar{x} = 2.3$ versus 2.0). Taken together, these findings are consistent with an antigen specific response to the OVA allergenic peptide induced by *E. coli*-OMPOVA (Fig. 6).

3.5. Specific IgE anti-OVA in sera

Specific IgE anti-OVA was measured by ELISA in serum of allergic mice before and after treatment with *E. coli*-OMP or *E. coli*-OMPOVA. Although mice treated with *E. coli*-OMPOVA presented slight decrease of IgE anti-OVA, the difference was non-significant in comparison with mice receiving *E. coli*-OMP (Fig. 7A). In addition, it

was not possible to demonstrate specific IgG2a anti-OVA after treatment with *E. coli*-OMPOVA, and only control mice immunized with OVA in complete Freund's adjuvant presented strong IgG2a production (Fig. 7B).

4. Discussion

Eosinophilic inflammation is a hallmark feature in immediate hypersensitivity reactions, such as allergic rhinitis, atopic dermatitis, sinusitis and asthma. Several experimental models have been developed to assess its physiopathological role in allergy. Mice sensitized to OVA develop localized allergic reactions in the skin, nose or lung depending on the administration route and immunization schedule [1]. In the present study, BABL/c mice developed an allergic airway inflammation in response to OVA as demonstrated by the presence of specific IgE antibodies against OVA, eosinophilia in BAL, mucus overproduction, goblet cells hyperplasia and intense lung perivascular and peribronchial infiltrates of eosinophils, lymphocytes and macrophages. This model was selected since pulmonary inflammation and mucus overproduction are two main components of asthma.

