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Systemic IL-2/anti-IL-2Ab complex combined with sublingual immunotherapy suppresses experimental food allergy in mice through induction of mucosal regulatory T cells

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Abstract

Therapeutic tolerance restoration has been proven to modify food allergy in patients and animal models and although sublingual immunotherapy (SLIT) has showed promise, combined therapy may be necessary to achieve a strong and long-term tolerance. In this work, we combined SLIT with systemic administration of IL-2 associated with an anti-IL-2 monoclonal antibody (IL-2/anti-IL-2Ab complex or IL-2C) to reverse the IgE-mediated experimental allergy.

Balb/c mice were sensitized with cholera toxin and milk proteins and orally challenged with allergen to elicit hypersensitivity reactions. Then, allergic mice were treated with a sublingual administration of very low amounts of milk proteins combined with intraperitoneal injection

of low doses of IL-2C. The animals were next re-exposed to allergens and mucosal as well as systemic immunological parameters were assessed *in vivo* and *in vitro*.

The treatment reduced serum specific IgE, IL-5 secretion by spleen cells and increased IL-10 and TGF-β in the lamina propria of buccal and duodenal mucosae. We found an augmented frequency of IL-10-secreting CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells (Treg) in the submaxilar lymph nodes and buccal lamina propria. Tregs were sorted, characterized and adoptively transferred to naïve mice, which were subsequently sensitized. No allergy was experienced in these mice and we encouragingly discovered a faster and more efficient tolerance induction with the combined therapy compared with SLIT.

In conclusion, the combination of two therapeutic strategies rendered Treg-mediated tolerance more efficient compared to individual treatments and reversed the established IgE-mediated food allergy. This approach highlights the ability of IL-2C to expand Tregs, and it may represent a promising disease-modifying therapy for managing food allergy.

Introduction

Cow's milk allergy (CMA) has evolved as an emerging epidemic during the last decades (1,2). The allergy is mediated by adaptive immune responses characterized by a Th2-skewed T cell response (3,4) that drives high IgE secretion which may result in the risk of life-threatening anaphylaxis in a proportion of allergic patients (5). One of the potential causes of this aberrant immune response to harmless dietary antigens has been attributed to a failure to induce regulatory T cell (Treg)-mediated tolerance (6–8). Patients suffering from polyendocrinopathy X-linked syndrome (IPEX) have a deficit in Tregs due to a mutation of

the FOXP3 gene, causing a loss of tolerance with subsequent severe autoimmunity, food allergy and infection (9). In addition, Foxp3 polymorphism and differential epigenetic control of gene expression have been described in food allergic patients (10–12). Remarkably, induction of Tregs is a critical marker of spontaneous resolution of food allergy (8,13) and of successful specific disease-modifying immunotherapy (14,15). We have previously demonstrated in an IgE-mediated food allergy mouse model, (that closely recapitulates food allergy in humans), that Tregs are reduced in sensitized animals and the adoptive transfer of these cells reversed mucosal inflammation and allergic immune responses (16). We observed that the step-wise oral administration of cow´s milk proteins (CMP) to sensitized mice induced intestinal Tregs, which suppressed the Th2-mediated mechanisms (including IgE, cytokines, skin mast cell activation).and the clinical symptoms. Based on these findings, we were prompted to down-modulate clinical signs by sublingual administration of very low doses of CMP, compared to the doses administered by gavage.

A recent therapeutic approach for several immunological diseases (autoimmunity, GVHD, etc) has been the *in vivo* or *ex vivo* expansion and transfer of Tregs (17–20) using low doses of the T cell growth factor IL-2 (21,22). It has been therapeutically used in immunological disorders in human and experimental models (23–27) with differing degrees of success . (28,29). Low doses of IL-2 increased *in vivo* the frequency of Tregs expressing the high affinity IL-2 receptor (IL-2R) (30), and different strategies have been investigated to optimize its bioavailability (31–33). In a pilot study using low doses of human IL-2 (25000 IU), along with sublingual immunotherapy (10 ng of allergen), we failed to suppress hypersensitivity symptoms in animals that were intragastrically sensitized (unpublished results). Based on the fact that particular high affinity anti-IL-2 monoclonal antibody improves IL-2 bioavailability and targets IL-2 (preferentially to CD25 on Tregs) (34,35,45), we next assessed the

therapeutic potential of low doses of IL-2 (15000 IU) combined with the monoclonal antibody (IL-2/anti-IL-2 complex; IL-2C) in the experimental model of food allergy. We found that the intraperitoneal administration of IL-2C successfully enhanced the tolerogenic mechanisms induced by very low doses of sublingual antigen and reversed food allergy.

