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Hydrolysed ovalbumin offers more effective preventive and therapeutic protection against egg allergy than the intact protein

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Summary

Background The application of specific immunotherapy to stimulate oral tolerance towards food allergens is hampered by the high frequency of adverse side effects and the excessive duration of the treatments.

Objective In this work, a hydrolysate of ovalbumin with pepsin (OP), selected for its low IgE reactivity and Th2-stimulating capacity, was assayed for its ability to prevent and treat allergy to egg white (EW).

Methods As a first step, the safety of OP, in terms of the absence of sensitizing and eliciting potential, was evaluated in BALB/c mice. Then, its suitability for prophylactic and therapeutic applications was compared with that of the intact allergen, paying attention to the molecular and cellular mechanisms involved in the control of the allergic process. To this aim, IgE, IgG1, IgG2a and IgA levels, allergic

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reactions, expression of genes related to Th1, Th2, Th17 and Treg responses, dendritic and T cell populations were assessed in intestinal tissues and spleens of EW-allergic mice, either untreated or treated with intact ovalbumin (OVA) or OP.

Results OP was hypoallergenic, lacked sensitizing potential and offered preventive and therapeutic protection against allergy to EW through the induction of Treg cells and the upregulation of TGF- β , IL-10, IL-17, Foxp3 and ROR γ t in intestinal tissues. This restrained the expression of GATA3 and the differentiation of Th2 cells, leading to low cytokine responses following *ex vivo* spleen cell stimulation. As compared with intact OVA, OP was more effective against sensitization. In addition, in the therapeutic setting, OP provided quicker desensitization that lasted for at least 3 weeks after discontinuation of the therapy.

Conclusions and Clinical Relevance This study provides evidence for the superior role of hydrolysed, as compared to intact allergens, in the prevention of allergy development and in the promotion of long term desensitization, as well as of intermolecular tolerance.

Keywords peptide immunotherapy, hydrolysed ovalbumin, egg white, BALB/c, Foxp3, ROR γ t.

Introduction

Allergen-specific immunotherapy stands among the most promising approaches currently assayed against IgE-mediated allergic diseases, but despite continuous attempts to desensitize food allergic patients, there are still no validated therapies to induce tolerance, mainly because of the high frequency of adverse side effects and the excessive duration of the treatments [1]. This has prompted the investigation of strategies aimed at reducing the allergenicity of the treatment preparations, while maintaining their immunogenicity or even improving their immunomodulating potential, to stimulate oral tolerance towards food allergens or to prevent allergic sensitization [2]. Allergen-derived peptides can redirect the immune response of specific cell types and reduce inflammation, and thus, enhance the effectiveness of immunotherapy [3]. However, while the use of peptides has demonstrated

sustained tolerance with fewer adverse events than conventional allergen immunotherapy in clinical trials for cat, house dust mite and pollen allergy, it is in a much more exploratory stage in the field of food allergy [4]. Moreover, almost no studies compared the therapeutic efficacy of food peptides or hydrolysates with that of intact allergens or re-assessed protection from anaphylaxis after discontinuation of the treatment [5].

The aim of this investigation was to estimate the applicability of a hydrolysate of the egg allergen ovalbumin (OVA) to prevent the development of allergic sensitization to whole egg white (EW) and to achieve sustainable desensitization once allergy was established in BALB/c mice. OVA hydrolysed with pepsin (OP) was beforehand a good candidate to be used for immunotherapy on the grounds of its low IgE reactivity and its potential to reduce Th2 responses induced by egg allergens on splenocytes from sensitized mice, but also to enhance Th1 responses [6, 7]. As a first step, the safety of OP, in terms of the absence of sensitizing and eliciting potential, was evaluated. The results showed that the preventive and therapeutic administration of OP was more effective than that of the intact allergen in promoting intermolecular tolerance to EW allergens by virtue of its low allergenicity and its ability to induce the development of regulatory T (Treg) cells.

Materials and methods

Proteins, hydrolysate and mice

EW was obtained from fresh eggs. Bovine serum albumin (BSA), OVA grade VI and porcine pepsin (EC 3.4.23.1, 3440 U/mg) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and, when required, purified from lipopolysaccharide to less than 3 EU/mg by size exclusion chromatography [8]. OP was prepared by treating OVA with 172 U/mg of pepsin at pH 1.5 and 37°C for 24 h, followed by neutralization to pH 7.0 and heating at 95°C for 15 min. EW and OP were lyophilized and their protein content was determined by the Kjeldahl method. The peptide composition of the hydrolysate was reported in [6]. Female BALB/c mice were from Charles River Laboratories (Saint Germain sur l'Arbresle, Rhône, France). All protocols involving animals followed the European

legislation (Directive 2010/63/UE) and were approved by Comunidad de Madrid (Ref PROEX 089/15).

Experimental design

The sensitizing and eliciting capacities of OP, as compared to those of EW, were evaluated in 6 week-old mice subjected to a sensitizing protocol consisting of the oral administration of the amount equivalent to 5 mg of protein per mouse and 10 μ g of cholera toxin (CT, List Biologicals, Campbell, CA, USA) during 3 consecutive days on the first week and once per week during the following 5 weeks. Mice were distributed in 4 groups (n=5) (Supplementary Fig. 1 A), which received EW+CT (2 groups), OP+CT (1 group) or just CT in PBS (sham-sensitized group). One week after the last sensitization dose, mice administered EW+CT were challenged with either EW or OP, mice administered OP+CT were challenged with OP and sham-sensitized mice were challenged with EW. Oral challenge (50 mg per mouse on a protein basis) was followed by an intraperitoneal (i.p.) challenge (100 μ g per mouse) 90 min apart.

The capacity of OVA and OP to prevent EW allergy was evaluated in 4 groups of 3 week-old mice (n=5) (Supplementary Fig. 1 B). Two groups were orally pre-treated with 5 mg of protein per mouse of either OVA or OP and 2 groups were administered PBS, 3 times per week during 3 weeks. One week after the last pre-treatment dose, the groups pre-treated with OVA and OP, as well as one group administered PBS, were sensitized to EW, while the remaining PBS group was sham-sensitized. All groups were orally and i.p. challenged with EW.

Finally, the therapeutic capacity of OVA and OP was evaluated in 5 groups of 6 week-old mice (n=5) (Supplementary Fig. 1 C). Four groups were sensitized to EW and one group was sham-sensitized. One week apart, 2 groups of EW-sensitized mice were orally treated with 5 mg of protein per mouse of OVA or OP, 3 times per week during 3 weeks, while another group received 5 mg per mouse of a non-related protein, BSA, for comparison. The remaining EW-sensitized group and the sham-sensitized group received PBS during the therapy. Three days after, all groups were orally and i.p. challenged with EW. Mice treated with OVA and OP, as well as EW- and sham-sensitized mice that

only received PBS, were kept for a further 3 weeks without any treatment, in order to re-evaluate the allergic status by new oral and i.p. challenges with EW. This experiment was repeated 3 times.

Thirty min after each challenge, anaphylactic responses were evaluated by scoring clinical signs and rectal temperature [8]. After euthanasia by CO₂ inhalation, samples from duodenum, Peyer's Patches (PPs), mesenteric lymph nodes (MLNs) and spleens were collected. EW- and OP-specific antibodies: serum IgE, IgG1, IgG2a and fecal IgA were quantified by ELISA [8]. Mast cell degranulation was evaluated in sera collected after i.p. challenge by determining the concentration of mouse mast cell protease-1 (MCP-1) using a commercial ELISA kit (eBioscience, San Diego, CA, USA). Spleen cells (4×10^6 cells/mL) were stimulated with RPMI-1640, EW or OP (200 µg protein /mL) for 72 h at 37°C in 5% CO₂ and cytokines were analysed in the supernatants (Luminex, eBioscience).

For the *ex vivo* co-culture systems of dendritic cells (DCs) and T cells, MLN CD11c⁺ and splenic CD4⁺ T cells were isolated using EasySep Negative selection kits (StemCell Technologies, Vancouver, Canada). 1×10^5 CD11c⁺ cells from sham-sensitized mice and EW-sensitized mice, either untreated (PBS) or treated with OP, were incubated with 1×10^6 CD4⁺ T cells derived from untreated EW-sensitized mice (Supplementary Fig. 1 C) in the absence (RPMI) or presence of EW (200 µg protein/mL). After 3 days, cultures were stimulated with 3 µg/mL of anti-CD3 and anti-CD28 (eBioscience) for 3 additional days. The supernatants were collected and IL-5, IFN-γ and IL-10 were quantified by ELISA (eBioscience).

Passive cutaneous anaphylaxis assay (PCA)

Naïve BALB/c mice (2 per test) were injected intradermally in the ear pinna with 20 µL of either unheated or heat-inactivated (56°C for 3 hours) [9] pooled serum from EW-sensitized mice and with pooled serum from sham-sensitized mice in the other ear pinna. Twenty four hours later, mice were injected intravenously with 200 µg of EW in 100 µL of 1% Evans blue dye (Sigma-Aldrich). One hour after, mice were euthanized, the ears were individually collected and incubated overnight in N,N-dimethylformamide (Sigma-Aldrich) and the absorbance of the supernatant was measured at 655 nm.

Gene expression

Total RNA was individually extracted by using NucleoSpin RNA Kit (Macherey-Nagel GmbH & Co., Düren, Germany) and cDNA was synthesized using PrimeScript RT reagent kit (TaKaRa Bio Inc., Shiga, Japan). qPCR was performed in a real-time thermocycler (ViiA7 Real-Time PCR System, Applied Biosystems, Foster, USA) using SYBR Premix ExTaq II (TaKaRa). Primer pairs and optimized thermal cycling conditions are included in Supplementary Table 1. Relative gene expression was calculated by normalizing data to the expression of the reference gene β -actin, using the sham-sensitized group as calibrator [10].

