

Sublingual Omp16-driven redirection of the allergic intestinal response in a pre-clinical model of food allergy

Short running title: Sublingual Omp16 controls experimental food allergy

Maria Lucía Orsini Delgado¹, Gastón Pascual Rizzo¹, Carlos Alberto Fossati¹,
Karina Alejandra Pasquevich², Juliana Cassataro², Paola Lorena Smaldini^{1*} and
Guillermo Horacio Docena^{1*}

¹Instituto de Estudios Inmunológicos y Fisiopatológicos-IIFP, Facultad de Ciencias Exactas, Universidad Nacional de La Plata y Consejo Nacional de Investigaciones Científicas y Técnicas, La Plata, Argentina.

²Instituto de Investigaciones Biotecnológicas “Dr. Rodolfo A. Ugalde”, Universidad Nacional de San Martín y Consejo Nacional de Investigaciones Científicas y Técnicas, San Martín, Argentina.

**PLS and GHD share senior authorship.*

Corresponding author:

Guillermo H Docena, PhD

Instituto de Estudios Inmunológicos y Fisiopatológicos IIFP, Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas, Universidad Nacional de La Plata UNLP - CONICET, Calle 120 nro 1489, CP 1900 La Plata, Argentina.

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Corresponding author mail id: guillermo.docena@gmail.com

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**PLS and GHD share senior authorship.*

Corresponding author:

Guillermo H Docena, PhD

Instituto de Estudios Inmunológicos y Fisiopatológicos IIFP, Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas, Universidad Nacional de La Plata UNLP - CONICET, Calle 120 nro 1489, CP 1900 La Plata, Argentina.

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Abstract

Background: IgE-mediated food allergy remains a significant and growing worldwide problem. Sublingual immunotherapy (SLIT) shows an excellent safety profile for food allergy, but the clinical efficacy needs to be improved. This study assessed the effects of the Toll-like receptor 4 agonist outer membrane protein (Omp) 16 from *Brucella abortus* combined with cow's milk proteins (CMP) through the sublingual route to modulate cow's milk allergy in an experimental model.

Methods: Mice sensitized with cholera toxin and CMP were orally challenged with the allergen to elicit hypersensitivity reactions. Then, mice were treated with a very low amount of CMP along with Omp16 as a mucosal adjuvant, and finally, animals were re-exposed to CMP. Systemic and mucosal immune parameters were assessed *in vivo* and *in vitro*.

Results: We found that the sublingual administration of Omp16+CMP induced a buccal Th1-immune response that modulated the intestinal allergic response with the suppression of symptoms, reduction of IgE and IL-5, and up-regulation of IgG2a and IFN- γ . The adoptive transfer of submandibular IFN- γ -producing $\alpha\beta\gamma^+CD4^+$ and $CD8^+$ cells conferred protection against allergic sensitization. The use of Omp16+CMP promoted enhanced protection compared to CMP alone.

Conclusion: In conclusion, Omp16 represents a promising mucosal adjuvant that can be used to improve the clinical and immune efficacy of SLIT for food allergy.

Keywords: Food allergy, Mouse model, Sublingual immunotherapy, Omp16, IgE

Introduction

Vaccination is the most successful application of immunological knowledge, and several vaccines were developed and tested against many infectious and non-infectious diseases. However, only a limited number came to the market, and new strategies have been developed in the last decades. The initial site of entry and immune recognition of the antigen should be considered to design an immune-intervention that promotes protection. As mucosa-associated inflammation is central to control several infectious and non-infectious disorders, the development of new mucosal vaccines that may provide protective immune responses both in the mucosal and systemic immune compartments is of paramount importance [1]. The mucosal vaccine efficacy is highly dependent on the use of potent adjuvants. The *Brucella* outer membrane proteins (Omp) Omp16 and Omp19 were employed as antigens -Ags- in vaccine formulations against *Brucella*, and both demonstrated to be effective [2–4]. Omp16 and Omp19 were also used as mucosal adjuvants. They showed to boost the mucosal immune system and increased the vaccine immunogenicity by targeting dendritic cells [5,6] and Th1-mediated immune responses against the co-administered Ags [3,6]. Also, the use of Omp16 as an adjuvant has shown promissory results in immunotherapies for the immunomodulation of inflammatory mucosal disorders such as food allergy [5,7].

Food allergies (FA) are immune-mediated adverse reactions to food proteins that can be mediated by IgE- or non-IgE-dependent mechanisms. The former is on the rise worldwide and constitutes the target for immune intervention as it can provoke life-threatening anaphylactic reactions. Despite advances made in understanding the underlying mechanisms of food allergy, currently approved treatments consist of strict avoidance of the allergen, nutritional assessment, and pharmacological management of symptoms in case of accidental exposure to the allergen. Recently, it has been recommended by the FDA treatment for life-threatening peanut allergies in children, which consists of oral or subcutaneous immunotherapy with peanuts. Nevertheless, most of the concerns are still focused on adverse reactions and safety.

In previous studies, we showed that the intragastric or intranasal administration of Omp16 along with CMP mitigated the allergic reaction against CMP through the induction of IFN- γ -producing mucosal T cells in an IgE-mediated food allergy mouse model. The induction of a Th1-dominated environment reversed allergic intestinal inflammation [5,7]. These studies characterized the pro-Th1 mucosal adjuvant properties of Omp16 to abrogate

the type 1 hypersensitivity reactions in sensitized mice. More recently, the sublingual immunization route has received increased attention in infectious and non-infectious diseases. It has been safely used in human and animal models for vaccination [8,9] and antigen-specific immunotherapy [10,11] to boost the local immune system and elicit a local and systemic response. It is known that the buccal mucosa is a site for the rapid absorption of antigens [12], and distribution into the systemic compartments [13,14] to promote effective adaptive immune responses [15]. Vaccine components are taken up by intraepithelial CD11c⁺ dendritic cells of the sublingual mucosa [16], which then migrate to the draining lymph nodes for antigen presentation and priming of lymphocytes with the capacity to migrate to related mucosa [17].