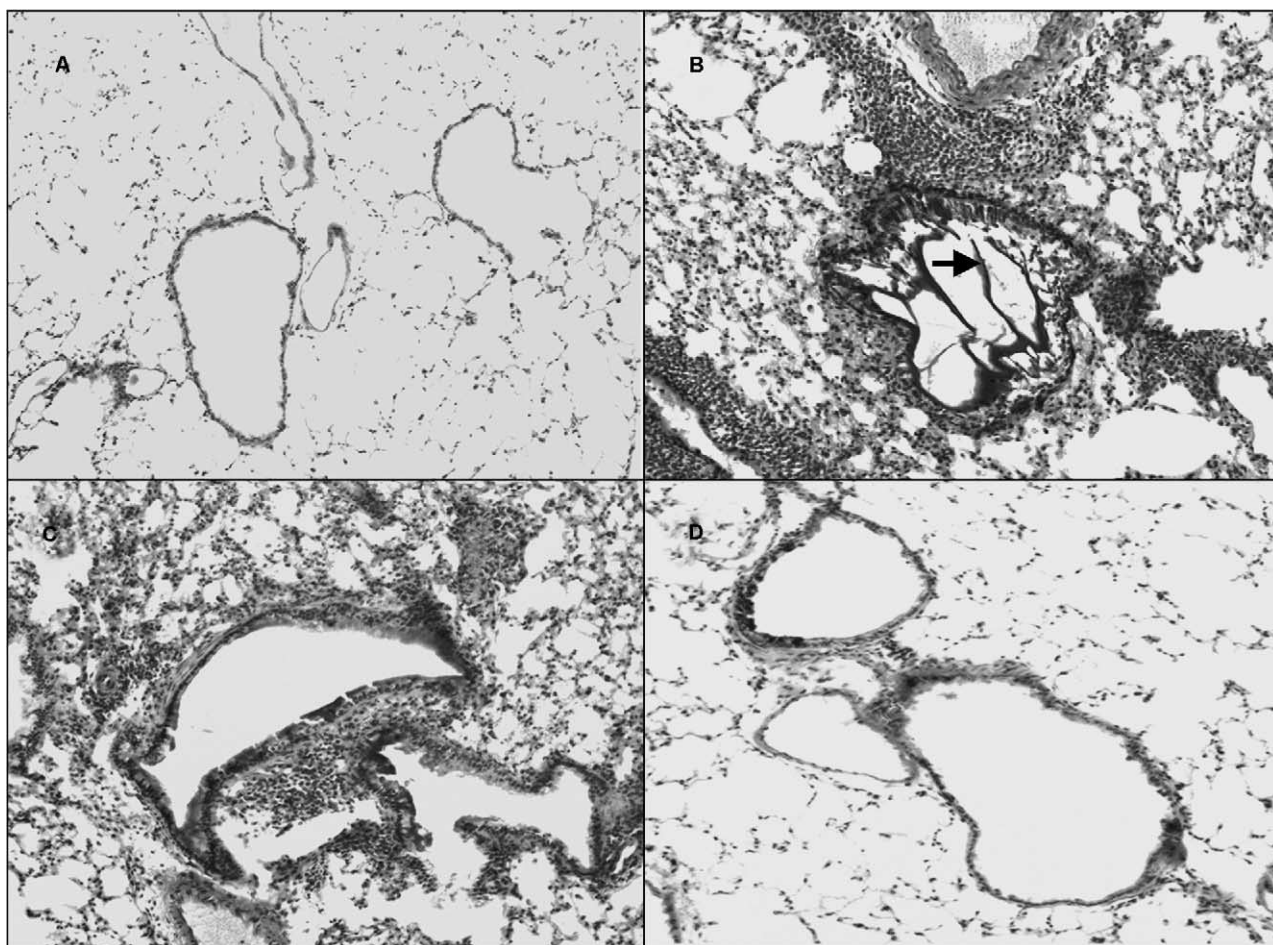


Fig. 4. Photomicrographs of lung sections from mice allergic to OVA and treated with *E. coli*-OMPOVA. Representative lung histopathology of sham and OVA sensitized mice. (A) Normal histological appearance from sham mouse. (B) OVA allergic mouse treated with saline solution shows thick mucus layer covering all the surface of the bronchial epithelium (arrows), and mononuclear inflammatory infiltrate surrounding the blood vessels and bronchial walls. (C) Intranasal administration of *E. coli*-OMP in OVA sensitized mice produced mild reduction of mucus production with similar amount of inflammatory cells than control OVA-sensitized animals treated with saline solution. (D) In contrast, *E. coli*-OMPOVA suppressed mucus overproduction and significantly decreased the inflammatory infiltrate. All sections PAS stained, 100 $\times$ magnification.

Once sensitized, the mice were treated with an *E. coli* bearing the allergenic peptide. The porinless *E. coli*-UH302 strain was transformed with a plasmid bearing a hybrid gene constructed by inserting the allergenic sequence of OVA (amino acid 323–339) in the external loop 5 of OmpC from *S. enterica* serovar *Typhi*. This porin was selected as carrier because it has been demonstrated to induce humoral and cellular protective immune responses in mice [20,21] and has permissive insertion sites for foreign antigens [14,22]. Allergic mice treated with the resulting *E. coli*-OMPOVA strain presented important reduction of eosinophilia in BAL, goblet cells hyperplasia, and lung inflammatory infiltrates. The decrease of IL-5 mRNA transcription is consistent with the improvement of inflammation because this cytokine plays an important role in eosinophil expansion and promotes local chemotactic signals [23]. Moreover, the increased IFN- γ mRNA expression suggests that this cytokine may have also contributed since it has been demonstrated to directly inhibit eosinophil expansion [24]. Although the fusion OmpC–OVA

protein expression was not measured, flow cytometry and western blot analyses strongly suggested that its expression was weaker as compared with the wild type OmpC and we could attribute this difference to codon usage by *E. coli*. This poor expression could have influence in the outcome of the immune response, since it has been described that the quantity of antigen and administration route are important factors for the modulation of Th1/Th2 profiles, suggesting that better Th1 responses are obtained from a smaller amount of antigen [25].

Nevertheless, the RT-PCR used in this report is a semi-quantitative method with important margin of error; therefore, more detailed studies are necessary to assess the influence of the bacterial vector on the Th1/Th2 cytokine profile modulation.