Materials & Methods

Reagents

Milk protein extract was prepared from skimmed dry milk (Svelty, Nestle). CMP were dissolved in PBS for sublingual administration or in buffer bicarbonate for oral administration. Cholera toxin (CT) (Sigma Aldrich, St. Louis, USA), used as mucosal adjuvant, was dissolved in bicarbonate buffer (14). IL-2C was prepared by mixing IL-2 (15000IU) (Proleukin, Novartis, San Diego, CA, USA) with anti-IL-2 (4.5µg) (clone 5344, BD, NJ, USA) at a 2:1 molar ratio and incubated 30 minutes at 37ºC before injected in the mice.

Animals, sensitization and challenge

For sensitization, 5- to 7-week old BALB/c male mice received 6 weekly intragastric (ig) doses of 20 mg of CMP and 10 µg of CT in 200 µl per mouse (n = 5/group) (16). Ten days after the final boost, mice were challenged by gavage with 10 mg of CMP in 200 µl on two consecutive days (2 intragastric challenges). Twenty-four hours later animals were sacrificed by cervical dislocation. Control group mice received 20mg CMP (without CT) during the sensitization phase. The experimental design is shown in Fig. S1.

Animal treatment

Sensitized mice were anesthetized and divided into 2 groups for sublingual (sl) treatment: a) mice receiving PBS_{sl} and b) mice receiving 10 pg/4 µl of CMP_{sl} twice a week during four weeks and 10 ng during the last four weeks. Groups were further divided in 2 sub groups for intraperitoneal treatment: i) mice receiving PBS_{ip} and ii) mice receiving IL-2C ip (Fig. S1). Thirty minutes after the sl administration mice were grouped and placed in clean cages to assure no cross-contamination between animals.

To characterize the mucosal induction of Tregs, mice received 175µg/4µl of CMP_{sl} or PBS_{sl} for 2 consecutive days, and thereafter they received intraperitoneal injections of IL-2C for 3 consecutive days. Finally, mice were sacrificed (day 5) and submaxilar lymph nodes (SLN) and sublingual mucosa were analyzed.

Ethics statement

All protocols were approved by the Institutional Committee for the Care and Use of Laboratory Animals of the School of Sciences (University of La Plata) (Protocol Number: 017-00-15).

In vivo evaluation of the allergic reaction

Clinical symptoms. Symptoms were observed 15-45 min following the second intragastric challenge in a blind fashion by 2 independent investigators. Clinical scores are as follows: 0= No symptoms; 1= Scratching and rubbing around the snout and head; 2= Puffiness around the eyes and mouth, pilo-erection, 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 contra-lateral footpad (negative control), and later were injected

intravenously (iv) with 100 μl of 0.1% Evans blue dye (Anedra, Buenos Aires, Argentina). The presence of blue color 10–20 min after the iv injection was considered a positive test and footpad swelling was measured with a digital micrometer.

In vitro evaluation of the allergic reaction

Serum specific antibodies. CMP-specific IgE was measured by EAST as previously described (36). All samples were run in the same experiment.

Cytokine quantification. Splenocytes ($4x10^6$ spleen cells/ml) were seeded in 96-well cell culture plates in complete medium (RPMI-1640 supplemented with 10% FBS, 100U/ml penicillin and 100μg/ml streptomycin) and stimulated with CMP (350μg/ml) for 72 h at 37ºC in controlled atmosphere. Cytokines (IL-5, IFN-γ) were measured in supernatants by ELISA (eBioscience, San Diego, CA, USA) following the manufacturer´s instructions (16).