T cell and dendritic cell subsets by flow cytometry

Spleen and MLN cells were recovered in PBS containing 2% FBS and 1mM EDTA. Fc receptors were blocked using anti-CD16/CD32 (clone 2.4G2, BD Biosciences) and live cells determined with LIVE/DEAD® Fixable Near-IR Dead Cell Stain Kit (Thermo Fisher Scientific, Waltham, US). Samples were stained with the antibodies listed in Supplementary Table 2. Approximately 1×10^5 cells were acquired using Gallios flow cytometer (Beckman Coulter, Krefeld, Germany) and analyses were performed with Kaluza Analysis software (version 1.3).

Statistical analyses

Results are presented as means $\pm$ SEM, excluding clinical signs, which are expressed as medians. Differences were determined by one-way ANOVA followed by Tukey post-hoc test, except for clinical sign scores and gene expression data, which were evaluated by Mann-Whitney U test. $P < 0.05$ was considered statistically significant. Statistical analyses were performed using GraphPad Prism v5 (GraphPad Software Inc., San Diego, USA).

Results

The hydrolysate of ovalbumin with pepsin lacks sensitizing and allergenic potential

Negligible EW-specific IgE, IgG1, IgG2a or IgA levels were detected in mice administered OP+CT, although these antibodies were significantly increased in mice administered EW+CT (Fig. 1 A), thus showing that the hydrolysate did not hold sensitizing potential to the intact protein from which it derived, that constitutes 54% (w/w) of EW. Administration of OP+CT gave rise to low, but detectable OP-specific IgG2a, but not to OP-specific IgE, IgG1 or IgA responses, which is indicative of low immunogenicity (Supplementary Fig. 2). IgG1 and IgG2a present in serum from mice sensitized to EW cross-reacted to OP, probably because the administration of EW+CT generated antibodies directed towards products of gastric digestion of OVA (Supplementary Fig. 2). PCA tests were conducted to assess the participation of IgE and IgG1 in the development of allergic responses to EW. Heat-treatment of pooled serum from EW-sensitized mice before its intradermal injection into the ears of naïve mice significantly reduced the extent of dye extravasation following intravenous challenge with EW, although it did not eliminate it (Supplementary Fig. 3). This suggests that, in addition to heat-sensitive IgE antibodies, IgG1 antibodies also contributed to anaphylaxis in this model.

Mice administered OP+CT did not present clinical signs or temperature changes following oral and subsequent i.p. challenges with OP, which reinforced that OP lacked sensitizing potential (Fig. 1 B). The assessment of the allergenic potential of the hydrolysate in mice sensitized to EW revealed that, while these animals experienced significant anaphylactic symptoms and rectal temperature drops upon challenge with EW, accompanied by the release of mast cell mediators, oral and i.p. challenges with OP did not cause allergic reactions (Fig. 1 B).

The expression levels of the intestinal Th2-skewing cytokines IL-33, IL-25 and TSLP, as well as the transcription factors GATA3, T-bet, Foxp3 and ROR γ t, which specify, respectively, the development of Th2, Th1, Treg and Th17 cells, were assessed in the duodenum (Fig. 1 C and D). A significant upregulation of IL-33, IL-25 and TSLP was detected in mice administered EW+CT and challenged with EW, as compared with sham-sensitized mice (Fig. 1 C). The different responses induced by EW

and OP in EW-sensitized mice indicate that intestinal injury resulting from mast cell activation due to the oral challenge influenced the gene regulation of these cytokines. However, challenge with OP in mice previously administered OP+CT also boosted intestinal IL-33 and GATA3 expression in the absence of detectable allergic symptoms (Fig. 1 C and D). In fact, the expression of GATA3 was significantly enhanced in mice administered EW+CT and OP+CT with respect to sham-sensitized mice, although it was the highest in mice sensitized to EW and challenged with EW. On the other hand, T-bet was upregulated in the duodenum of mice sensitized to EW regardless of the challenge outcome, whereas no changes were observed in the expression levels of either Foxp3 or ROR γ t with respect to sham-sensitized mice (Fig. 1 D).

In parallel with the regulation of the transcription factors GATA3, T-bet, Foxp3 and ROR γ t in the intestine, an increased percentage of activated Th2 and Th1 cells and no change in Treg cells were found in the spleen of mice administered EW+CT and challenged with EW, while challenge with OP led to significantly lower levels of activated Th2 cells. The proportion of activated Th2, Th1 and Treg cells was similar in the spleen of mice administered OP+CT and in sham-sensitized mice (Fig. 1 E). When the specific response of splenocytes from mice administered EW+CT and OP+CT was studied by re-stimulation with the protein preparation used to induce sensitization (Supplementary Fig. 4), a significant release of Th2 (IL-4 and IL-5), Th1 (IFN- γ), regulatory (IL-10) and Th17 (IL-17) cytokines was found in mice administered EW+CT; while no significant production of IL-4, IL-5, IFN- γ or IL-10 was detected from the spleen cells of mice administered OP+CT, which, however, released the highest levels of IL-17. Indeed, the spleen cells from mice administered OP+CT did not react to EW stimulation, except for the release of IL-17. On the other hand, mice administered EW+CT responded to re-stimulation with OP with lower levels of IL-4 and IL-5 than those induced by EW, but with equivalent levels of IFN- γ . Overall, OP was confirmed to be weakly immunogenic and hypoallergenic and to hold T cell epitopes that could help to prevent sensitization or restore tolerance to egg proteins. Therefore, we then explored the preventive potential of this hydrolysate.

The hydrolysate of ovalbumin with pepsin prevents more effectively than intact ovalbumin the development of allergy to egg white in mice

Treatment with OVA or OP for 3 weeks, before administration of EW+CT, prevented sensitization, as judged by significantly lower concentrations of serum EW-specific IgE, IgG2a and IgA at the end of the experimental period, although only OP induced reduced levels of EW-specific IgG1 (Fig. 2 A). In addition, pre-treated mice did not experience anaphylaxis following oral challenge with EW. However, subsequent i.p. challenge led to significant clinical symptoms, temperature drops and release of MCP-1 in mice previously treated with OVA, while mice treated with OP were effectively protected against anaphylactic reactions (Fig. 2 B).

Pre-treatment of mice with OVA or OP inhibited upregulation of duodenal Th2-skewing cytokines and of IL-9, a cytokine that influences mast cell recruitment and activation [11], following sensitization and challenge with EW (Fig. 2 C). In general terms, prevention of sensitization and anaphylaxis by OVA and its hydrolysate matched an increased expression of the regulatory cytokines IL-10 and TGF- β in the MLNs (Fig. 2 D). Of note was the upregulation of IL-17 in the duodenum, PPs and MLNs induced by both pre-treatments (Fig. 2 D). In parallel, we observed a decreased expression of GATA3 in the PPs and MLNs of the pre-treated animals, while T-bet was downregulated in the duodenum and upregulated in the PPs (Fig. 2 E). OP, which was most effective than OVA in inhibiting allergy development, distinctly upregulated IL-10 in PPs and TGF- β in the duodenum and PPs, leading to a significant increased expression of Foxp3 and ROR γ t in the intestinal tissues and to a further increase in that of T-bet in the MLNs (Fig. 2 E).

Consequently, both pre-treatments reduced the percentage of activated Th2 cells in the MLNs, although no changes were detected in the percentage of activated Th1 and Treg cells, compared with the untreated mice (Fig. 3 A). The study of the phenotype of MLN DCs revealed no significant effects in the proportion of CD11c⁺ cells or of lamina propria-derived CD103⁺ cells (not shown). Interestingly, pre-treatments with OVA and OP depressed, in the MLNs, the expression of DC genes that promote Th2 differentiation, such as OX40L and Jagged-2, but they did not modify that of IL-

12p40 or Delta-4, which prime T cells for Th1 responses [12, 13] (Fig. 3 B). Thus, pre-treatments with OVA and OP inhibited Th2-skewing in the MLNs induced by the subsequent administration of EW+CT and challenge with EW, an effect that could be traced to the downregulation of Th2-priming factors on DCs. In the spleen of pre-treated mice, OP concomitantly induced a decrease in activated Th2 cells and an increase in Treg cells (Fig. 3 C). Following incubation with EW, splenocytes from mice pre-treated with OVA and OP responded with significantly lower levels of IL-4, IL-5, INF- γ , IL-10 and IL-17 (Supplementary Fig. 5).

These results indicate that pre-treatment with OP prevented more effectively than intact OVA the development of allergy to EW in mice. Increased expression of the suppressor cytokines IL-10 and TGF- β , IL-17, as well as Foxp3 and ROR γ t in intestinal tissues, and modulation of DC genes that affect Th2 cell priming in the MLNs concurred with downregulation of GATA3 and reduction of Th2 cells, hindrance of sensitization and clinical protection against anaphylaxis.

The hydrolysate of ovalbumin with pepsin induces sustained desensitization to egg white in mice

In these experiments, once allergy to EW was established in mice by the administration of EW+CT, these were treated with OVA and OP for 3 weeks and challenged with EW. A non-related protein, such as BSA, was also used to exclude the contribution of non-specific effects. Of note was the appearance of diarrhea (score 3), without other noticeable allergic signs, during the first week of OVA therapy in 4 out of 5 mice. Determination of serum antibodies 3 days after the last treatment dose (week 9) showed that OP reduced significantly the levels of EW-specific IgE and IgG1, whereas neither BSA nor OVA changed the concentration of these antibodies with respect to the untreated mice (Fig. 4 A). None of the treatments altered the concentration of EW-specific IgG2a, although therapy with OVA and OP led to significantly increased levels of fecal IgA ($P < 0.05$ with respect to week 6) (Fig. 4 A). Unlike BSA and OVA, which did not avoid anaphylaxis, the hydrolysate abolished clinical signs, temperature changes or increase in serum MCP-1 levels upon oral and subsequent i.p challenge with EW (Fig. 4 B).