The contribution of the bacterial LPS to the overall phenomenon may be assumed by comparing the effects of *E. coli*-OMP and *E. coli*-OMPOVA on the inflammatory response. Whereas mice treated with *E. coli*-OMP presented

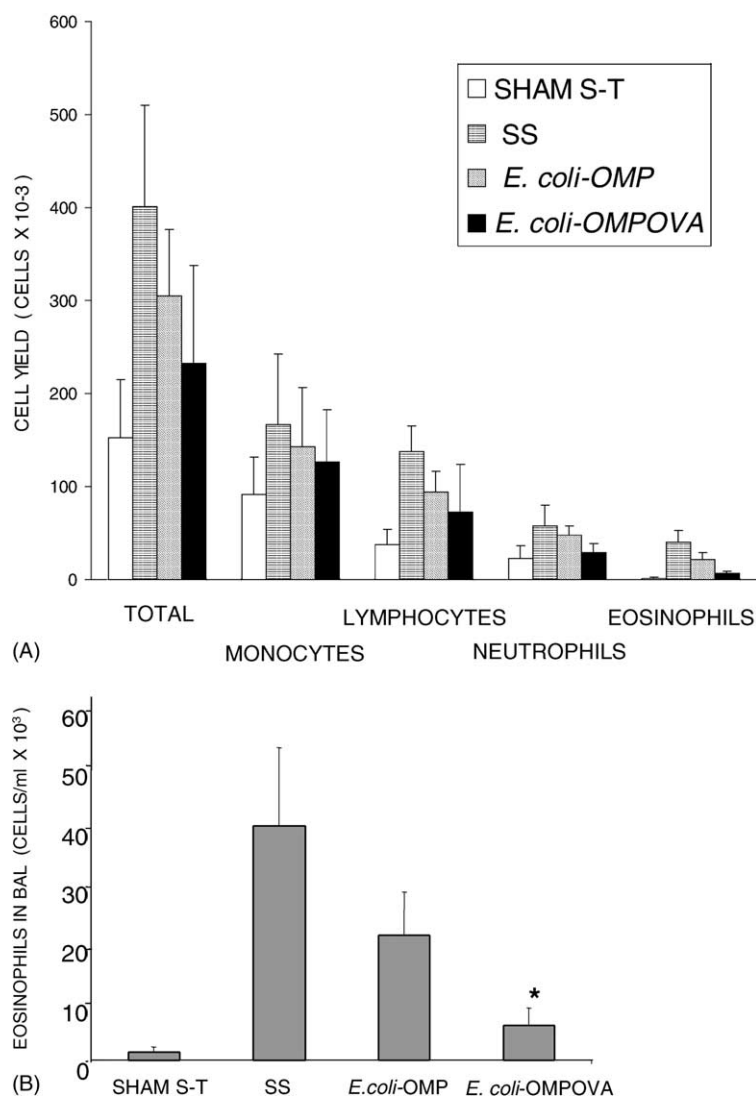


Fig. 5. *E. coli*-OMPOVA inhibited BAL eosinophils in mice allergic to OVA. (A) Cell composition in BAL. Total leukocytes and differential counts determined in BAL from mice allergic to OVA. (B) Eosinophils in BAL. Cytospin BAL preparations were stained with H/E and the differential count of 500 cells per mouse was done (100 $\times$). Results are presented as the average and standard deviations from five mice from each experimental group. * $P < 0.05$, comparison between groups (ANOVA; Kruskal–Wallis).

eosinophils reduction in BAL and induced IFN- γ mRNA expression, neither the inflammatory infiltrates nor the IL-5 mRNA expression were modified. These observations suggest that the therapeutic effect of *E. coli*-OPMOVA was achieved by eliciting both specific and non-specific immune responses. Although a Th1 profile was not demonstrated, IgE antibodies against OVA were not reduced, and no switching to IgG2a was obtained. The main finding is that the known allergenic epitope of OVA delivered in the context of a bacterial OMP produced a clear inhibition of the inflammatory response, IL-5 mRNA reduction and IFN- γ induction. The non-specific immune response was expected and may be explained by the immunomodulatory activity of several bacterial constituents. It has been demonstrated that the administration of whole bacteria, such as *Mycobacterium vaccae*,