Cytokine quantification in tissues. Harvested gut and sublingual mucosa were immediately frozen in liquid nitrogen and stored at -80°C. Sections of tissues were weighed, minced and treated with lysis buffer (10mM Tris-HCl, 150mM NaCl, 1% NP-40, 10% Glycerol, 5 mM EDTA and a protease inhibitor cocktail-Sigma). Homogenates were sonicated, spinnedand the supernatant was collected. IL-10 and TGF-β were determined by ELISA (eBioscience) following the manufacturer´s instructions.

Cell isolation from gut and sublingual mucosa. Cells were isolated from the lamina propria (LP) of the oral mucosa or small bowel as described by Di Sabatino et al. (37). In brief summary, the epithelial compartment was removed by incubation with HBSS and 1mM EDTA, followed by treatment with 1mg/ml of collagenase A (Roche, IN, USA) and 10U/ml of DNAse in complete RPMI-1640 medium. Cell debris was removed by filtration and finally cells from submaxilar lymph nodes (SLN) were obtained by digestion with collagenase in RPMI medium for 30 minutes at 37°C.

Flow cytometry for cell characterization. SLN and LP cells were stained using anti-CD4 (PerCyP 5.5), anti-CD25 (PE or FITC) and anti-α4β7 (APC) (eBioscience, UK). For intracellular staining, cells were washed, pre-incubated with fixation/permeabilization solution (eBioscience, UK) for 20 min at 4°C, then incubated with the Staining Intracellular kit (eBioscience, UK), anti-FoxP3 (APC or PE) or anti-IL-10 (APC) (eBioscience, UK). For intracytoplasmatic cytokine staining, cells were stimulated with mouse rIL-2 (20 ng/ml, Preprotech, NJ, USA) for 12 h at 37°C and 3 μg/ml of Brefeldin A (eBioscience, UK) for the last 4 h of cell culture. Fluorescence acquisition was performed with a FACScalibur cytometer using QuestProCell software. The gating strategy for cell analysis consisted of a lymphocyte gate based on SSC-H vs FSC-H parameters, followed by SSC-H vs CD4 fluorescence; CD4$^+$ lymphocyte subset was further gated as CD25 vs FoxP3, and CD4$^+$CD25$^+$FoxP3$^+$ cells were gated as FoxP3 vs IL-10 or α4β7. Finally, data was analyzed with FlowJo software.

Gating strategy for dendritic cell characterization was as follows: First we plotted SSC-H vs FSC-H followed by S CD11c vs MHC II I-A/I-E (eBioscience, UK) dot plot.

Treg purification and transfer to naïve mice. Donor CD4$^+$ cells were purified from SLN of treated (PBS$_{sl}$ or CMP$_{sl}$ + IL-2 C) mice using the CD4$^+$ T cells isolation kit (BD, Pharmigen, USA), followed by the sorting of CD4$^+$CD25$^+$CD62L$^+$ and CD4$^+$CD25$^+$CD62L$^-$ cells in a FACSAria II (BD, USA). The purity of sorted cells was evaluated by flow cytometry and characterized as IL-10-, FoxP3- or α4β7-expressing cells. A total of 1x10^5 Treg were intravenously transferred into naïve recipient mice, which were then orally sensitized with CMP + CT during a 4-week period. Seven days following the last boost mice were challenged by gavage with CMP and hypersensitivity symptoms were recorded with an accompanying skin test also being carried as described (16).

Ex vivo suppression assay. Suppressor activity of sorted Tregs was assessed by inhibiting cell proliferation (carried out by the CFSE dilution method). Spleen mononuclear cells (1×10^5 cells) used as responder were labeled with 3µM CFSE (Invitrogen, Carlsbad, CA, USA) and cultured with highly purified unlabeled $CD4^+CD25^+CD62L^+$ T cells or $CD4^+CD25^-CD62L^+$ T cells at decreasing responder: suppressor cell ratios (4:1, 8:1, 16:1, and 32:1). Responder cells were stimulated with anti-CD3/CD28 Abs (eBioscience, UK) and complete medium. Proliferation of CFSE-labeled cells was assessed by flow cytometry after 4 days of culture.