Interestingly, reassessment of anaphylaxis 3 weeks after withdrawal of therapy with OVA and OP (week 12) revealed similar responses in mice treated with OP, while those treated with OVA experienced no symptoms or temperature drops upon oral challenge and milder, but still significant, signs upon subsequent i.p. challenge with EW (Fig. 4 B). These findings overlapped, in both cases, with lower levels of EW-specific IgE and IgG1, as compared with the point when the treatments were discontinued, and also lower concentrations of EW-specific IgG2a and IgA in the case of OP ($P < 0.05$ with respect to week 9) (Fig. 4 A), showing a continued depletion of circulating and intestinal specific antibodies. Of note is the observation that serum levels of EW-specific IgE and IgG1 were maintained in EW-sensitized mice treated with PBS up to week 12, but those of IgG2 and IgA decreased ($P < 0.05$ with respect to week 6).

Examination of gene expression in intestinal tissues following the first challenge (week 9), revealed that treatments with OVA and OP reduced the expression of IL-33 and IL-25 in the duodenum induced by previous EW sensitization to levels which, in the case of IL-25, were equivalent to those of sham-sensitized mice, while they did not significantly change that of IL-9 (Fig. 4 C). The therapeutic effect of OP could be traced to the increased expression of IL-10 in PPs and of TGF- β in duodenum, PPs and MLNs observed in OP-treated mice in comparison with OVA-treated mice that, in general terms, concurred with the upregulation of IL-17 (Fig. 4 D). In fact, therapy with OP distinctively enhanced the expression of Foxp3 and ROR γ t in the intestinal tissues studied (Fig. 4 E). In particular, in the MLNs of the animals treated with OP, the expression of GATA3 and T-bet was deeply decreased, while the less effective OVA therapy did not exert any influence on the expression of these transcription factors (Fig. 4 E). This agreed with a reduced percentage of activated Th2 and Th1 cells and an increased percentage of Treg cells in the MLNs of mice from the OP-treated group (Fig. 5 A).

Therapy with OVA and OP increased the proportion of MLN CD11c⁺ cells, but while OVA enhanced CD103⁻ cells, OP augmented CD103⁺ cells and, particularly, the CD11b⁺ population within this subset (Supplementary Fig. 6). Both OVA and OP treatments, but mostly the latter, induced a significant downregulation of the expression of OX40L, Jagged-2 and Delta-4 in the MLNs (Fig. 5 B). In the

spleen, activated Th2 cells were reduced in both OVA and OP-treated mice, whereas activated Th1 cells were unchanged. However, the percentage of Treg cells was only enhanced after therapy with OP (Fig. 5 C and Supplementary Fig. 7). Accordingly, following *ex vivo* stimulation with EW, splenocytes from treated mice responded with a significantly lower level of Th2 and Th1 cytokines. Noteworthy, EW-induced secretion of IL-10 and IL-17 from splenocytes from OP-treated mice was higher than that from the spleen cells from the OVA-treated group (Supplementary Fig. 8). Hindrance of cytokine release from spleen cells upon EW stimulation was maintained in the OVA and OP groups 3 weeks after discontinuation of the respective therapies (Supplementary Fig. 8). Although splenocytes from PBS-treated sensitized mice produced less IL-4, IL-5 and IFN- γ than 3 weeks before, levels of Th2 and Th1 cytokines were still significantly higher than those produced by cells from OVA and OP-treated mice.

In order to discern if DCs from tolerized animals could influence the immune response to EW of T cells from sensitized mice by inducing a regulatory environment, CD4⁺ T cells from spleens of mice sensitized to EW were cocultured with DCs isolated from the MLNs of sham-sensitized, untreated (PBS) or OP-treated mice, and stimulated with EW (Supplementary Fig. 9). DCs from allergic (untreated) mice pulsed with EW induced a mixed response characterized by a significant release of IL-5, IFN- γ and IL-10 as compared with DCs from sham-sensitized mice. In the cocultures using DCs from tolerized (OP-treated) animals, we observed a significantly lower production of IL-5 (equivalent to the level induced by DCs from sham-sensitized mice) and IFN- γ , together with an increase in IL-10. These results indicate that DCs from mice tolerized by treatment with OP attenuated Th2 and Th1 cytokine responses to the allergen in CD4⁺T cells from sensitized mice, and that this effect was linked to the release of the regulatory cytokine IL-10.

In general terms, OP provided effective immunotherapy with durable effects in EW allergic mice. Unlike OVA, OP decreased the levels of EW-specific IgE and IgG1 and avoided anaphylaxis upon oral and systemic challenges. As it was the case of the preventive trial, enhanced intestinal expression of IL-10, TGF- β , IL-17, Foxp3 and ROR γ t, linked to increased Treg cell populations and reduced activated Th2 cells, appeared as responsible for the curative effects observed.

Discussion

In this work, a hydrolysate of OVA with pepsin was assayed for its ability to prevent and treat allergy to EW using a mouse model of oral hypersensitivity. Its suitability for prophylactic and therapeutic applications was compared with that of the intact allergen, paying attention to the molecular and cellular mechanisms involved in the control of the allergic process.

OP fulfilled the prerequisite of lacking sensitizing capacity in order to be used to prevent allergy development: OP administered orally with CT was weakly immunogenic and oral and i.p. challenges with OP did not induce allergic signs. Furthermore, administration of OP for 3 weeks avoided subsequent allergy to EW. It has been reported that partially hydrolysed cow's milk proteins deliver protective responses against milk allergy in mice, although the intact proteins are more effective in preventing sensitization due to a more pronounced reduction in the levels of allergen-specific IgE or IgG1 [14-17]. Nevertheless, in our study, pre-treatment with OVA did not hinder anaphylaxis following i.p. challenge with EW, likely because of the absence of a significant lowering effect on serum IgG1 concentration. Through PCA experiments with heated and unheated sera from EW-sensitized mice, we demonstrated that both specific IgE and IgG1 could participate in anaphylaxis in this model. Since much higher antibody and antigen concentrations are required to induce anaphylaxis by the IgG1-mediated pathway, parenteral administration of a relatively large quantity of antigen, as in our i.p. challenge tests, after CT-induced sensitization, would largely drive IgG1-mediated anaphylaxis [18].

Our results associate the preventive effects of OVA and OP with a reduced expression, in mouse intestinal tissues, of GATA3, the transcription factor induces the development of Th2 and group 2 innate lymphoid cells (ILC2s) [19]. This resulted in decreased numbers of activated Th2 cells in the MLNs and spleens, as well as in decreased Th2 responses upon stimulation of the splenocytes with the allergen used for sensitization, and explains the positive effects of the pre-treatments on serum concentrations of IgE and IgG1 antibodies, in agreement with similar approaches using hydrolysates or peptides [16, 17, 20]. However, changes in the Th1 response were less consistent, with upregulated

expression of T-bet in PPs and MLNs, but no change in the proportion of activated Th1 cells in the MLNs or spleen and diminished allergen-stimulated production of IFN- γ .

Intestinal CD103⁺ DCs, bearing the integrin α -chain CD103 marker are crucial in the immune response to oral antigens. They migrate from the lamina propria to the MLNs, where they promote tolerance through the differentiation of gut homing Treg cells, mainly via production of TGF- β and the dietary metabolite retinoic acid [21]. However, CT-induced intestinal sensitization is also mediated by the active migration of CD103⁺ DCs expressing OX40L and Jagged-2 from the lamina propria to the MLNs [22]. Although we could not detect an effect of OVA and OP on the CD103⁺ DC population, the observed downregulation of the DC genes OX40L and Jagged-2 points out that the pre-treatments counteracted the influence of CT on intestinal DCs that leads to Th2-priming and allergic sensitization. In parallel, OP, unlike OVA, upregulated Foxp3 in intestinal lymphoid tissues and increased the percentage of Treg cells in the MLNs and spleens, in line with other studies that showed a key role of Treg cells in prevention of sensitization and development of food allergy [15, 17, 20, 23].

As for its use as a therapeutic agent once EW allergy was established, assessment of the anaphylactic potential of OP by challenge to mice sensitized to EW confirmed that it was hypoallergenic. Moreover, when administered to EW-sensitized mice for 3 weeks, OP diminished adverse reactions upon oral and subsequent i.p. challenges with the allergen and protection was sustained 3 weeks after termination of the treatment, along with a gradual decrease in EW-specific IgE and IgG1 levels. Conversely, therapy with intact OVA for 3 weeks did not change the concentration of EW-specific IgE and IgG1, nor did it avoid anaphylaxis following challenge with EW. Of note was the observation that, in the animals treated with OVA, desensitization was observed after the 3 week off-therapy period, also in parallel with a reduction in the level of EW-specific antibodies, while the concentration of serum specific IgE and IgG1 was maintained in untreated mice sensitized to EW, in agreement with [24].

Successful desensitization with immunomodulatory hydrolysates or peptides is usually accompanied by a significant reduction in allergen-specific IgE levels [5, 25-28]. Furthermore, in view of the participation of IgG1 in anaphylaxis in our mouse model, decreased serum concentrations of this isotype are also likely to account for clinical benefits. On the other hand, effective therapy has been attributed to increases in allergen-specific IgG2a or IgA, which act as protective antibodies in the mouse [5, 26-28], although, evidence for induction of blocking antibodies through peptide immunotherapy is scarce, because, unlike intact allergens, short peptides hold a restricted potential to drive antibody production [29, 30]. In line with its weak immunogenicity, treatment with OP did not increase the concentration of IgG2a, albeit it enhanced fecal IgA. Nevertheless, the property of IgA to block systemic anaphylaxis by binding to allergens appears to belong to serum IgA and not to IgA contained in the gut lumen [31].