BCG or *Listeria monocytogenes* promotes the differentiation of Th1 CD4⁺ T cells [26,27] and that CpG oligonucleotides can inhibit allergic pulmonary inflammation and airway hyper-reactivity [28]. Moreover, it has been demonstrated that at least one porin (OmpA) induces dendritic cells maturation and IL-12 production [29]. On the other hand, the role of LPS is controversial. Some studies suggest that airborne LPS is a risk factor for asthma severity [30,31], and can trigger bronchiospasm [32], LBP-deficient mice do not develop airway hyper-reactivity to aerosolized allergens and produce insufficient iNOS and peroxynitrites; nevertheless, they develop pulmonary inflammation [33]. However, in the present study whole bacteria were used as antigen vector. Thus, since LPS is linked to bacterial proteins and other immunomodulators could expect a predominant Th1 response.

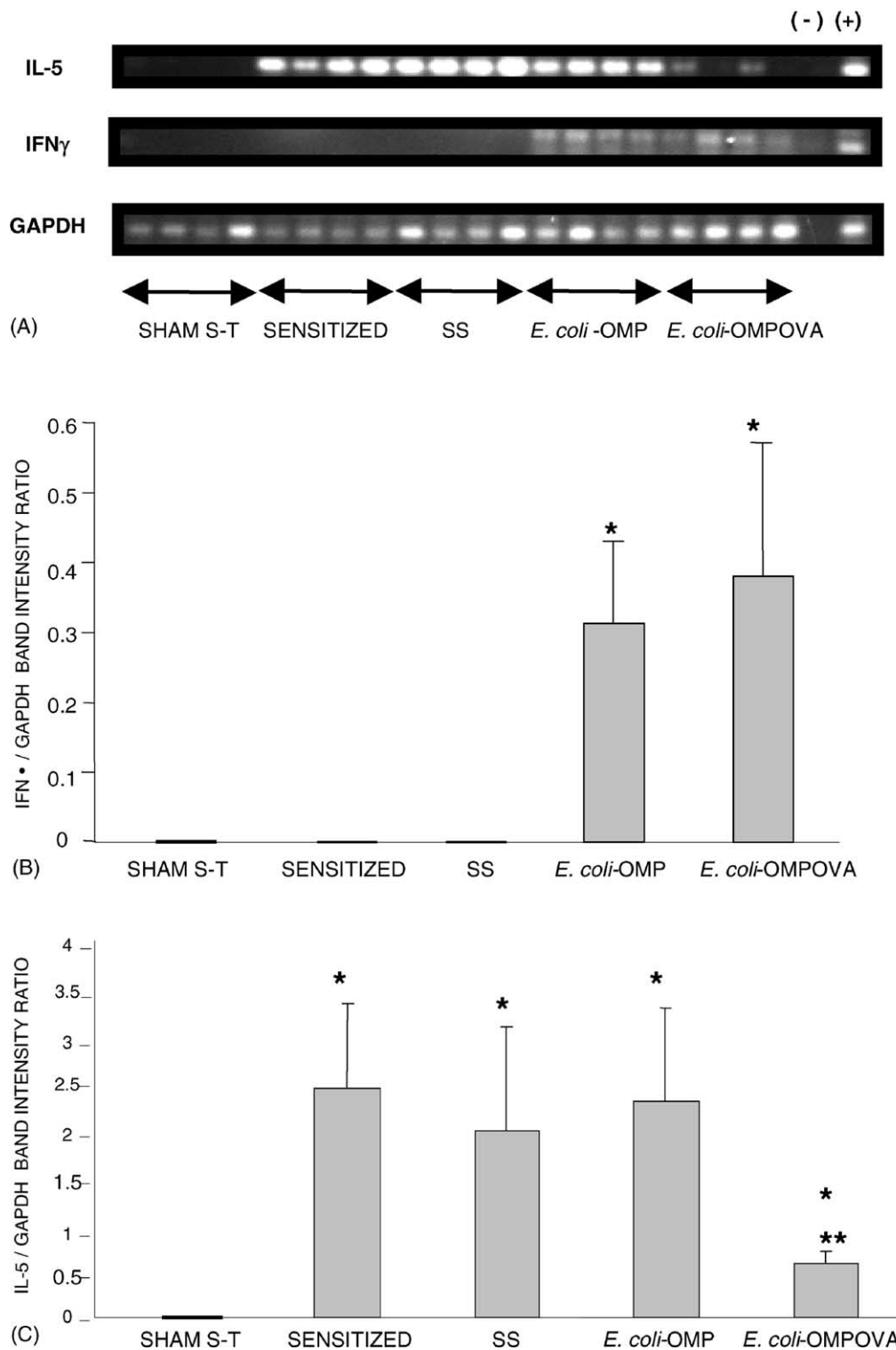


Fig. 6. *E. coli*-OMPOVA reduces IL-5 and enhances IFN γ transcription. (A) Transcription analysis was performed by RT-PCR in mRNA purified from BAL. Individual amplifications from four mice for each experimental group are presented. The constitutive gene GAPDH was amplified as control for RNA integrity. Negative (–) and positive (+) controls are shown to demonstrate absence of DNA contamination. (B and C) Densitometry analysis of RT-PCR bands, intensity of cytokine and GAPDH PCR bands in the original photographs of the agarose gels, were quantified by scanning with a contrast scanner using the Sigma Gel optical software, averages were obtained and the ratios against GAPDH were calculated. (B) IFN- γ /GAPDH ratio. (C) IL-5/GAPDH ratio. * $P < 0.05$ comparisons with control groups (*U* Mann–Whitney). ** $P < 0.05$ comparison between groups (ANOVA).