Statistical analysis

Experimental values were expressed as means ± SD and statistical significances between groups were evaluated using Mann-Whitney *U* test, or ANOVA followed by post hoc analysis with Bonferroni or Dunn´s comparison test. *P* values were considered significant if $p < 0.05$; $p < 0.01$; $p < 0.001$ and analysis was performed using GraphPad 5 software (San Diego, CA).

Results

Administration of IL-2C ameliorates symptoms and reduces skin mast cell degranulation in sensitized mice

We tested the effect of the sublingual administration of CMP, CMPsl, IL-2 ip and CMPsl combined with IL-2Cip in IgE-sensitized mice. After the second oral challenge to the sensitized mice we observed a high clinical score (Fig. 1A) and a positive skin test (Fig. 1B and C), thus suggesting the induction of IgE and activation of sensitized cells. Then, sensitized animals (from now referred as CMP+CT) received treatments for two months. After the first round of treatment, all the mice showed significantly lower clinical scores compared to the sensitized mice that received PBS as; $CMP_{sl}+IL-2C_{ip}$ (combined SLIT)

proved the most effective (p<0.001). In addition, combined SLIT reduced symptoms more efficiently than SLIT (p<0.05). Following the second round of treatments, combined SLIT again proved the most efficient treatment compared to the sensitized mice that were administered with PBS and those mice also showed less intense skin test and swelling of footpads. No significant improvement regarding clinical scores was observed between the first and second round of treatment with $CMP_{sl}+IL-2C_{ip}$. As control, animals that received only CMP during the complete protocol did not show any hypersensitivity or sensitization (Control Ag group). These findings demonstrated that sensitized mice that received the combined SLIT reduced in the clinical score (p<0.05), skin test and foot swelling (p<0.01) faster and more efficiently than mice that received only SLIT.

Systemic Th2 immune response is effectively suppressed with treatments

To elucidate on the immunological response that mediates the control of food allergy, Th2-mediated mechanisms were analyzed. We first observed that CT-driven sensitization to CMP induced a significant increase of serum CMP-specific IgE, which was reversed after the first round of treatment (day 76) with CMP_{sl} (p<0.05) and $CMP_{sl}+IL-2C_{ip}$ (p<0.01) (Fig. 1D). Furthermore we observed that IL-2C alone did not suppress IgE secretion after the first round of treatment. Nevertheless, the analysis of serum specific IgE after the second round of treatments (day 110) revealed a significant reduction of antibody secretion with all treatments, $CMP_{sl}+IL-2C_{ip}$ being the most efficient desensitization protocol. Moreover, IL-5, a prototype type 2 cytokine, was substantially inhibited from spleen cells in animals treated with IL-2C and $CMP_{sl}+IL-2C_{ip}$ (p<0.001), and it was suppressed with CMP_{sl} (p<0.01) after the first round of treatment. The second round of treatment sustained the inhibition of IL-5 secretion with $CMP_{sl}+IL-2C_{ip}$, whereas CMP_{sl} induced a more profound effect (Fig. 1E). Controls showed no induction of splenocyte IL-5 secretion in mice that received CMP,

whereas high levels of the cytokine were detected in the supernatants of cells from sensitized mice (administered with PBS_{sl}). No changes in IFN-γ levels were observed in all conditions (data not shown). These findings suggest a more effective attenuation of the Th2-sensitization in allergic mice when treated with combined SLIT.

Treatments induced regulatory cytokines and Tregs in the intestinal lamina propria

To understand the clinical control of sensitized mice with treatments, we analyzed the intestinal level of tolerogenic cytokines and we found a significant increase in IL-10 and TGF-β in mice treated with CMP_{sl}+IL-2C_{ip} (2 rounds) compared to control mice (sensitized mice that received PBS) (Fig. 2A). We observed that treatment with IL-2C did not induce an increase in TGF-β, and SLIT led to a significant up-regulation of IL-10 and TGF-β, although symptoms were not reduced. Notably, IL-2C alone induced IL-10 secretion. After the second round of treatments combined SLIT induced higher levels of intestinal IL-10 and TGF-β compared to SLIT ($p<0.05$). Assessment of cell frequency in the intestinal LP revealed that $CD4^+$ cells was increased for $CD25^+FOXP3^+$ cells in mice that received CMPsl+IL-2C after 2 rounds of treatment (figure 2B), compared to sensitized mice that received PBS or CMP_{sl} ($p<0.05$). The combined SLIT induced a 5.15 and 2.45 fold-increase of $CD4^+CD25^+FOXP3^+$ cells compared to sensitized mice and SLIT, respectively. These findings showed that the combined SLIT induced more efficiently Tregs and tolerogenic intestinal cytokines compared to SLIT.