Thus, in this work, we investigated the capacity of Omp16 as an adjuvant for the sublingual administration of low amounts of the allergen to suppress the local intestinal and systemic inflammation and restore homeostasis. We showed that the formulation containing Omp16 and the allergen delivered sublingually inhibited the production and secretion of IgE, and consequently negativized the skin test, and reduced allergic symptoms following the oral exposure to milk proteins.

Methods

Reagents and adjuvants

The milk protein extract was prepared as described by Candreva et al. [18]; cholera toxin (CT) (Sigma Aldrich, St. Louis, USA) used as a mucosal adjuvant was dissolved in bicarbonate buffer [19], and OVA (Sigma Aldrich, St. Louis, USA) was used as a control antigen.

Recombinant unlipidated outer membrane protein 16 of *Brucella abortus* or Omp16 was obtained as previously described [5], and detoxified with a Sepharose polymyxin B resin (Sigma-Aldrich). Endotoxin level was assessed with the Limulus amoebocyte chromogenic assay (LONZA), and protein extracts with less than 0.10 endotoxin U/mg protein were used in all the experiments.

Animals

Female specific pathogen-free five to seven-week-old BALB/c mice were purchased from the School of Animal Science at the University of La Plata. Animals were housed in a conventional animal care facility and manipulated following international guidelines of animal experimentation. The Institutional Committee for the Care and Use of Laboratory Animals of the School of Sciences (University of La Plata) approved the protocol (Protocol Number: 017-00-15).

Sensitization and challenge

Sensitization was done according to Smaldini et al. 2012 [19]. Briefly, mice (n=5/group) received 6 weekly intragastric (ig) immunizations with 10 µg CT and 20 mg CMP in 200 µl of bicarbonate buffer. Ten days following the final boost, mice were ig challenged with CMP (10mg/200 µl) in two consecutive days (Sensitized group). As a control, age- and sex-matched naïve mice received 6 weekly intragastric CMP without CT (Control group). The experimental design is shown in **Fig. 1A**.

Sublingual treatment

Sensitized mice were anesthetized and received 4µl of different sublingual treatments twice a week for eight weeks (2 rounds of treatments separated by oral challenges). Mice were administered with: a) PBS_{sl} (Sensitized group), b) 10pg of CMP_{sl} for the first round and 10ng

of CMP_{sl} for the second round (CMPdes group), c) same doses of CMP plus 50µg of Omp16 (CMP+Omp16 group), and d) same doses of ovalbumin (OVA), as a non-related protein, with 50µg Omp16 (OVA+Omp16 group).

In vivo evaluation of the allergic reaction

-*Clinical signs*: Mice were ig challenged with 10 mg CMP/200 µl in two consecutive days, and the clinical signs were scored 15-45 minutes following the second ig challenge in a blinded fashion by 2 independent investigators. Clinical scores were as follows: 0= No symptoms; 1= Scratching and rubbing around the snout and head; 2= Puffiness around the eyes and mouth, piloerection, reduced activity and/or decreased activity with increased respiratory rate; 3= Hyperreactivity followed by respiratory distress, cyanosis around the snout and tail; 4= No activity upon stimuli, convulsion; 5= Death. Death of mice is infrequently observed since it is not an anaphylaxis model.

-*Cutaneous tests*: Mice were injected with 20 µg of CMP in 20 µl of sterile saline in one footpad, and saline in the contralateral footpad (negative control), and then were injected intravenously (iv) with 100µl of 0.1% Evans blue dye (Anedra, Buenos Aires, Argentina). The presence of blue color in the skin 10–20 min after the iv injection was considered as a positive test. The footpad thickness was measured with an electronic caliper.

In vitro evaluation of the allergic reaction

-*EAST*: Serum CMP-specific IgE antibodies were assessed as previously described [19]. Samples from all mice were run in the same experiment.

- *ELISA*: Serum CMP-specific IgG1 and IgG2a antibodies were measured by ELISA, as described in Smaldini et al. [19].

-*Cytokine quantification*: Twenty-four hours following the last oral challenge, mice were sacrificed, and different tissues were removed. Cell suspension from the spleen was prepared, and splenocytes (4×10^6 cells/well) were plated in complete media (RPMI-1640 supplemented with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin) with 350µg/ml CMP or medium for 72 h. Cytokines in the supernatants were quantified by ELISA.

Sections of jejunum were frozen in liquid nitrogen and then minced using scalpels. Cells were lysed using a lysis buffer (10mM Tris-HCl, 150mM NaCl, 1% NP-40, 10% Glycerol, 5mM EDTA, and protease inhibitor cocktail-Sigma), homogenized and sonicated. After

centrifugation, the supernatants were collected, and cytokines were quantified by ELISA: IL-5, IL-13, IFN- γ (eBioscience, San Diego, CA, USA).

- *Flow cytometry*: Cell suspensions from the submandibular lymph nodes (SLN) and lamina propria (LP) were obtained. Lamina propria cells were isolated as described in Smaldini et al.⁵ from the jejunum. Briefly, the tissue was removed and digested with 400 U/mL Collagenase Type IV and 50 mg/mL DNase in RPMI 1640 for 30 minutes at 37°C. Cell suspensions were filtered and washed in PBS solution. SLN cells were mechanically disaggregated and then filtered.

The SLN cell suspension was incubated with anti-CD4 (PerCyP 5.5), anti-CD8, or anti- $\alpha\beta 7$ (PE or APC) (eBioscience) for membrane staining during 40 min at 4°C. For intracytoplasmatic cytokine detection, cells were stimulated with mouse rIL-2 (20 ng/ml, Preprotech, NJ, USA) for 12 h at 37°C and 3 μ g/ml of Brefeldin A (eBioscience, UK) for the last 4h of cell culture. Then, cells were washed, pre-incubated with the fixation/permeabilization solution (eBioscience, UK) for 20 min at 4°C, and then incubated with the Staining Intracellular kit (eBioscience, UK) with anti-IFN γ (FITC) (eBioscience, UK).