Whereas the role of blocking antibodies appears less important in peptide immunotherapy than in whole allergen immunotherapy; similarly to conventional immunotherapy, downregulation of Th2 proliferative and cytokine responses to the allergen is usually reported [5, 25-28]. However, different studies refer either an associated increase in Th1 cytokines [26-28] or a concomitant Th1 decrease [25]. Treatment with both OVA and OP decreased the expression of GATA3 in the duodenum and PPs and the percentage of splenic activated Th2 cells, with no change in the percentage of activated Th1 cells. OP additionally increased the CD103⁺ CD11b⁺ subset of DCs in the MLN, and reduced the expression of the Th2 and Th1 skewing factors OX40L, Jagged-2 and Delta-4. Although, as mentioned, evidence so far argues against a functional specialization of DC subsets, the effects of OP on DCs could be linked to the downregulation of GATA3 and T-bet in the MLN and to the observed reduction in the percentage of activated Th2 and Th1 cells, indicating that OP balanced more efficiently than OVA the immunostimulating effects of CT, which leads to mixed Th2 and Th1 local and systemic responses [22]. Furthermore, upregulation of Foxp3 in intestinal tissues and of the percentage of Treg cells in the MLNs and spleens of mice treated with OP point to a crucial role of these cells in the inhibition of Th1 and Th2 responses and highlight induction of Treg cells as the

main mechanism underlying the efficacy of tolerance induction by food peptides [25, 26, 28], as also shown in *ex vivo* experiments.

In contrast to our study, immunotherapy with pepsin-hydrolysed cashew was reported to work through the same mechanism as immunotherapy with the intact allergen due to the strong Th2-stimulating properties of the hydrolyzate [5]. However, as it was previously shown, OP, in addition to low IgE-binding, exhibits comparable Th1 stimulating properties than OVA in the absence of Th2 inducing capacity [7]. Furthermore, diarrhea during the first week of therapy with OVA was a symptomatic feature of IgE-mediated reactions, suggesting that OVA gavage during immunotherapy, unlike OP gavage, would have worked as a repeated oral challenge. It is known that stimulation of mouse mast cells by IgE cross-linking releases Th2-driving cytokines, promoting the expansion of mucosal ILC2s and the collaboration between these innate cells and allergen-specific Th2 cells, ultimately acting, through IL-4, as a Foxp3 inhibitor in Treg cell differentiation [11, 24, 32, 33]. Therefore, the lack of IgE-binding and Th2-priming capacity of OP may not only increase safety, but also efficacy of immunotherapy.

In addition, therapy with OP provided extended desensitization. While this observation contrasts with other reports describing the reestablishment of clinical reactivity in mice once therapy is stopped, it is recognized that the induction of Treg cells can provide long lasting beneficial effects [34, 35]. Furthermore, it is noteworthy that peptides resulting from OVA fragmentation offered protection against challenge with whole EW. In this respect, it is also accepted that the most effective way to provide tolerance towards multiple epitopes within an allergen, or even towards other allergens normally co-exposed with it, is through the induction of a regulatory function in the T cells primed by the tolerogenic substance, as the regulatory environment can then be extended to T cells specific for other epitopes [36]. Indeed, there are cases of preventive and therapeutic uses of single food peptides that reduce sensitization and allergic responses to the whole intact protein in mice [23, 27]. However, this is, to the best of our knowledge, the first example of oral tolerance to non-cross reacting proteins not present in the therapy, an effect which was previously shown in peptide immunotherapy experiments in mouse models of allergic airways disease [37]. Still, whereas Treg cells can suppress

the effector responses of T cells with different antigen specificities, the generation of allergen-specific Treg cells is a requisite, as shown by the lack of effect of therapy with a non-related protein such as BSA.

The superior potential of OP versus OVA for allergy prevention and therapy linked to Treg cell development paralleled a more efficient upregulation of the cytokines TGF- β and IL-10 in the intestinal tissues. In addition, it is noteworthy that IL-17 and ROR γ t, the Th17 master transcription factor, were also overexpressed. Treg and Th17 cells, which apparently exert opposite functions, as the latter are associated with pathogenic proinflammatory phenotypes, arise through a common intermediate that simultaneously upregulates both Foxp3 and ROR γ t [38]. Tregs that constitutively express Foxp3 and ROR γ t have been found in human and mice and it is postulated that they can contribute to the control of exacerbated Th2 responses [39-41]. Furthermore, while IL-17 signaling is required to develop allergic asthma, an inverse correlation between IL-17 levels and food allergy has been reported [42-44]. Therapy with OP increased the DC CD103⁺ CD11b⁺ population in the MLNs, which can promote the development of Treg cells through the production of TGF- β , but also produce of IL-6 that, besides inducing B cell activation to secrete IgA, leads to Th17 cells [45]. Therefore, it can be hypothesized that the concomitant upregulation of TGF- β , IL-10, IL-17, Foxp3 and ROR γ t observed in the intestinal tissues of mice, either preventive or therapeutically treated with OP, had a joined effect in the inhibition of Th2 and inflammatory responses contributing to a balanced immune protection.

Disclosures

The authors have no financial conflicts of interest.

Acknowledgments

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LEGENDS TO THE FIGURES

Figure 1. Sensitizing and eliciting capacity of OP. Mice were administered CT, EW+CT or OP+CT. (A) EW-specific IgE, IgG1, IgG2a and IgA antibodies. (B) Anaphylactic scores and body temperature after oral and i.p. challenges with EW or OP, and levels of MCP-1 in serum samples collected after sacrifice. Relative gene expression of (C) IL-33, IL-25 and TSLP; and (D) GATA3, T-bet, Foxp3 and ROR γ t determined in duodenum samples (the CT group, used as calibrator, is represented as a discontinuous line in the figure). (E) Activated Th2, Th1 and Treg cells within the CD4⁺ population in the spleens. Data are expressed as means $\pm$ SEM, except for clinical scores, that are represented as medians (n=5). Different letters indicate statistically significant differences (P< 0.05) and in (A) they designate differences between groups of mice at the same experimental point. * indicates differences (P< 0.05) compared with the calibrator.

Figure 2. Prevention of allergy to EW by pre-treatment with OVA and OP. Mice were either treated with OVA or OP or left untreated (PBS), before being sensitized to EW or sham-sensitized (CT). (A) EW-specific IgE, IgG1, IgG2a and IgA antibodies. (B) Anaphylactic scores and body temperature after oral and i.p. challenges with EW, and levels of MCP-1 in serum samples collected after sacrifice. Relative gene expression of (C) IL-33, IL-25, TSLP and IL-9 determined in duodenum (DD); (D) IL-10, TGF- β and IL-17; and (E) GATA3, T-bet, Foxp3 and ROR γ t determined in DD, PPs and MLNs (the CT group, used as calibrator, is represented as a discontinuous line in the figure). Data are expressed as means $\pm$ SEM, except for clinical scores, that are represented as medians (n=5). Different letters indicate statistically significant differences (P< 0.05) and in (A) they designate differences between groups of mice at the same experimental point. * indicates differences (P< 0.05) compared with the calibrator.

Figure 3. Dendritic and T cell responses of mice pre-treated with OVA and OP before sensitisation to EW. Mice were either treated with OVA or OP or left untreated (PBS), before being sensitized to EW or sham-sensitized (CT). (A) Activated Th2, Th1 and Treg cells within the CD4⁺ population in MLNs. (B) Relative gene expression of OX40L, Jagged-2, IL-12p40 and Delta-4 in MLNs (the CT group, used as calibrator, is represented as a discontinuous line in the figure). (C) Activated Th2, Th1 and Treg cells within the CD4⁺ population in spleens. Data are expressed as means $\pm$ SEM (n=5). Different letters indicate statistically significant differences (P< 0.05). * indicates differences (P< 0.05) compared with the calibrator.

Figure 4. Therapy of allergy to EW by treatment with OVA and OP. Mice were sensitized to EW or sham-sensitized (CT) before being treated with either BSA, OVA or OP or left untreated (PBS). (A) EW-specific IgE, IgG1, IgG2a and IgA antibodies. (B) Anaphylactic score and body temperature after oral and i.p. challenges with EW, and levels of MCP-1 in serum samples collected after sacrifice. Relative gene expression of (C) IL-33, IL-25, TSLP and IL-9 determined in duodenum (DD); (D) IL-10, TGF- β , and IL-17; and (E) GATA3, T-bet, Foxp3 and ROR γ t determined in DD, PPs and MLNs (the CT group, used as calibrator, is represented as a discontinuous line in the figure). Different letters indicate statistically significant differences (P< 0.05) and in (A) they designate differences between groups of mice at the same experimental point. * indicates differences (P< 0.05) compared with the calibrator. This experiment was repeated 3 times with consistent results and data from a representative experiment are reported.

Figure 5. Dendritic and T cell responses of mice sensitized to EW and treated with OVA and OP. Mice were sensitized to EW or sham-sensitized (CT) before being treated with either BSA, OVA or OP or left untreated (PBS). (A) Activated Th2, Th1 and T reg cells within the CD4⁺ population in MLNs. (B) Relative gene expression of OX40L, Jagged-2, IL-12p40 and Delta-4 in MLN samples (the CT group, used as calibrator, is represented as a discontinuous line in the figure). (C) Activated Th2, Th1 and Treg cells within the CD4⁺ population in spleens. Data are expressed as means $\pm$ SEM (n=5). Different letters indicate statistically significant differences (P< 0.05). * indicates differences

($P < 0.05$) compared with the calibrator. This experiment was repeated 3 times with consistent results and data from a representative experiment are reported.