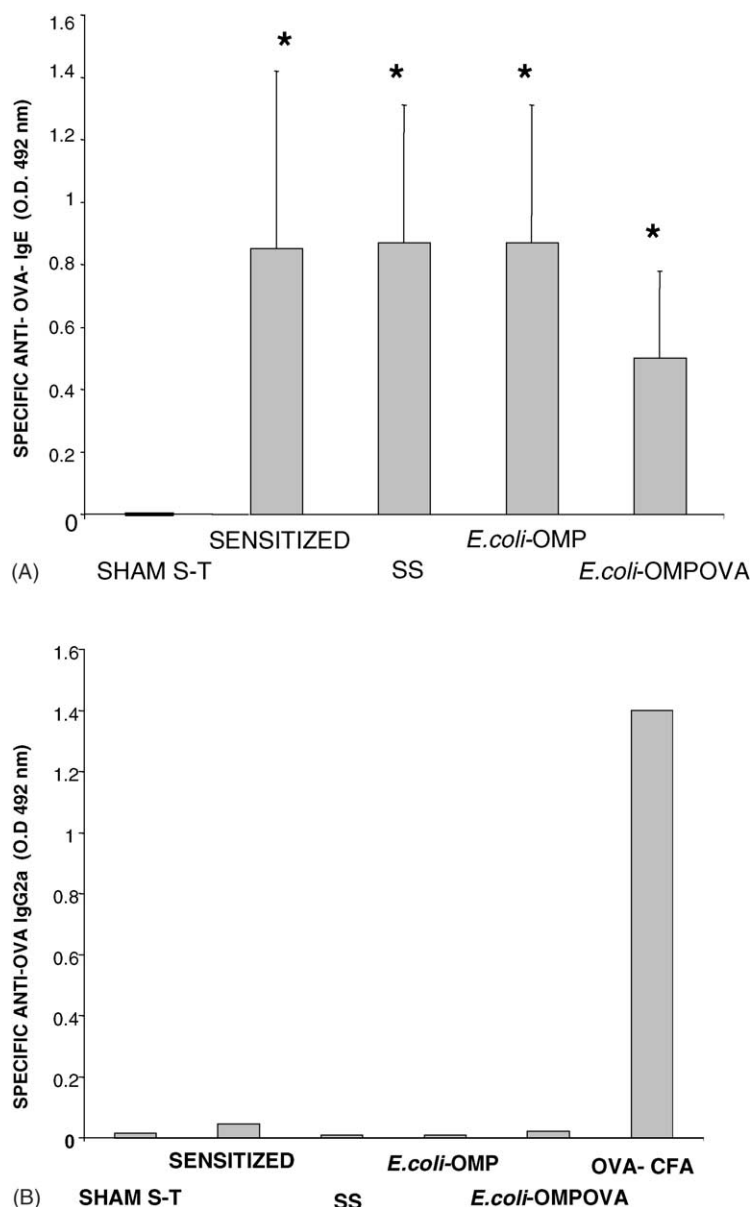


Fig. 7. IgE and IgG2 determination. (A) Specific IgE and (B) IgG2a were determined by ELISA. The results are presented as average and standard deviations of 10 mice for each experimental group. * $P < 0.05$ comparisons with the control groups (U Mann–Whitney). $P = 0.08$, comparisons between groups (ANOVA; Kruskal–Wallis).

Although the bacterial vector was able to moderate the pulmonary inflammatory response, more extensive studies are necessary to assess its effectiveness in asthma. The physiopathological association between airway inflammation and hyper-reactivity and the eosinophil role in this process remain controversial [34,35]. Some authors identify temporal association between Th2 cytokine production, tissue eosinophil infiltration and development of airway hyper-reactivity [36], whereas others find that infiltration precedes the development of airway hyper-reactivity and mucosal exudation [37], or airway hyper-reactivity before development of an eosinophilic airway inflammation [38], and airway responsiveness in the absence of edema [39].

Moreover, eosinophilic airway inflammation may not account for all the clinical manifestations of asthma, which may result from airway remodeling and irreversible airflow obstruction [40].

We have also evaluated whether the administration of the bacterial vector bearing the OmpC–OVA fusion protein before sensitization with OVA, could prevent the development of allergy. Our results in two independent experiments were consistent and showed that despite a clear tendency to diminish the inflammatory pulmonary response, goblet cells hyperplasia, eosinophilia and IgE anti-OVA, the pre exposition treatment did not reach statistical significance. Therefore, different immunization schedules should be attempted.

In summary, we demonstrated that an *E. coli* strain carrying an allergenic peptide as fused protein to OmpC administered i.n. to BALB/c mice with allergic airway inflammatory response to OVA, was able to modulate the inflammatory infiltrate, lung eosinophilia, mucus overproduction and goblet cells hyperplasia. IL-5 mRNA as diminished and IFN- γ mRNA was induced. These observations suggest that bacterial vector based approaches may be attempted for the treatment of allergic disorders.