The therapeutic control of food allergy correlated with the induction of Tregs in the oral mucosa

To elucidate whether SLIT and combined treatment induced Tregs in the oral mucosa, we assessed the frequency of $CD4^+CD25^+FOXP3^+$ cells in the SLN and sublingual mucosa. We

found an increase of Tregs in SLN (p<0.01) in sensitized mice that received CMP$_{sl}$+IL-2C (Fig. 2C), whereas no increase was observed after SLIT, neither in SLN nor in the buccal mucosa. Interestingly, sensitized animals that received CMP$_{sl}$ showed the highest amount (12.7±0.8%) of IL-10-producing CD4$^+$CD25$^+$FOXP3$^+$ T cells (p<0.01) in the SLN compared to other treatments (Fig. 2D). We could not document an induction in TGF-β-producing Tregs neither in the SLN nor in the buccal lamina propria (not shown). The combined treatment showed the highest frequency of CD4$^+$CD25$^+$FOXP3$^+$ cells in the buccal mucosa following the whole treatment, compared to sensitized animals (p<0.01), indicating that these cells accumulated in this tissue (Fig. 2E). We also examined other cell subsets in the buccal mucosa and we found that the I-A/I-E^{high}CD11c$^+$ cells (comprising conventional and inflammatory DCs (38)), lost the MHC expression with all treatments (p<0.001). The frequency of I-A/I-E^{high}CD11c$^+$ cells was 7.1±3.1% in sensitized mice that received PBS$_{sl}$, 3.2±0.3% in sensitized mice treated with CMP$_{sl}$, 1.4±0.1% of mice treated with IL-2C$_{ip}$ and 1.1±0.1% in mice treated with CMP$_{sl}$+IL-2C$_{ip}$ (Fig 3A). Interestingly, the abrogation of I-A/I-E^{high}CD11c$^+$ myeloid cells was specific of the buccal mucosa (Fig. 3B).

The protective effect of CMP+IL-2C treatment initiated in the oral mucosa and correlated with the induction of suppressive Tregs

In assessing the immunological mechanism underlying the suppression of food allergy, a treatment with CMP$_{sl}$+IL-2C$_{ip}$ was designed to purify and characterize Tregs (Fig. 4A). We observed that 2 administrations of CMP$_{sl}$ followed by 3 systemic boosters with IL-2C$_{ip}$ increased IL-10 and TGF-β levels in the buccal lamina propria, compared to control mice only receiving PBS $_{sl}$+IL-2C$_{ip}$ (Fig. 4B). After CD4$^+$CD25$^+$CD62L$^+$ and CD4$^+$CD25$^-$CD62L$^+$ cells were sorted from SLN of mice treated with CMP$_{sl}$+IL-2C (figure 4C), we found that the frequencies of cells expressing IL-10, FOXP3$^+$ and surface α4β7$^+$ were significantly

increased in $CD4^+CD25^+CD62L^+$ cells, but not in those from mice that received $PBS_{sl}+IL-2C_{ip}$ (not shown). Finally, the functional role of sorted cells was examined *in vitro* and *in vivo*. Figure 5A and B show cell proliferation of responder CFSE-labeled $CD4^+$ T cells from untreated mice (activated with anti-CD3/anti-CD28), and co-cultured with SLN sorted cells. The staining of CFSE demonstrated that proliferation of responder cells was maximum with medium (i), not suppressed by $CD4^+$ T cells from untreated mice (ii), or by $CD4^+CD25^-CD62L^+$ from mice receiving CMP+IL-2C (iii); whereas $CD4^+CD25^+CD62L^+$ cells from CMP+IL-2C-treated mice suppressed up to a responder:suppressor cell ratio of 4:1 (only this condition is depicted) (iv); responder:suppressor ratio of 32:1 with no effect (v) (Figure 5A and Fig. 5B). Moreover, sorted $CD4^+CD25^+CD62L^+$ and $CD4^+CD25^-CD62L^+$ cells from mice that received PBS+IL-2Cdid not exert any inhibitory effect at the same cell ratios (not shown).