Gating strategy for lamina propria dendritic cell characterization was as follows: First, we plotted SSC vs FSC-H where lymphocytes were excluded, followed by CD11c vs MHC II I-A/I-E dot plot. CD11c⁺ MHC II⁺ subset was further gated as CD11b vs CD8 α . Cells were marked with conjugated antibodies anti-CD11c (eBioscience, UK), anti- MHC II I-A/I-E (eBioscience, UK), anti-CD11b (eBioscience, UK) and anti-CD8 α (eBioscience, UK). Fluorescence acquisition was performed with a FACScalibur cytometer using QuestProCell software.

T cell purification and adoptive transfer to naïve mice

Donor CD4⁺ or CD8⁺ T cells were sorted from SLN of treated (CMP_{sl} or CMP_{sl}+ Omp16) mice with a FACSaria Fusion (BD, USA), and its purity was evaluated by flow cytometry. The strategy of gating consisted of delimiting the cells in the lymphocyte gate and then staining with CD4 and CD8 conjugated antibodies. Sorted cells were characterized as IFN- γ -expressing cells, and a total of 1x10⁶ CD4⁺ T cells and 1x10⁶ CD8⁺ T cells were intravenously transferred into naïve recipient mice. Animals were then orally sensitized with CMP+CT for 4 weeks, and 7 days following the last boost, they underwent oral gavage with

CMP. Hypersensitivity symptoms were scored, and the skin test was carried out as previously described.

In addition, sorted CD4⁺ cells were stained with carboxy-fluorescein diacetate succinimidyl ester (CFSE) 3μM (Invitrogen, USA) for 30 min at 37°C and then washed with PBS. Each naïve mouse was injected intravenously with 10⁶ CFSE CD4⁺ T cells in 0.2 ml PBS on day -1; on day 0, they received CT+CMP, and twenty-four hours later, mice were sacrificed, and CD4⁺ T cells were tracked in the small intestinal inductive sites.

Statistical analysis

All statistical analysis was carried out using GraphPad Prism 5 software. The significance of the difference was determined using an independent-sample t-test or ANOVA test. A p-value <0.05 was considered as statistically significant. All experiments were performed at least twice to achieve reproducibility.

RESULTS

Sublingual co-administration of the allergen with Omp16 reduces symptoms in allergic mice

To investigate if the immunotherapy could control the hypersensitivity reaction, mice were intragastrically sensitized with cholera toxin and milk proteins (Figure 1A); then, oral challenges were carried out with the allergen, and the sensitization status of mice was evaluated. We found consistent clinical signs of immediate hypersensitivity in all mice at day 45; the control group that received only CMP showed no clinical signs, indicating that the exposure to CMP did not induce allergic sensitization. Most of the clinical signs induced were systemic (scratching, conjunctive inflammation, piloerection, reduced response upon stimuli, and respiratory distress). Treatment with CMP+Omp16 was initiated after the oral challenge and was performed in two rounds. After the first round of therapy (day 79), mice showed significantly lower clinical scores following the oral challenges compared to mice of the sensitized group (sensitized mice treated with PBS) and to mice that received 10pg of CMP alone, or OVA+Omp16, as controls. Amelioration of symptoms was sustained in mice of the CMP+Omp16 group after the second round of treatment (day 120) compared to sensitized mice ($p < 0.001$). At this time point, clinical scores between sensitized mice that received PBS, 10ng of CMP, or OVA+Omp16 showed no significant differences (Figure 1B). In conclusion, sensitized mice that received CMP+Omp16 showed a significant reduction of symptoms. Furthermore, the comparison of the clinical outcome of mice treated with sublingual CMP with those treated with sublingual CMP+Omp16 indicated that a more efficient reduction of symptoms was accomplished using Omp16 ($p < 0.05$).

Mice treated with CMP+Omp16 show skin test negativization

To further elucidate the mechanisms underlying sensitization and treatment, we evaluated the activation of the IgE-sensitized skin mast cells after the second oral challenge. As depicted in Figure 1C and D, sensitized mice showed a positive skin test (blue-colored skin), and significantly increased swelling of the footpad injected with the allergen, compared with the contralateral footpad injected with PBS. Of note, sensitized mice that were treated with CMP+Omp16 showed a negative skin test and a reduced footpad swelling compared to sensitized mice that received PBS ($p < 0.0001$).

As controls, sensitized mice treated with CMP_{sl} showed a significant reduction of the footpad swelling and a slightly positive cutaneous test, whereas sensitized mice that received OVA+Omp16 showed a positive skin test and a footpad swelling.

Our findings in sensitized mice that were treated with CMP+Omp16 indirectly demonstrated lower IgE-mast cell sensitization or reduced number of skin sensitized mast cells compared to sensitized mice. In addition, the desensitization with CMP+Omp16 was more efficient than the sublingual immunotherapy with CMP.

The sublingual CMP+Omp16 treatment reduces serum milk-specific IgE and increases specific IgG2a

To gain more insight into the immunological response that mediates the *in vivo* control of food allergy, Th2-mediated mechanisms were analyzed. After the sensitization, mice showed significantly increased serum IgE levels (day 46). Following the two rounds of treatment, we observed increased serum CMP-specific IgE in sensitized mice compared to mice that only received CMP (control group) ($p < 0.0001$) (Fig. 2A). Sensitized mice that received sublingual CMP or CMP+Omp16 as treatments showed significantly decreased levels of specific IgE ($p < 0.001$), whereas sustained levels were observed in animals that received OVA+Omp16 (Fig. 2A). Besides, CMP-specific IgG2a level was significantly increased in sensitized animals treated with CMP+Omp16, and the IgG1/IgG2a ratio was lower in mice treated with CMP+Omp16 compared to the sensitized animals that received PBS ($p < 0.001$), CMP ($p < 0.05$), or OVA+Omp16 ($p < 0.001$), which exhibited a reduction comparable to naïve mice. These findings indicated a Th1-dependent humoral immune response induction with Omp16.