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References

- [1] Leong KP, Huston DP. Understanding the pathogenesis of allergic asthma using mouse models. *Ann Allergy Asthma Immunol* 2001;87(2):96–109.
- [2] Renaud JC. New insights into the role of cytokines in asthma. *J Clin Pathol* 2001;54(8):577–89.
- [3] Kim TS, DeKruyff RH, Rupper R, Maecker HT, Levy S, Umetsu DT. An ovalbumin-IL-12 fusion protein is more effective than ovalbumin plus free recombinant IL-12 in inducing a T helper cell type 1-dominated immune response and inhibiting antigen-specific IgE production. *J Immunol* 1997;158(9):4137–44.
- [4] Hofstra CL, Van A I, Hofman G, Kool M, Nijkamp FP, Van Oosterhout AJ. Prevention of Th2-like cell responses by coadministration of IL-12 and IL-18 is associated with inhibition of antigen-induced airway hyperresponsiveness, eosinophilia, and serum IgE levels. *J Immunol* 1998;161(9):5054–60.
- [5] Lack G, Bradley KL, Hamelmann E, Renz H, Loader J, Leung DY, et al. Nebulized IFN- γ inhibits the development of secondary allergic responses in mice. *J Immunol* 1996;157(4):1432–9.
- [6] Kung TT, Stelts DM, Zurcher JA, Jones H, Umland SP, Egan RW, et al. Interferon-gamma and antibodies to interleukin-5 and interleukin-4 inhibit the pulmonary eosinophilia in allergic mice. *Inflamm Res* 1995;44(Suppl 2):S185–6.
- [7] Woodruff PG, Fahy JV. Asthma: prevalence, pathogenesis, and prospects for novel therapies. *JAMA* 2001;286(4):395–8.
- [8] Bumann D, Hueck C, Aebischer T, Meyer TF. Recombinant live *Salmonella* spp. for human vaccination against heterologous pathogens. *FEMS Immunol Med Microbiol* 2000;27(4):357–64.
- [9] Medzhitov R. CpG DNA: security code for host defense. *Nat Immunol* 2001;2(1):15–6.
- [10] Cowan SW, Schirmer T, Rummel G, Steiert M, Ghosh R, Pauptit RA, et al. Crystal structures explain functional properties of two *E. coli* porins. *Nature* 1992;358(6389):727–33.
- [11] Charbit A, Newton SM, Klebba PE, Clement JM, Fayolle C, Lo-Man R, et al. Expression and immune response to foreign epitopes in bacteria. Perspectives for live vaccine development. *Behring Inst Mitt* 1997;98:135–42.
- [12] Agüero J, Mora G, Mroczenski-Wildey MJ, Fernandez-Beros ME, Aron L, Cabello FC. Cloning, expression and characterization of the 36 KDa *Salmonella typhi* porin gene in *Escherichia coli*. *Microb Pathog* 1987;3(6):399–407.
- [13] Cole ST, Sonntag I, Henning U. Cloning and expression in *Escherichia coli* K-12 of the genes for major outer membrane protein OmpA from *Shigella dysenteriae*, *Enterobacter aerogenes*, and *Serratia marcescens*. *J Bacteriol* 1982;149(1):145–50.
- [14] Paniagua J, Martin N, Ortiz V, Ramirez G, Gonzalez CR, Isibasi A. Predicted epitopes of *Salmonella typhi* OmpC porin are exposed on the bacterial surface. *Immunol Infect Dis* 1995;5:244–9.