To test the importance of sorted cells *in vivo*, $CD4^+CD25^-CD62L^+$ or $CD4^+CD25^+CD62L^+$ cells (10^5) were adoptively transferred into naïve mice, which were then sensitized with CT+CMP (Fig. 5C). After the intragastric challenge, animals transferred with $CD4^+CD25^+CD62L^+$ cells showed a lower clinical score and a weaker skin test, with reduced footpad swelling compared to animals injected with $CD4^+CD25^-CD62L^+$ cells or PBS (Fig. 5D and E). This led us to conclude that the therapeutic intervention with the combined therapy induces $CD4^+CD25^+CD62L^+$ cells in SLN of sensitized mice, which exerts a suppressor effect and in turn controls the allergic reaction.

Discussion

Genetic and immunological evidence proposes that the pathogenesis of allergic disease is closely related with a breakdown in oral tolerance to foods. The expansion of Tregs balances the immune system, restraining the specific Th2 cell response at mucosal sites (39).

Currently, interesting research is being focused on developing therapies that modify the allergic immune response through the restoration of tolerance in patients (40,41). The oral allergen specific approach seems to be promising with a recent study that compared oral and sublingual immunotherapies showing that the controlled sublingual administration of allergens is safer, although less effective, compared to oral immunotherapy (42,43). Although SLIT has proven to be clinically effective in asthma and rhinoconjunctivitis, in food allergy it has been proposed the allergen should be administered accompanied with a pro-tolerogenic adjuvant for the induction of robust tolerance (44). Another therapeutic option is combined therapies. Bonnet et al. recently showed that a low-dose IL-2 (50,000IU) regime controlled experimental food allergy through the induction of Tregs (45). Systemically sensitized mice were given human IL-2 in a preventive treatment protocol and the intestinal expansion of $CD4^+$ and $CD8^+$ $FOXP3^+$ Tregs controlled anaphylaxis upon allergen challenge. Again, an alternative strategy was explored by Wilson et al., who reported that human IL-2 complexed with an anti-IL-2 mAb (clone JES6-1A12) reduced lung inflammation, eosinophilia and bronchial hyperreactivity in experimental asthma, with an increase of tissue IL-10-producing Treg (46). Similar to us, they found no beneficial effect with the administration of IL-2 on airway inflammation. In our mouse model of intragastric sensitization, hypersensitivity reactions were evidenced following two intragastric challenges. Treated animals showed that the most effective therapeutic procedure consisted in the sublingual administration of very low doses of allergen (10 pg and 10 ng of CMP per administration) combined with low doses of IL-2 (15000 IU/injection) complexed with anti-IL-2. We observed that the combined therapy reversed the Th2-mediate immune response, along with the expansion of Treg cells in the buccal mucosa, thus meaning that the antigen is absorbed (Although we cannot evidence that mice retained the allergen under the tongue for a period). Our results suggest that the

combined immunotherapy can improve the safety and efficacy of the sublingual therapy and hopefully create further clinical opportunities for new treatment in food allergy.