The sublingual treatment with CMP+Omp16 reduces the secretion of type 2 cytokines and induces the production of IFN- γ

To evaluate the cellular immune response and cytokines responsible for the allergic reaction, we assessed the cytokine secretion of antigen-activated spleen cells. As shown in Figure 2B, cells isolated from non-sensitized mice showed the secretion of basal levels of IL-5, IL-13, and IFN- γ , whereas cells from sensitized mice showed a significant rise in the IL-5, and IL-13 production, along with the basal secretion of IFN- γ . Sensitized animals that were treated with CMP or CMP+Omp16 showed decreased IL-13 production compared to non-treated

sensitized mice ($p < 0.001$), while only mice treated with CMP+Omp16 also experienced a significant downregulation of IL-5 secretion compared to non-treated sensitized mice ($p < 0.05$). Finally, IFN- γ secretion was significantly increased in sensitized mice that received sublingual CMP+Omp16 treatment compared to PBS-treated sensitized mice ($p < 0.05$). As a control, cells isolated from the OVA+Omp16 group did not show differences in cytokine secretion compared to sensitized mice, thus indicating that the immunomodulation promoted by the administration of Omp16 is antigen-specific.

The production of cytokines in the gut was also evaluated after allergen challenge. Sensitized mice showed significantly increased levels of IL-5 (pg/gr of the jejunum) compared to non-sensitized control mice ($p < 0.001$) and mice treated with CMP ($p < 0.01$) or CMP+Omp16 ($p < 0.001$). Also, sensitized mice showed significantly lower levels of tissue IFN- γ than control mice ($p < 0.01$); this cytokine was significantly increased upon CMP+Omp16 treatment ($p < 0.001$) or restored to control levels in CMP treated mice (Fig. 2C). Moreover, the IL-5/IFN- γ ratio was markedly reduced in sensitized mice that received sublingual CMP+Omp16 ($p < 0.001$), indicating a switch in the cytokine production (Fig. 2C).

The combined treatment induces the expression of gut-homing adhesion molecules on sublingual T cells

To understand the clinical control of hypersensitivity, we analyzed T cells in the SLN, and we found a significantly increased frequency of CD4⁺IFN γ ⁺ T cells in CMP+Omp16 treated mice compared with sensitized animals or animals that received only CMP as treatment (Figure 3A). In addition, a significantly higher proportion of these CD4⁺IFN γ ⁺ T cells expressed $\alpha_4\beta_7$ (Figure 3B), and we assessed the immunomodulatory properties of these IFN γ -producing T cells in an adoptive transfer experiment (Figure 4). Sorted CD4⁺ and CD8⁺ lymphocytes showed a higher frequency of IFN γ -producing lymphocytes in mice that received CMP+Omp16 compared with those that were given sublingual CMP (Figure 4B). These cells were adoptively transferred to naïve mice that were further sensitized with CT+CMP (Figure 4C). Following the oral challenge with CMP, we found significantly decreased symptoms in mice transferred with CD4⁺IFN γ ⁺ or CD8⁺IFN γ ⁺ cells isolated from animals treated with CMP+Omp16, whereas only animals that were transferred with CD4⁺IFN γ ⁺ isolated from mice treated with CMP showed higher efficacy in mitigating allergy compared with CD8⁺ cells ($p < 0.05$) (Figure 4D). Finally, skin test was assessed, and

we found that mice that received PBS prior to sensitization exhibited inflammation, whereas mice transferred with CD4⁺ or CD8⁺ T cells isolated from mice treated with CMP+Omp16 showed decreased swelling ($p<0.01$) compared with control mice (Figure 4E). Mice transferred with CD4⁺ or CD8⁺ T cells isolated from mice treated with CMP showed similar swelling to those treated with PBS and then sensitized. Representative pictures showing the dye leakage are also depicted.

An additional group of mice was transferred with CFSE-stained CD4⁺ T cells, and following the sensitization, cells were found in Peyer's patches and MLN, indicating that SLN-induced cells homed to the inflamed intestinal tissue (Figure 4F).

We also analyzed the presence of CD4⁺CD25⁺Foxp3⁺ T cells in the jejunum lamina propria, and we only observed an increased frequency of these regulatory cells in mice treated with CMP_{sl}. Mice treated with CMP+Omp16 showed reduced Treg frequencies compared with CMP-treated mice ($p<0.01$) (Supplementary Figure).

The combined treatment induces a specific subset of dendritic cells in the lamina propria

To further dissect the immunomodulatory mechanism exerted by Omp16, we analyzed dendritic cell subsets in the lamina propria of the jejunum. We found that mice treated with CMP or CMP+Omp16 showed a higher frequency of CD11c⁺CD11b⁻CD8 α ⁺ cells than sensitized animals ($p<0.01$) (Figure 3C), whereas CD8 α ⁻CD103⁺CD11c⁺MHC II⁺ cells were only significantly increased in mice treated with CMP_{sl} compared with control and CMP+Omp16-treated mice ($p<0.01$) (Supplementary figure).