Fig. 1

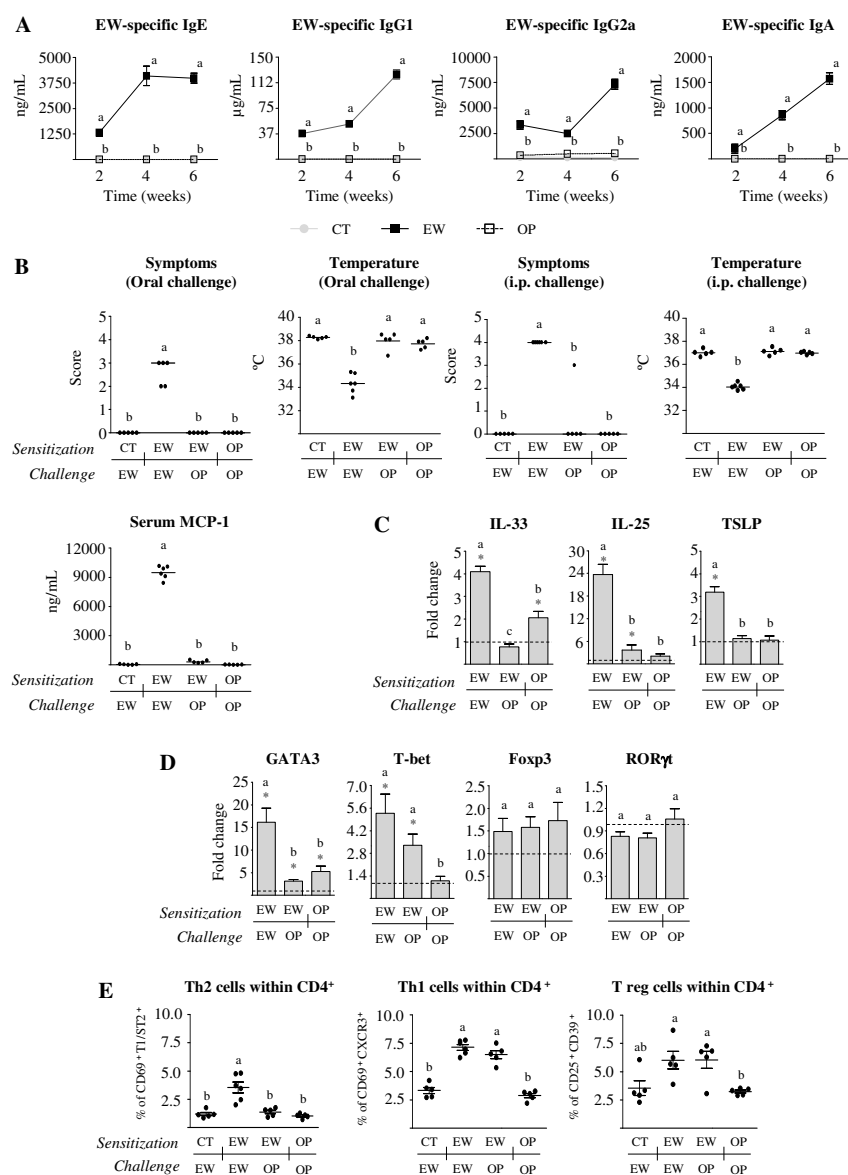


Fig. 2

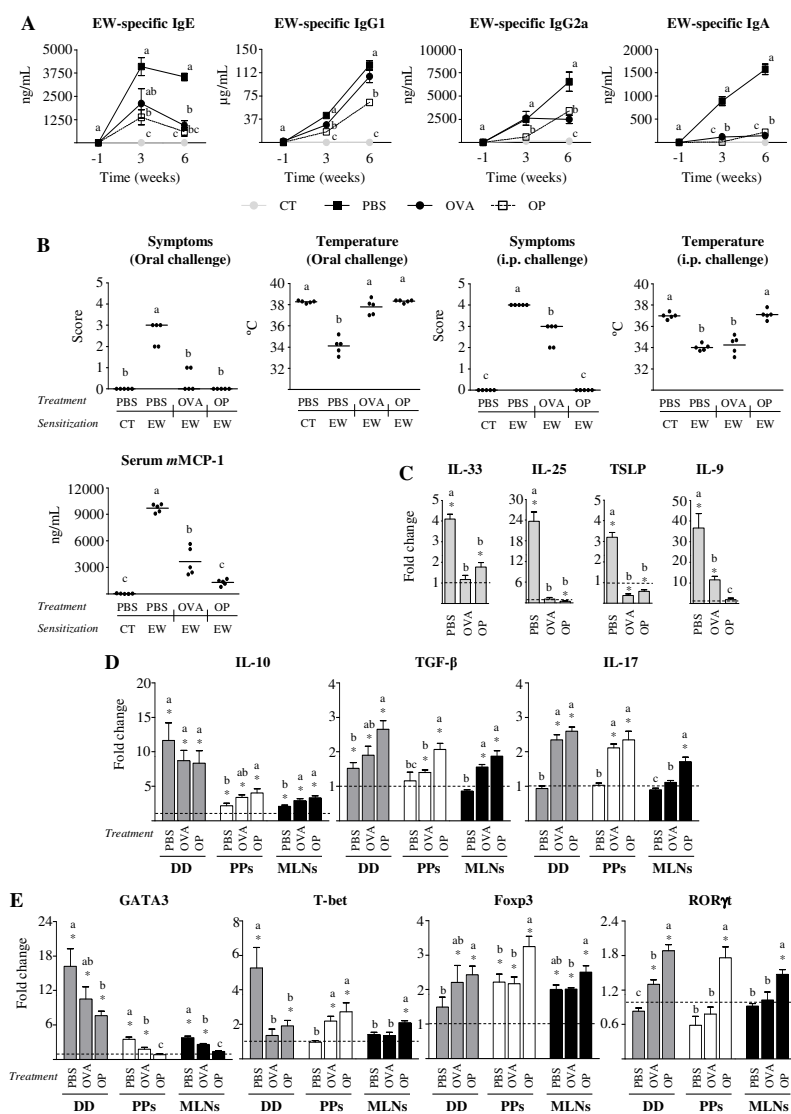


Fig. 3

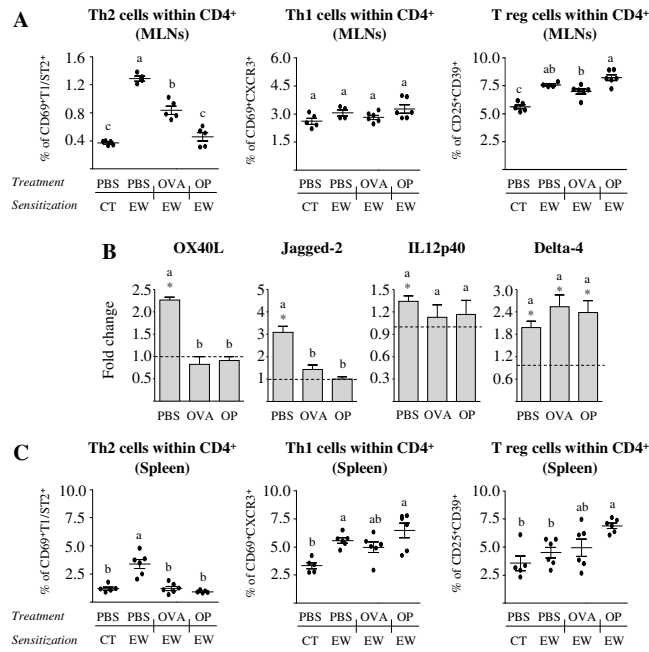


Fig. 4

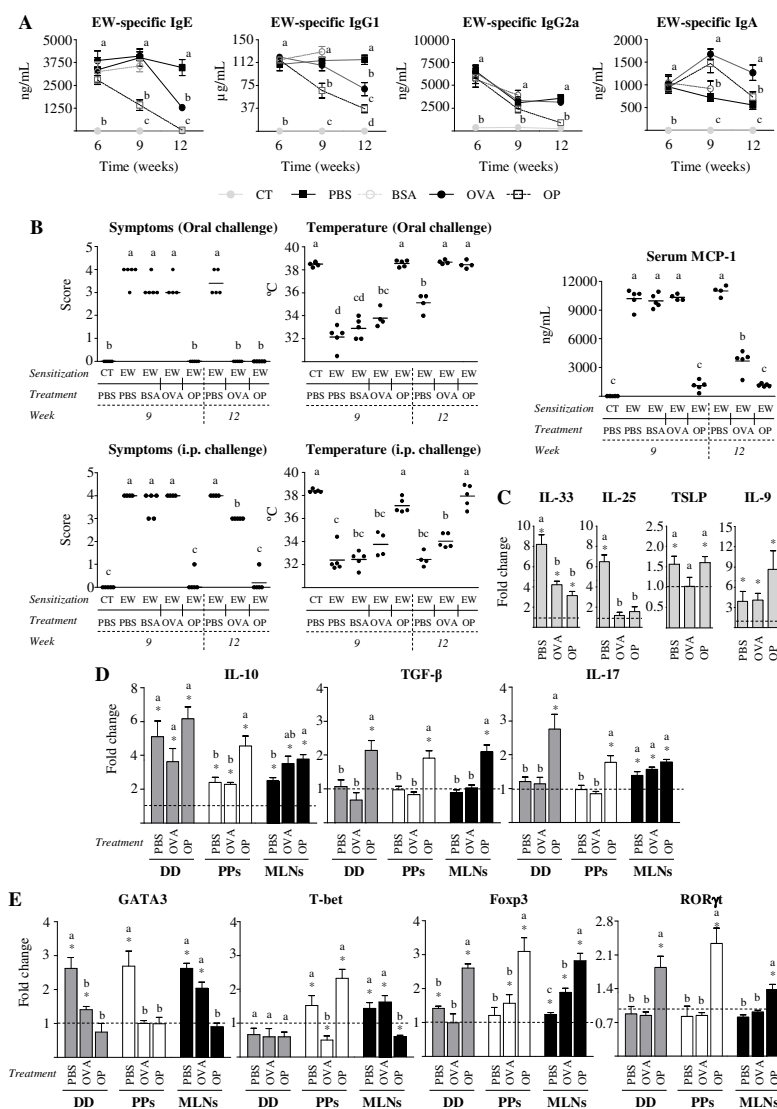
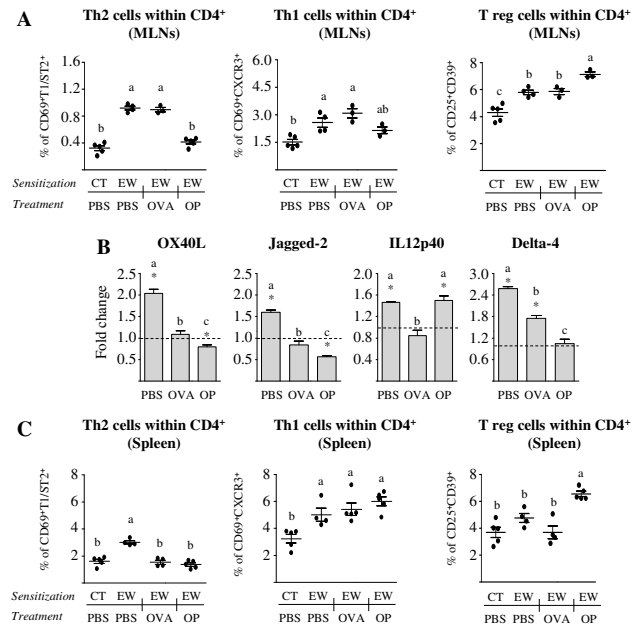


Fig. 5



SUPPLEMENTARY MATERIALS

Supplementary Table 1. Primer sequences used in RT-qPCR analyses of gene expression.