As IL-2C was here shown to be more effective at inducing Tregs when the antigen was administered at the buccal mucosa compared with PBS administration, the combined use of antigen and IL-2C seems to promote immune tolerance compared to antigen or IL-2C alone. Moreover, our findings highlight the functional role of buccal-induced Tregs, which favored a mucosal environment that restored intestinal tolerance, and thus nullifying the side effects that might occur with the intragastric allergen challenge. Remarkably, as Tregs are reduced in sensitized mice both at the buccal mucosa (14.4±1.7%) and intestinal lamina propria (13.6±0.9), compared with mice treated with the combined therapy (93.3±1.7% vs 56.7±2.3% cells, respectively). The tolerogenic environment induced at the buccal mucosa promoted a significant reduction in the frequency of the pro-inflammatory DC-expressing $CD11c^+MHCII^{+.}$(7.1±3.1% vs 1.1±0.1% cells in sensitized and treated mice, respectively) ($p<0.05$). The frequency of intestinal DC-bearing $CD11c^+MHC\ II^+$ remained statistically unchanged (11.6±0.8 vs 8.1 0.1% cells in sensitized and treated mice, respectively). In addition, the SLN-induced $\alpha4\beta7$-expresing $CD4^+CD25^+CD62L^+Foxp3^+$ Tregs migrated to the gut and induced the local production of IL-10 and TGF-β. This tolerogenic environment controlled the intestinal and systemic Th2-biased allergic immune reaction.

In conclusion, we have improved the SLIT treatment using very low amounts of allergen through the mucosal route combined with the systemic administration of Treg cell-expanding IL-2C, which proved to be efficient to reverse IgE-mediated experimental food and this combined therapy may represent a promising treatment for managing food allergy.

Conflict of Interest Statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

Author contributions

-Conception and design of study: GHD, EP, JLC, PLS

-Acquisition of data: PLS, FT

-Analysis and/or interpretation of data: GHD, EP, JLC, PLS

-Drafting the manuscript: GHD, PLS

-Revising the manuscript critically for important intellectual content: GHD, EP, JLC, PLS, FT

-Approval of the version of the manuscript to be published: GHD, EP, JLC, PLS, FT

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Legends to figures

Figure 1. *In vivo* and *in vitro* immune response to oral CMP administration in the food allergy mouse model. A. Clinical scores corresponding to symptoms elicited following the intragastric challenges (at the end of the first and second rounds of treatments) with CMPs. **B.** Skin tests; control Ag corresponds to animals that were administered with CMP only (not sensitized), CMP+CT corresponds to sensitized and non treated animals; PBS_{sl} corresponds to sensitized animal that were treated with PBS as control and the other pictures correspond to treated sensitized mice. Results correspond to a single experiment (n=5 per group), which is representative of two separate experiments **C**. Swelling of footpath; difference in thickness corresponds to the footpad injected with PBS as control with the contralateral footpad injected with CMP in the same mouse. **D**. CMP-specific serum IgE monitoring during sensitization and treatments steps. **E**. IL-5 in supernatants of spleen cells stimulated with CMP or medium for 72h. These results are representative of two independent experiments (n=5 per group) with similar results. Data are expressed as means ± SEM. Statistical

significance was assessed using ANOVA test (*p<0.05; **p<0.01; ***p<0.001) for comparison with sensitized mice (CMP+CT).

Figure 2. Regulatory cytokines and Treg analysis in the intestinal and oral mucosa. **A**. IL-10 and TGF-β protein levels in jejunum were determined by ELISA. **B**. Representative staining of CD25^{+} FOXP3^{+} cells gated on CD4^{+} lymphocytes in the lamina propria by flow cytometry. **C.** Representative staining of CD25^{+}FOXP3^{+} cells gated on CD4^{+} lymphocytes in SLN and cell frequencies. **D.** Representative staining of FOXP3^{+}IL-10^{+} cells gated on CD4^{+}CD25^{+} lymphocytes in SLN and cell frequencies. **E.** Representative staining of CD25^{+}FOXP3^{+} cells gated on CD4^{+} lymphocytes in sublingual lamina propria and cell frequencies. These results are representative of two independent experiments (n=5 per group) with similar results. Data are expressed as means ± SEM. Statistical significance was assessed using ANOVA two way (*p<0.05; **p<0.01; ***p<0.001) for comparison with sensitized mice (CMP+CT), or Mann-Whitney for comparison between SLIT and combined SLIT (# p<0.05).