DISCUSSION

Food allergy is an emerging health problem worldwide with increasing incidence and severity. Therefore, there is an urgent need to develop treatments that redirect the immune system and promote sustained unresponsiveness to prevent severe food-induced allergic responses. Although oral and sublingual immunotherapies have shown promise, no procedure has demonstrated long-term protection in patients. Most of the clinical trials and surveys published over at least 20 years showed that sublingual immunotherapy is a safe and effective alternative for the treatment of rhinitis and asthma caused by dust mites, grass, ragweed, cat dander, and tree pollens [9,20,21]. Compared with subcutaneous immunotherapy, high cumulated doses of allergen are needed in sublingual immunotherapy (SLIT) to induce allergen-specific tolerance, likely due to the absence of any adjuvant [11]. Therefore, the use of a mucosal adjuvant in SLIT may reduce the dose of allergen and improve the safety of the therapy. With regards to food allergies, the first immunotherapy approved in 2020 is AR101* or PALFORZIA, which consists of oral immunotherapy for peanut allergy (www.fda.gov/vaccines-blood-biologics/allergenics/palforzia). It is mainly focused on avoiding life-threatening reactions when patients are accidentally exposed to peanut. As anaphylaxis has been reported during all phases of PALFORZIA dosing, sublingual immunotherapy may be a safer alternative compared with oral immunotherapies (very low amounts of the allergen are needed), although with a lower tolerogenic capacity [22]. Very rarely, anaphylaxis has been reported using SLIT, and the use of adjuvants may potentiate the efficiency of the immunomodulatory mechanisms to reverse the Th2-mediated immune response. The present study investigates the combination of SLIT with the use of a potent mucosal adjuvant that was previously demonstrated to confer allergen-specific Th1 immunity through the nasal and oral administration [5,7]. Here, we found that the sublingual co-administration of the allergen with Omp16 triggered a specific Th1-mediated immune response at the site of allergen up-take.

The re-direction of the intestinal allergen-specific Th2 responses significantly protected against oral milk challenge. The reduced allergic hypersensitivity achieved was not only due to desensitization with milk exposure since mice treated only with CMP showed still a mild allergic reaction. The combined use of the Omp16 improved the control of symptoms and promoted a Th1-biased intestinal microenvironment that controlled the local allergic reaction. We observed an increased frequency of intestinal CD11c⁺ CD11b⁻ CD8α⁺ dendritic cells,

which were probably involved in the immune response responsible for the significantly diminished level of IL-5 and increased IFN- γ , along with a significant decrease of milk-specific IgE and increase of milk-specific IgG2a. Animals that received only CMP sublingually also prevented the hypersensitivity reactions following the oral challenge, along with a reduction of IgE production and type-2 cytokine secretion through tolerance induction, although with a lower efficacy compared with sensitized animals that received the combined SLIT. We finally studied the IFN- γ -producing T cells induced in the inductive site of the buccal mucosa that migrated to the intestinal inductive sites in a preventive protocol of adoptive cell transfer experiments. Although this experiment does not strictly evoke the suppressive properties of these cells, the intestinal traffic of SLN-induced T cells was demonstrated. Further investigations are needed to fully elucidate the suppressive mechanisms exerted by cells induced through the combined sublingual administration. With our findings, we hypothesize that the sublingual Omp16/CMP induced milk specific IFN- γ -producing $\alpha 4\beta 7^+CD4^+$ and $\alpha 4\beta 7^+CD8^+$ cells that homed to the gut. The oral CMP activates these T cells in the gut and the secretion of IFN- γ , which promoted the induction of $CD11c^+CD11b^-CD8\alpha^+$ dendritic cells. Another potential explanation would be that the sublingual Omp16 is absorbed and reach the gut through the blood. Pasquevich et al. reported that Omp16 expands the $CD11c^+CD11b^-CD8\alpha^+$ dendritic cell subset through the binding to TLR4 and promotes the activation and differentiation of Th1 and $CD8^+$ T cells [3].

The literature shows that the use of TLR agonists has been reported in pre-clinical models and patients to enhance immunity that modulates the Th2-mediated airway hyperresponsiveness [23], or in sublingual vaccines [24] to prevent infectious diseases. Rodriguez et al. reported in a mouse model of anaphylaxis that the allergen-specific SLIT with CpG and a peptide derived from Pru p3 controlled the allergic reaction, although it is not a model of food allergy [25]. Pfaar et al. reported the first clinical trial of adjuvanted SLIT. They used monophosphoryl lipid A combined with grass pollen allergens to test the safety and clinical and immunological efficacy in patients with seasonal allergic rhinitis. Patients showed a diminished reactivity in the nasal challenge along with a significative increase in serum specific IgG antibodies.

Nevertheless, the increase in blocking IgG4 was not statistically significant [26]. Furthermore, several interventional studies have demonstrated the safety and efficacy of blocking IL-5 in patients with asthma [27,28] or eosinophilic esophagitis [29,30], blocking the IL-4 and IL-13 action with dupilumab in patients with conjunctivitis [31] or atopic

dermatitis [32], and blocking IgE in food allergic patients [33,34]. These studies reflect the crucial role of these mediators in allergic reactions, and in this sense, the immunotherapeutic protocol here used diminished the secretion of IgE, IL-5, and IL-13.

In summary, the use of Omp16 and milk proteins in a sublingual immunotherapy procedure is a more efficient treatment for IgE-mediated food allergy compared with SLIT with CMP. Very low amounts of the allergen were employed to induce sublingual Th1 cells, which migrated to the gut to promote a Th1-dominated cytokine environment that suppressed the secretion of type-2 cytokines, responsible for eosinophil attraction and class-switch recombination to ϵ chain and IgE, two cardinal features of food allergy. Also, the reduced cell sensitization prevented allergic reactions when animals were challenged with the allergen. In conclusion, our findings may pave the way for the use of a mucosal adjuvant in allergen-specific sublingual immunotherapy that mitigates symptoms in a food allergy setting.

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LEGEND OF FIGURES

Figure 1. *In vivo* responses of the food allergy mouse model. **A**, Schematic drawing of the experimental protocol in BALB/c mice ($n=6/\text{group}$). **B**, Clinical scores corresponding to signs elicited following the oral challenges with milk proteins. **C**, Skin tests in control, sensitized, and treated mice. **D**, Swelling of the footpad, the difference in thickness corresponds to the footpad injected with PBS as control with the contralateral footpad injected with CMP. Results correspond to a single experiment, representative of three separate experiments that gave similar results. One and two-way ANOVA was used because all data had a normal distribution and equal variances **** $P<0.0001$, *** $P<0.001$, ** $P<0.01$, * $P<0.05$. CMP, cow's milk proteins; CMP+Omp16, sublingual treatment with CMP and outer membrane protein 16 (Omp16) of *Brucella abortus*; OVA+Omp16, sublingual treatment with ovalbumin (OVA) and Omp16.