- [15] Kissel V, Gonzalez C, Astudillo M, Godard A, Wachman B, Cabello FC. *Salmonella*-specific monoclonal antibodies against recombinant *Salmonella typhi* 36-kilodalton porin. *Clin Diagn Lab Immunol* 1994;1(2):250–2.
- [16] Hernandez-Pando R, Rook GA. The role of TNF-alpha in T-cell-mediated inflammation depends on the Th1/Th2 cytokine balance. *Immunology* 1994;82(4):591–5.
- [17] Chomczynski P. A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *Biotechniques* 1993;15(3):532–7.
- [18] Miralles GD, Stoeckle MY, McDermott DF, Finkelman FD, Murray HW. Th1 and Th2 cell-associated cytokines in experimental visceral leishmaniasis. *Infect Immun* 1994;62(3):1058–63.
- [19] Sugaya H, Aoki M, Abe T, Ishida K, Yoshimura K. Cytokine responses in mice infected with *Angiostrongylus cantonensis*. *Parasitol Res* 1997;83(1):10–5.
- [20] Isibasi A, Ortiz-Navarrete V, Paniagua J, Pelayo R, Gonzalez CR, Garcia JA, et al. Active protection of mice against *Salmonella typhi* by immunization with strain-specific porins. *Vaccine* 1992;10(12):811–3.
- [21] Isibasi A, Paniagua J, Rojo MP, Martin N, Ramirez G, Gonzalez CR, et al. Role of porins from *Salmonella typhi* in the induction of protective immunity. *Ann NY Acad Sci* 1994;730:350–2.
- [22] Puente JL, Juarez D, Bobadilla M, Arias CF, Calva E. The *Salmonella ompC* gene: structure and use as a carrier for heterologous sequences. *Gene* 1995;156(1):1–9.
- [23] Foster PS, Mould AW, Yang M, Mackenzie J, Mattes J, Hogan SP, et al. Elemental signals regulating eosinophil accumulation in the lung. *Immunol Rev* 2001;179:173–81.
- [24] Cohn L, Herrick C, Niu N, Homer R, Bottomly K. IL-4 promotes airway eosinophilia by suppressing IFN- γ production: defining a novel role for IFN- γ in the regulation of allergic airway inflammation. *J Immunol* 2001;166(4):2760–7.
- [25] Power CA, Wei G, Bretscher PA. Mycobacterial dose defines the Th1/Th2 nature of the immune response independently of whether immunization is administered by the intravenous, subcutaneous, or intradermal route. *Infect Immun* 1998;66(12):5743–50.
- [26] Erb KJ, Holloway JW, Sobeck A, Moll H, Le Gros G. Infection of mice with *Mycobacterium bovis*-Bacillus Calmette-Guérin (BCG) suppresses allergen-induced airway eosinophilia. *J Exp Med* 1998;187(4):561–9.
- [27] Yeung VP, Gieni RS, Umetsu DT, DeKruyff RH. Heat-killed *Listeria monocytogenes* as an adjuvant converts established murine Th2-dominated immune responses into Th1-dominated responses. *J Immunol* 1998;161(8):4146–52.
- [28] Serebrisky D, Teper AA, Huang CK, Lee SY, Zhang TF, Schofield BH, et al. CpG oligodeoxynucleotides can reverse Th2-associated allergic airway responses and alter the B7.1/B7.2 expression in a murine model of asthma. *J Immunol* 2000;165(10):5906–12.
- [29] Jeannin P, Renno T, Goetsch L, Miconnet I, Aubry JP, Delneste Y, et al. OmpA targets dendritic cells, induces their maturation and delivers antigen into the MHC class I presentation pathway. *Nat Immunol* 2000;1(6):502–9.