Gene	Primer pairs	Reference	Cycling conditions
β -actin	fw 5' AGCTGCGTTTTACACCCTTT 3' rv 5' AAGCCATGCCAATGTTGTCT 3'	[1]	<i>Pre-Incubation</i> 2 min 50°C
IL-10	fw 5' GCCTTATCGGAAATGATCCA 3' rv 5' AGGGGAGAAATCGATGACAG 3'	[2]	<i>Incubation</i> 10 min 95°C
IL-22	fw 5' CATGCAGGAGGTGGTACCTT 3' rv 5' CAGACGCAAGCATTCTCAG3'	[3]	<u>40 cycles:</u>
OX40L	fw 5' GGGATGCTTCTGTGCTTCATCT 3' rv 5' TTTGGATTGGAGGGTCCTTTG 3'	[4]	<i>Denaturation</i> 15 s 95°C
TGF- β	fw 5' TTGCTTCAGCTCCACAGAGA 3' rv 5' TACTGTGTGTCCAGGCTCCA 3'	[2]	<i>Annealing/Extension</i> 45 s 58°C + 15 s 60°C
Delta-4	fw 5' ACCTTTGGCAATGTCTCCAC 3' rv 5' TTGGATGATGATTGGCTGA 3'	[5]	<i>Pre-Incubation</i> 2 min 50°C <i>Incubation</i> 10 min 95°C <u>40 cycles:</u> <i>Denaturation</i> 15 s 95°C <i>Annealing/Extension</i> 60 s 60°C
Foxp3	fw 5' ACAACCTGAGCCTGCACAAGT 3' rv 5' GCCCACCTTTTCTTGGTTTTG 3'	[1]	
GATA3	fw 5' CCTTAAACTCTTGGCGTCC 3' rv 5' AGACACATGTCATCCCTGAG 3'	[6]	
IL12p40	fw 5' AGGTGCGTTCCTCGTAGAGA 3' rv 5' AAAGCCAACCAAGCAGAAGA 3'	[5]	
IL-9	fw 5' GTCCGTCCTTTTCTGCGAA 3' rv 5' TCTGTCTTCATGGTCGGCTT 3'	This work	
IL-17	fw 5' TGCCTGTGGCACTGAAGTAG 3' rv 5' TTCATGGCTGCAGTGAAAAG 3'	[7]	
IL-33	fw 5' ATTTCCCCGGCAAAGTTCAG 3' rv 5' AACGGAGTCTCATGCAGTAGA 3'	[8]	
IL-25	fw 5' ACAGGGACTTGAATCGGGTC 3' rv 5' TGGTAAAGTGGGACGGAGTTG 3'	[8]	
Jagged-2	fw5' GGCAAAGAATGCAAAGAAGC 3' rv 5' GCTCAGCATTGATGCAGGTA 3'	[5]	
ROR γ t	fw5' TCACCTGTGAGGGGTGCAAG 3' rv 5' GTTCGGTCAATGGGGCAGTT 3'	[9]	
T-bet	fw 5' GTATCCTGTTCCCAGCCGTTTC 3' rv 5' ACTGTGTTCCCGAGGTGTCC 3'	[10]	
TSLP	fw 5' AGGCTACCCTGAAACTGAGA 3' rv 5' GGAGATTGCATGAAGGAATAC 3'	[11]	

fw, forward; rv, reverse

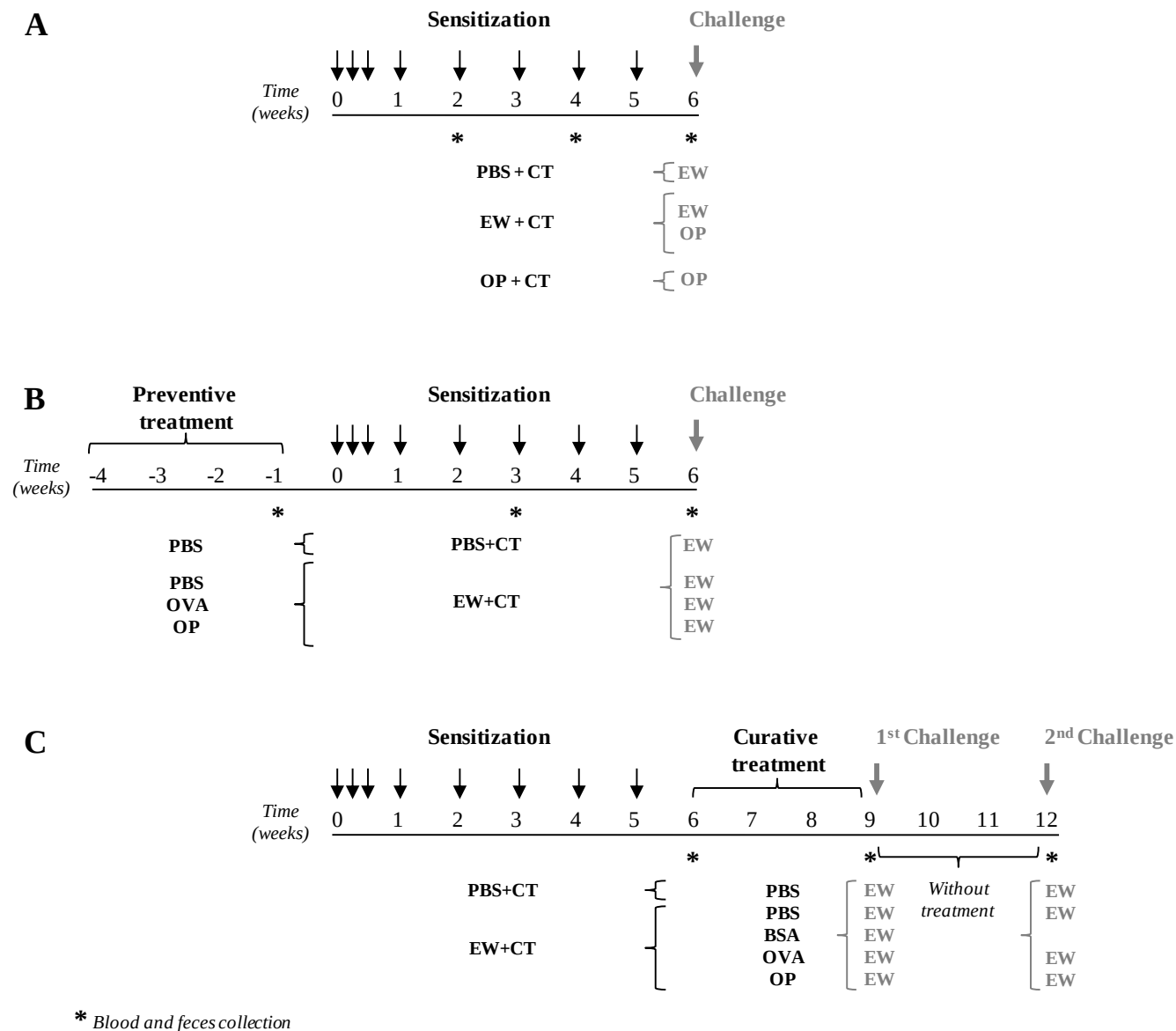
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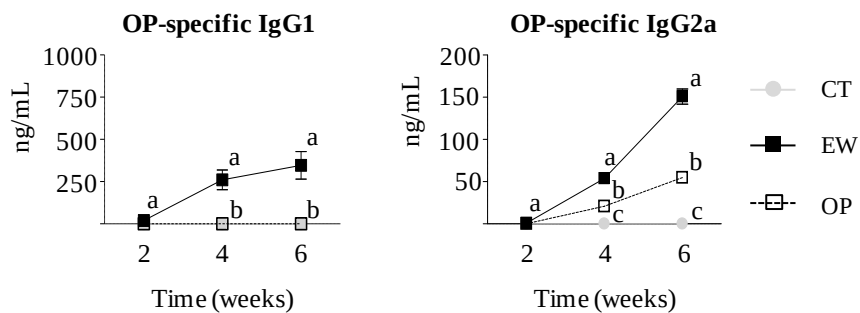
Supplementary Table 2. Antibodies used for the assessment of T cell and dendritic cell subsets by flow cytometry.