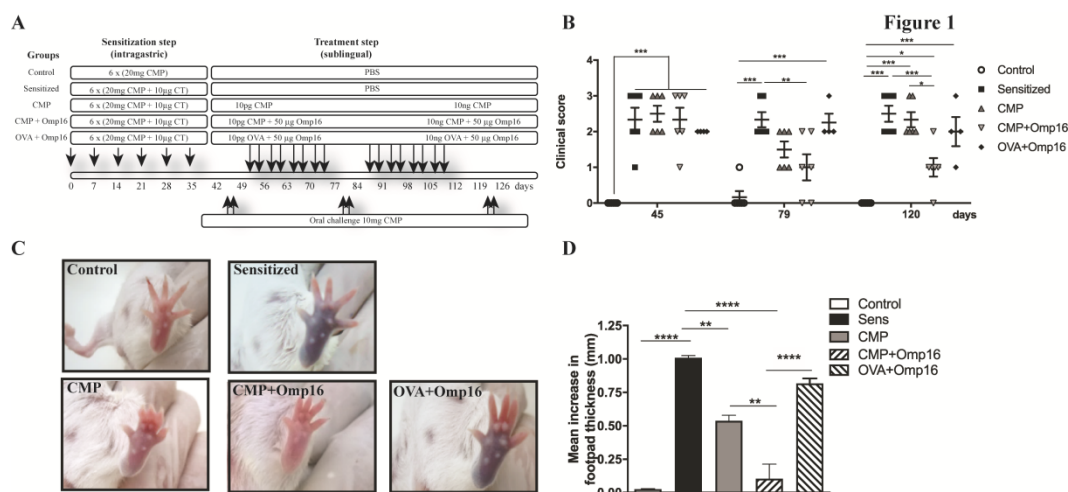
Figure 2. Specific serum isotypes and cytokines by ELISA. **A**, CMP-specific serum IgE, IgG1, and IgG2a during the whole experiment. **B**, Cytokines in supernatants of spleen cells stimulated with CMP or medium during 72 h. **C**, IL-5, and IFN- γ protein levels in jejunum homogenates were determined by ELISA. These results are representative of two independent experiments with similar results. Data are expressed as mean values $\pm$ SEM. One and two-way ANOVA was used because all data had a normal distribution and equal variances **** $P<0.0001$, *** $P<0.001$, ** $P<0.01$, * $P<0.05$.

Figure 3. SLN and intestinal mucosa. **A**, Percentage of CD4⁺ IFN γ ⁺ in SLN **B**, Expression in $\alpha_4\beta_7$ in LT CD4⁺ IFN γ ⁺. **C**, Representative plot of CD11c⁺ CD11b⁻ CD8a⁺ in intestinal mucosa. These results are representative of two independent experiments with similar results. One-way ANOVA was used because all data had a normal distribution and equal variances **** $P<0.0001$, *** $P<0.001$, * $P<0.05$.

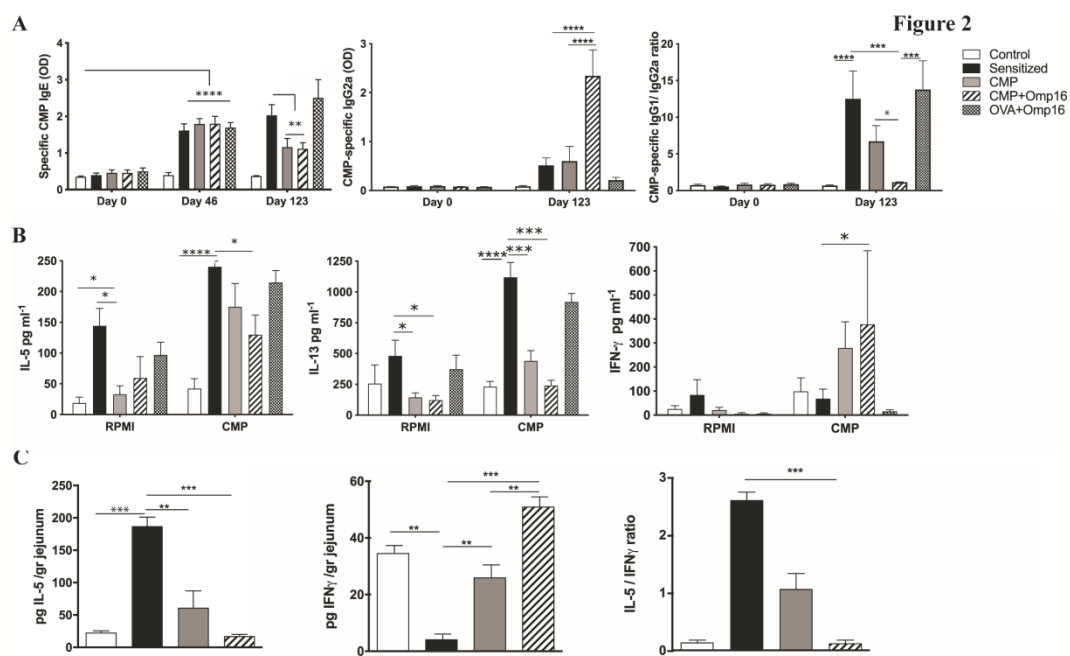
Figure 4. Experimental design and T cells adoptive transfer. **A**, Schematic drawing of the experimental treatment protocol. **B**, T cells sorted as CD4⁺ and CD8a⁺ from animals treated with CMP+Omp16 or CMP alone. **C**, Schematic drawing of the experimental protocol after T cells adoptive transfer. Naïve mice that were transferred with sorted cells were subsequently sensitized with CMP and CT and then orally challenged. **D**, Clinical score after CMP oral challenge. **E**, cutaneous test, and the swelling of footpad. **F**, CFSE-stained sorted CD4⁺ cells