- [30] Reed CE, Milton DK. Endotoxin-stimulated innate immunity: a contributing factor for asthma. *J Allergy Clin Immunol* 2001;108(2):157–66.
- [31] Schwartz DA. Does inhalation of endotoxin cause asthma? *Am J Respir Crit Care Med* 2001;163(2):305–6.
- [32] Michel O, Ginanni R, Le Bon B, Content J, Duchateau J, Sergysels R. Inflammatory response to acute inhalation of endotoxin in asthmatic patients. *Am Rev Respir Dis* 1992;146(2):352–7.
- [33] Strohmeier GR, Walsh JH, Klings ES, Farber HW, Cruikshank WW, Center DM, et al. Lipopolysaccharide binding protein potentiates airway reactivity in a murine model of allergic asthma. *J Immunol* 2001;166(3):2063–70.
- [34] Webb DC, McKenzie AN, Matthaie KI, Rothenberg ME, Foster PS. Distinct spatial requirement for eosinophil-induced airways hyper-reactivity. *Immunol Cell Biol* 2001;79(2):165–9.
- [35] Danahay H, Broadley KJ, McCabe PJ, Nials AT, Sanjar S. Temporal relationships between leukocytes, IL-5 and IL-8 in guinea pig lungs, plasma cortisol and airway function after antigen challenge. *Inflamm Res* 1999;48(1):41–7.
- [36] Tomkinson A, Cieslewicz G, Duez C, Larson KA, Lee JJ, Gelfand EW. Temporal association between airway hyperresponsiveness and airway eosinophilia in ovalbumin-sensitized mice. *Am J Respir Crit Care Med* 2001;163(3 Pt 1):721–30.
- [37] Van Oosterhout AJ, Fattah D, Van A I, Hofman G, Buckley TL, Nijkamp FP. Eosinophil infiltration precedes development of airway hyper-reactivity and mucosal exudation after intranasal administration of interleukin-5 to mice. *J Allergy Clin Immunol* 1995;96(1):104–12.
- [38] To Y, Dohi M, Tanaka R, Sato A, Nakagome K, Yamamoto K. Early interleukin-4-dependent response can induce airway hyper-reactivity before development of airway inflammation in a mouse model of asthma. *Lab Invest* 2001;81(10):1385–96.
- [39] Peebles Jr RS, Wagner EM, Liu MC, Proud D, Hamilton RG, Togias A. Allergen-induced changes in airway responsiveness are not related to indices of airway edema. *J Allergy Clin Immunol* 2001;107(5):805–11.
- [40] Homer RJ, Elias JA. Consequences of long-term inflammation. Airway remodeling. *Clin Chest Med* 2000;21(2):331–43.