Antibody^a	Clone	Cell population
Anti-CD4-Alexa Fluor 700	GK1.5	T cells
Anti-CD69-PerCP-Cy5.5	H1.2F3	T cells
Anti-CXCR3-APC	CXCR3-173	Th1 cells
Anti-ST2-PE	RMST2-2	Th2 cells
Anti-CD25-FITC	3C7	Treg cells
Anti-CD39-PE-Cy7	Duha59	Treg cells
Anti-CD11c-PE-Cy7	N418	DCs
Anti-CD11b-Alexa Fluor 700	M1/70	DCs
Anti-CD103-PE	2E7	DCs

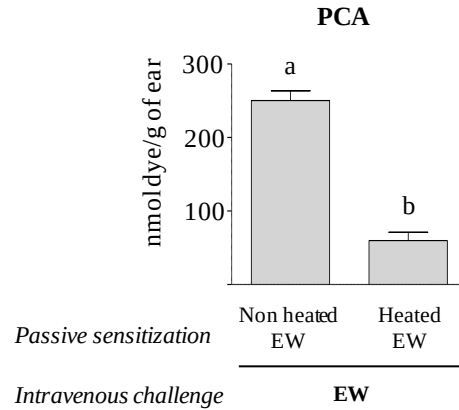
^a All from Biolegend (San Diego, CA, USA)



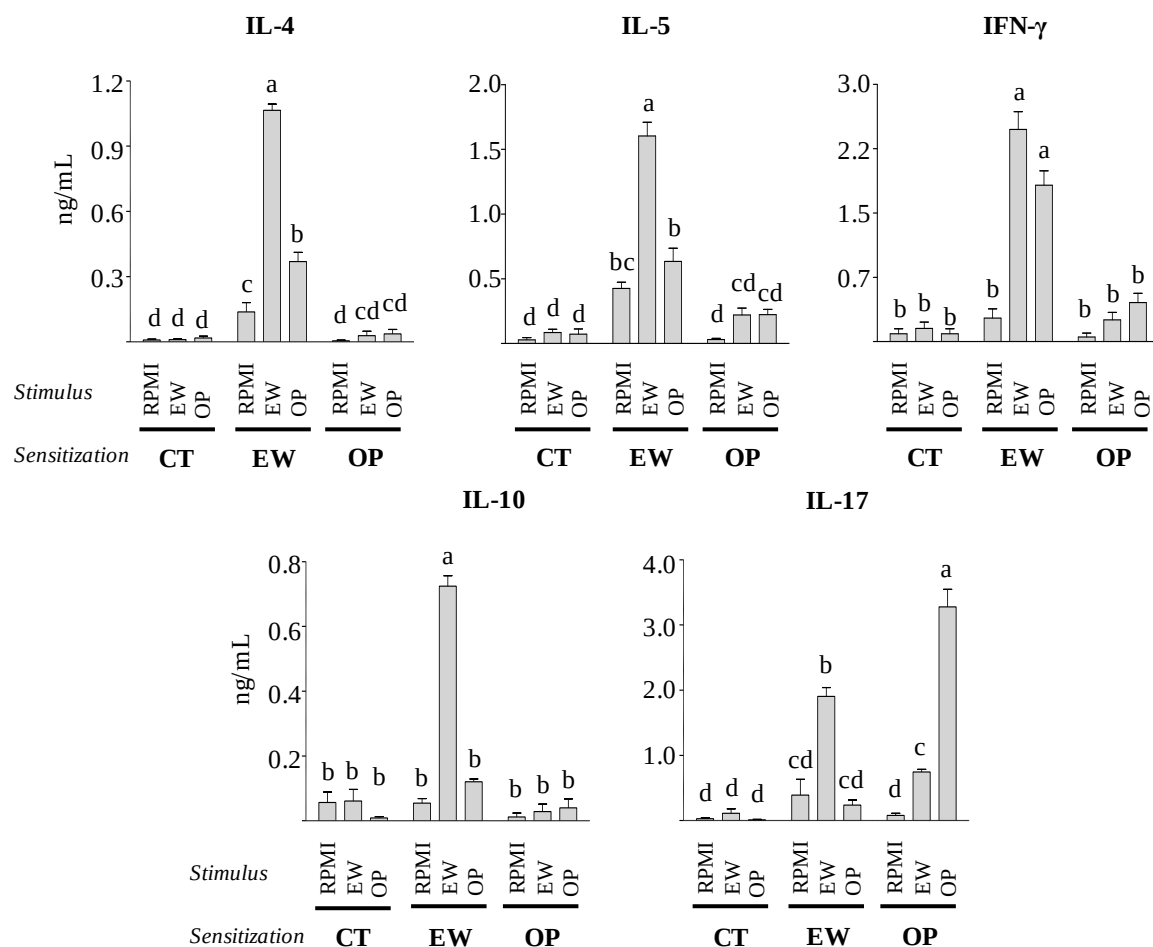
Supplementary figure 1. Experimental procedures followed in the study of the (A) sensitizing and eliciting capacity, and (B) preventive and (C) therapeutic effects of OP in BALB/c mice.



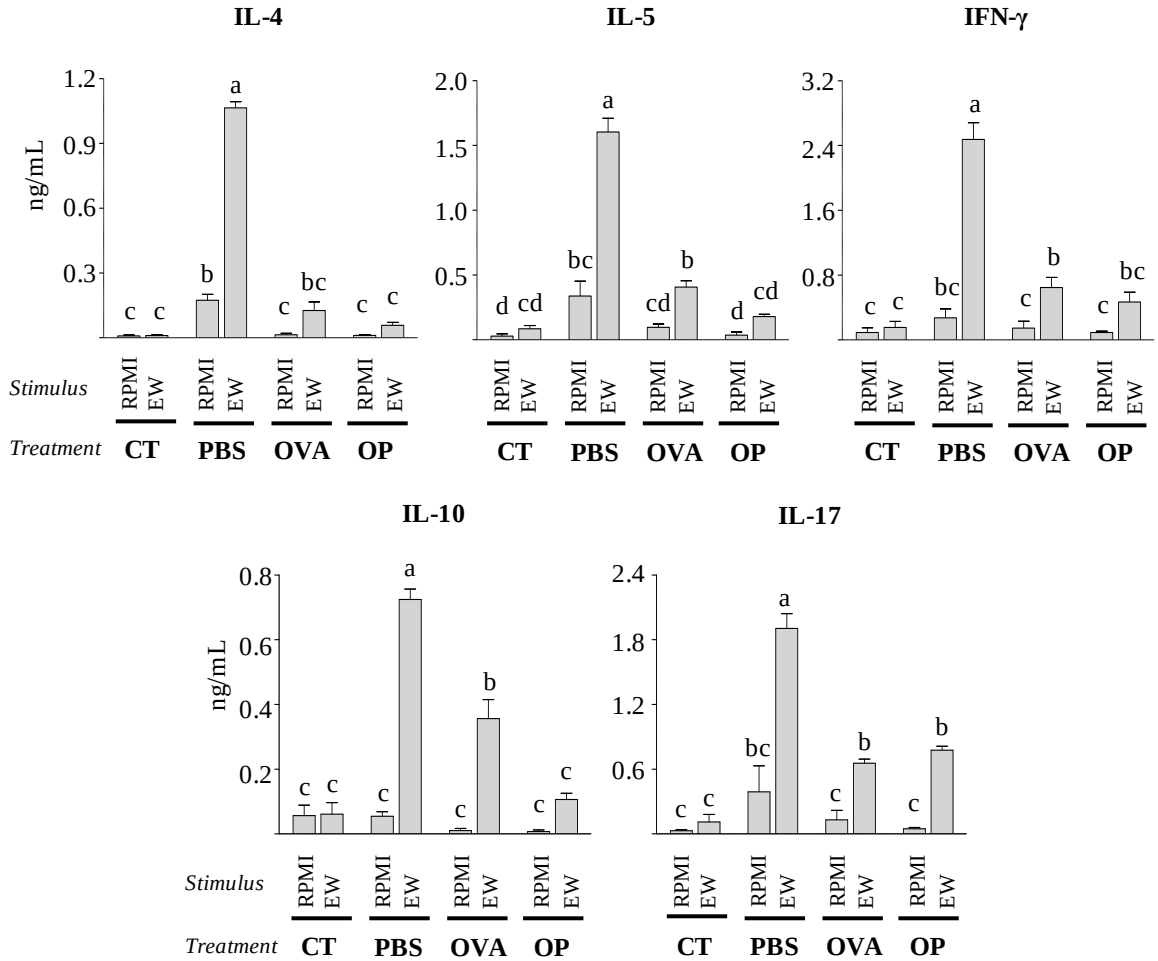
Supplementary figure 2. OP-specific IgG1 and IgG2a antibodies in mice administered CT, EW+CT or OP+CT. Data are expressed as means $\pm$ SEM (n=5) and different letters designate differences ($P < 0.05$) between groups of mice at the same experimental point.



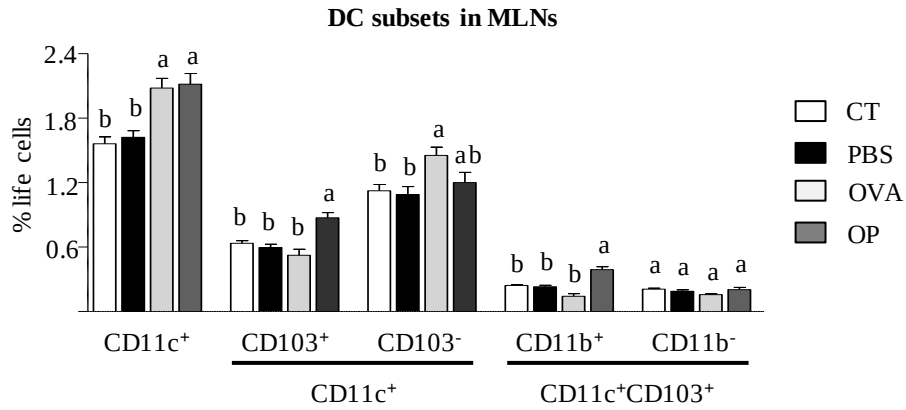
Supplementary figure 3. PCA assay with unheated and heated (56°C for 3 h) pooled serum obtained at sacrifice (week 6) from mice administered EW+CT (n=5). Dye extravasation was calculated by correcting the absorbance induced by EW inoculation with the absorbance of the supernatant of the ear injected with pooled serum from sham sensitized mice, extrapolation from a reference curve with known amounts of Evans blue and normalization considering the weight of each ear. Data are means $\pm$ SEM (n= 2) and different letters indicate statistically significant differences ($P < 0.05$).