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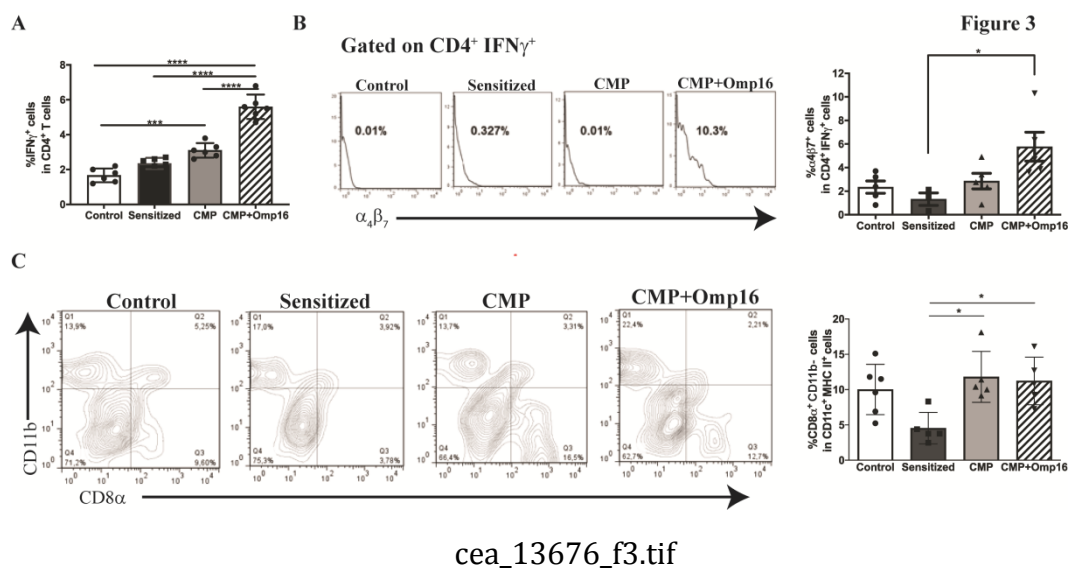
were transferred to naïve mice and were tracked in the small intestine mesenteric lymph nodes (MLN) and Peyer's patches (PP). PBS was used as control. Lymphocyte gate is depicted in the SSC vs FSC dot-plot. Data are expressed as means $\pm$ SEM. Statistical significant differences between the groups were determined using one-way ANOVA; **** $P < 0.0001$, ** $p < 0.01$, * $p < 0.05$.