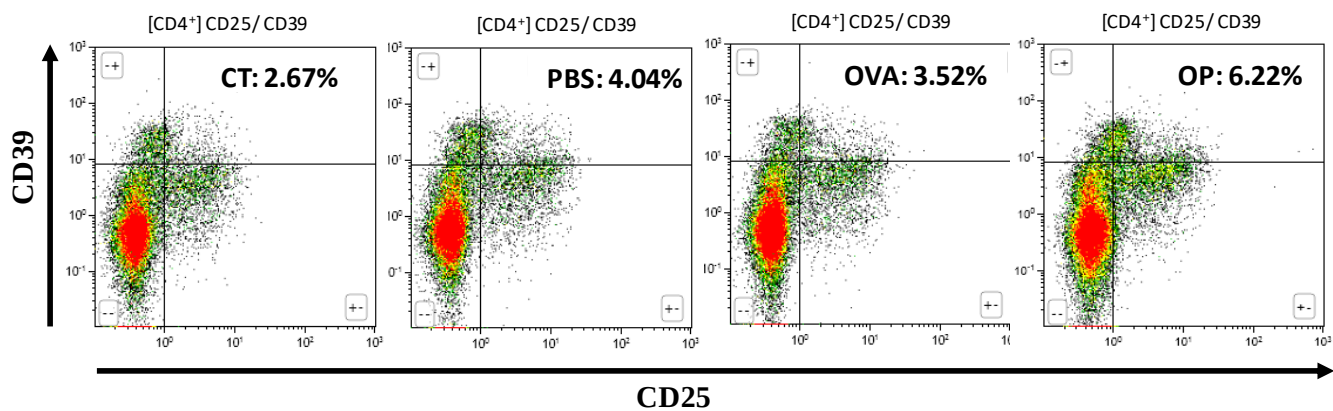
Supplementary figure 4. Effects of the *ex vivo* stimulation with EW and OP on the secretion of IL-4, IL-5, IFN-γ, IL-10 and IL-17 by splenocytes from mice administered CT, EW+CT and OP+CT. Data are expressed as means $\pm$ SEM (n=5) and different letters indicate statistically significant differences ($P < 0.05$).



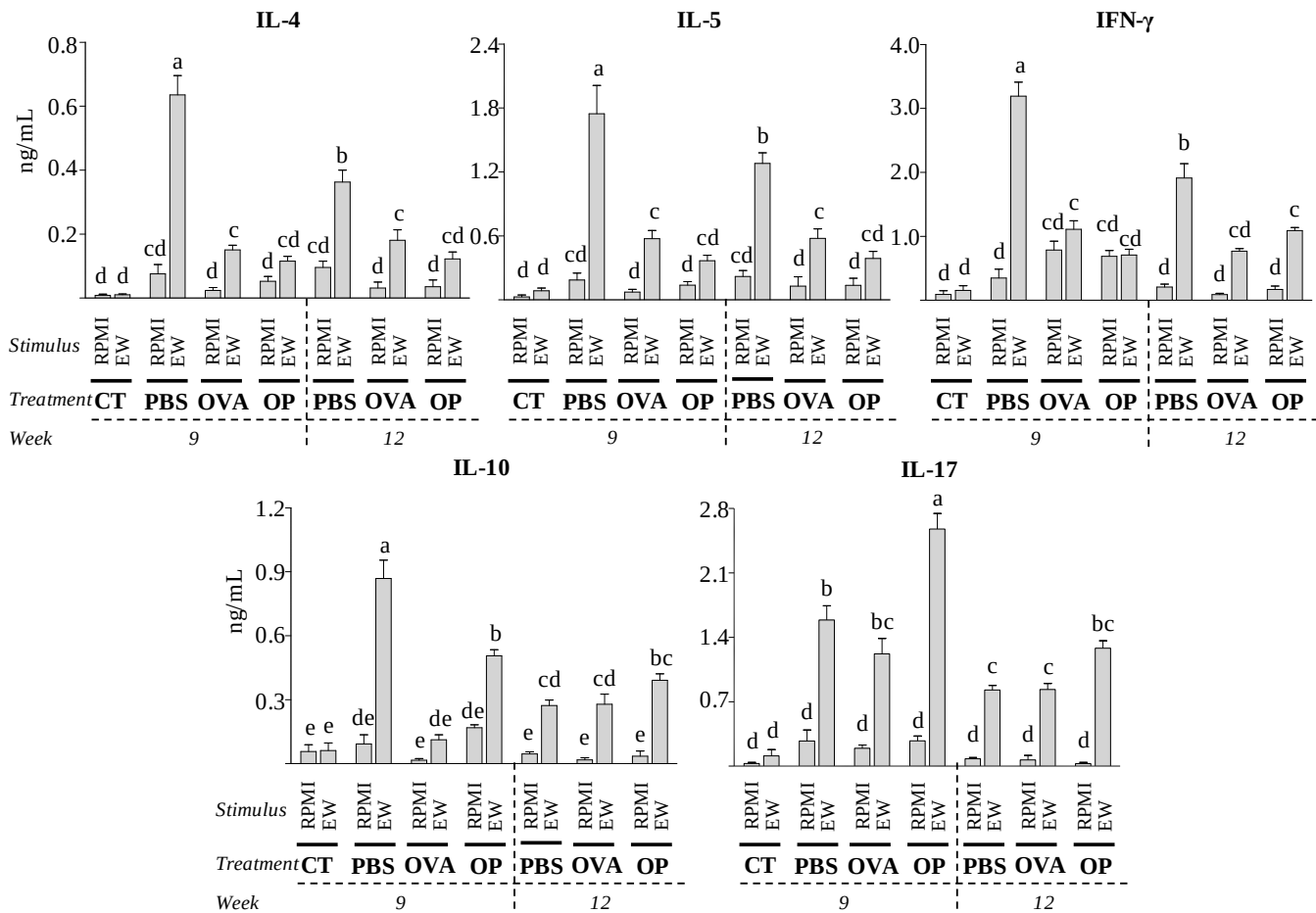
Supplementary figure 5. Effects of the *ex vivo* stimulation with EW on the secretion of IL-4, IL-5, IFN-γ, IL-10 and IL-17 by splenocytes from mice either treated with OVA or OP or non-treated (PBS), before being sensitized to EW or sham-sensitized (CT). Data are expressed as means $\pm$ SEM (n=5). Different letters indicate statistically significant differences (P < 0.05).



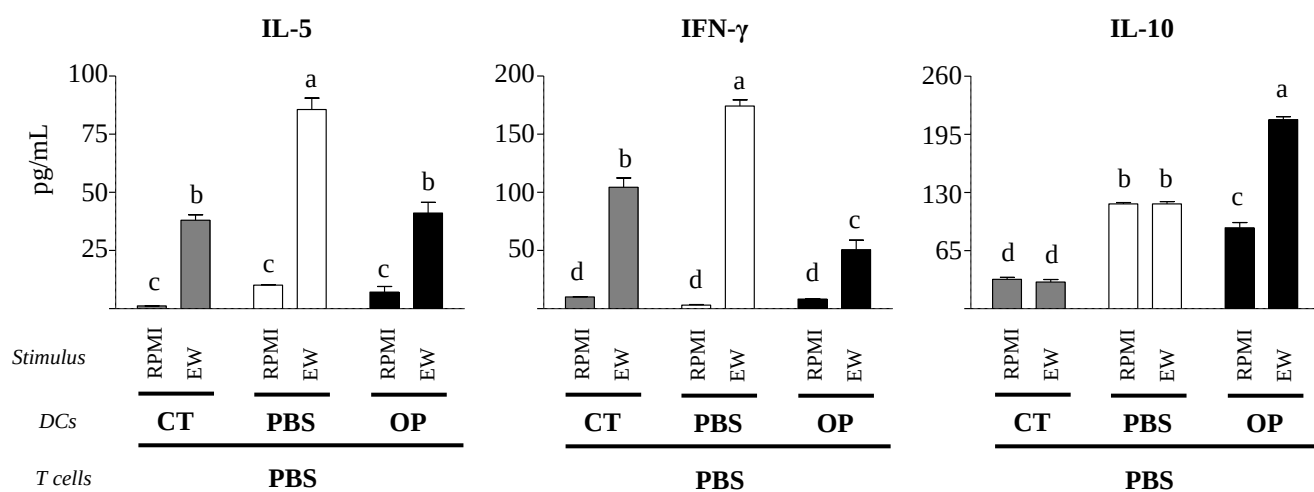
Supplementary figure 6. Distribution of dendritic cell (CD11c⁺) subsets (defined on the basis of the expression of surface markers CD103 and CD11b) in the MLNs of mice sensitized to EW or sham-sensitized (CT) before being treated with either OVA or OP or non-treated (PBS), estimated by flow cytometry. Data are expressed as means $\pm$ SEM (n=5). Different letters indicate statistically significant differences ($P < 0.05$).



Supplementary figure 7. Representative flow cytometry analyses of CD25⁺ CD39⁺ cells (Treg cells) within the CD4⁺ population in spleens from mice sensitized to EW or sham-sensitized (CT) before being treated with either OVA or OP or non-treated (PBS).



Supplementary figure 8. Effects of the *ex vivo* stimulation with EW on the secretion of IL-4, IL-5, IFN-γ, IL-10 and IL-17 by splenocytes from mice sensitized to EW or sham-sensitized (CT) before being treated with either OVA or OP or non-treated (PBS), 3 days (week 9) and 3 weeks after the end of the treatment (week 12). Data are expressed as means $\pm$ SEM (n=5). Different letters indicate statistically significant differences ($P < 0.05$).



Supplementary figure 9. Secretion of IL-5, IFN- γ and IL-10 by CD4⁺ T cells isolated from mice sensitized to EW co-cultured with DCs isolated from sham-sensitized mice (CT) and EW-sensitized mice, either untreated (PBS) or treated with OP and stimulated with EW. Data are expressed as means $\pm$ SEM (n=5). Different letters indicate statistically significant differences ($P < 0.05$).