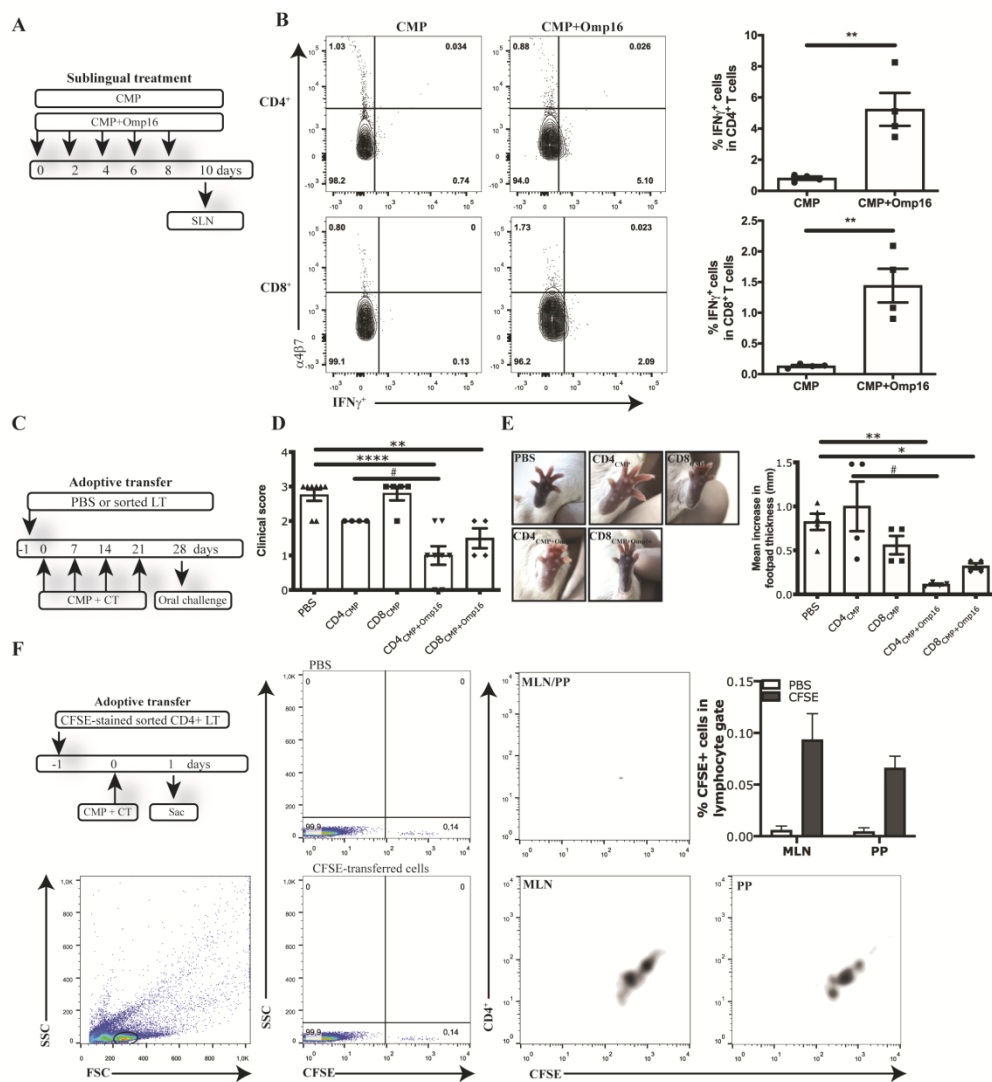


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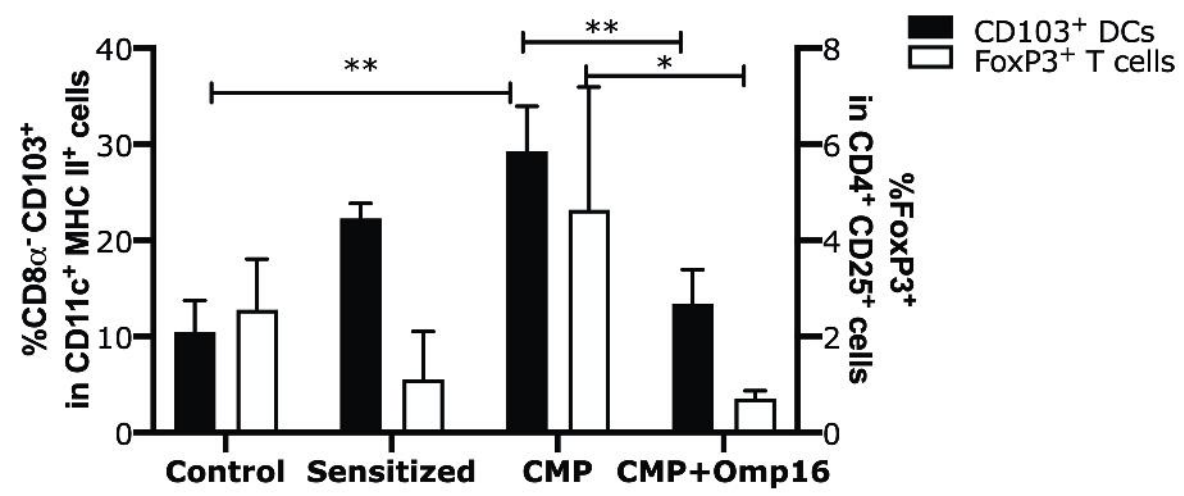


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SUPPLEMENTARY MATERIAL

Sublingual Omp16-driven redirection of the allergic intestinal response in a pre-clinical model of food allergy

Maria Lucía Orsini Delgado¹, Gastón Pascual Rizzo¹, Carlos Alberto Fossati¹, Karina Alejandra Pasquevich², Juliana Cassataro², Paola Lorena Smaldini^{1*} and Guillermo Horacio Docena^{1*}

METHODS

Flow cytometry analysis of Treg cells: Lamina propria cells were stained with anti-CD4 (PerCyP 5.5) and anti-CD25 (PE) (eBioscience) for membrane staining during 40 min at 4°C. For intracytoplasmatic FoxP3 detection, cells were incubated with the fixation/permeabilization solution (eBioscience, UK) for 20 min at 4°C, and then incubated with the Staining Intracellular kit (eBioscience, UK) with anti-FoxP3 (APC) (eBioscience, UK). Fluorescence acquisition was performed with a FACScalibur cytometer using QuestProCell software.

Flow cytometry analysis of dendritic cells in intestinal lamina propria: Lamina propria cells were stained with anti-CD8α (APC), anti-CD103 (FITC), anti-CD11c (PE) and anti-MHC II (I-A/I-E) (PerCy5). Cells were washed and fluorescence acquisition was performed with a FACScalibur cytometer using QuestProCell software for analysis.

SUPPLEMENTARY FIGURE

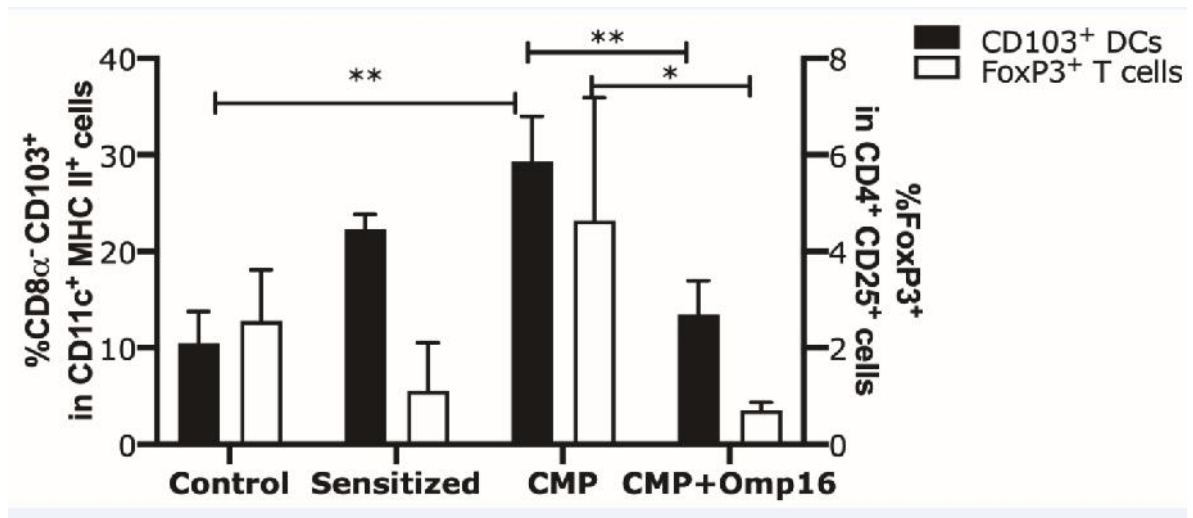


Figure S1. Lamina propria FoxP3⁺ T lymphocytes and CD8α⁻CD103⁺ dendritic cells in mice. Dendritic cells (black bars) were analyzed by gating on CD11c⁺ vs MHC II⁺ followed by CD8α vs CD103; Tregs (white bars) were analyzed by gating lymphocytes in the FSC vs SSC dot-plot, followed by SSC vs. CD4 fluorescence; the CD4⁺ lymphocyte subset was further gated as CD25 vs. FoxP3. Results were obtained from two independent experiments (n=5 mice per group). Data are expressed as means ± SEM. Statistical significant differences between the groups were determined using one-way ANOVA; **p<0.01, *p<0.05.