Figure 3. Analysis of cells in the lamina propria of the buccal and intestinal mucosa. Representative staining of CD11c and Class II MHC cells in the lamina propria of the buccal mucosa (**A**) and gut (**B**) of naïve, control (sensitized) and sensitized/treated mice and frequencies. These results are representative of two independent experiments (n=5 per group) with similar results. Data are expressed as means ± SEM. Statistical significance was assessed using Mann-Whitney test (*p<0.05) for comparison with sensitized mice (CMP+CT), or Mann-Whitney for comparison between SLIT and combined SLIT (# p<0.05).

Figure 4. Experimental design and characterization of sorted Treg. A. Schematic drawing of experimental protocol in BALB/c mice that received CMP or PBS through the sublingual route followed by intraperitoneal IL-2C. **B.** IL-10 and TGF-β levels in the buccal lamina propria by ELISA. **C.** Purified $CD4^+$ T cells sorted as $CD25^+CD62L^+$ or $CD25^-$ $CD62L^+$ cells characterized with IL-10, FOXP3 and α4β7 by flow cytometry. Data are expressed as means ± SEM. Statistical significant differences between the groups were determined using Mann-Whitney test (*p<0.05).

Figure 5. *In vitro* and *in vivo* functional characterization of sorted cells. A. Representative CFSE dilution of labeled $CD4^+$ T cells $(1x10^5)$ assessed after 4 d of anti-CD3/anti-CD28-stimulated co-culture with sorted cells (ratios of responder activated-T cells:sorted T cells is indicated). **B.** Capacity of sorted cells to s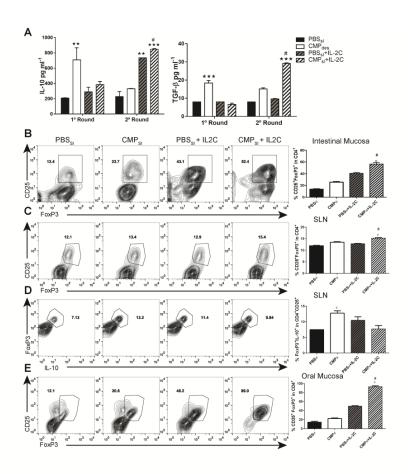uppress cell proliferation (ratios of responder T cells:sorted T cells is indicated). **C.** Schematic drawing of Treg- adoptive transfer experimental protocol (n=4 per group). Naïve mice that were transferred with sorted cells, were subsequently sensitized with CMP and CT, and finally challenged intragastrically. **D.** Symptoms scored after the intragastric challenge. **E**. Skin test and swelling of footpad in the different group of mice. Data are expressed as means ± SEM. Statistical significant differences between the groups were determined using Mann-Whitney test (*p<0.05; **p<0.01).

Supplementary figures

Figure S1. Diagram of the experimental protocol. Mice received CMP_{ig} as control or were sensitized with $CMP+CT_{ig}$; intragastric challenges with CMP_{ig} were performed to visualize and score symptoms; animals were treated with PBS_{sl} as control, CMP_{sl}, $PBS_{sl}+IL-2C_{ip}$ or $CMP_{sl}+IL-2C_{ip}$. The whole experimental protocol was repeated twice with n=5 mice per group.

A

1°Round

2°Round

Control Ag | CMP+CT | Control Ag | PBS$_{sl}$ | CMP$_{sl}$ | PBS$_{sl}$+IL-2 | CMP$_{sl}$+IL-2 | PBS$_{sl}$+IL-2C | CMP$_{sl}$+IL-2C | Control Ag | PBS$_{sl}$ | CMP$_{sl}$ | PBS$_{sl}$+IL-2 | CMP$_{sl}$+IL-2 | PBS$_{sl}$+IL-2C | CMP$_{sl}$+IL-2C

B

Control Ag | CMP+CT | PBS$_{sl}$ | CMP$_{sl}$
PBS$_{sl}$+IL-2 | CMP | PBS$_{sl}$+IL-2C | CMP$_{sl}$+IL-2C

C

Control Ag | CMP+CT | PBS$_{sl}$ | CMP$_{sl}$ | PBS$_{sl}$+IL-2 | CMP$_{sl}$+IL2 | PBS$_{sl}$+IL2C | CMPsl+IL-2C

D

CMP-specific IgE (OD)

□ Control Ag
■ PBS$_{sl}$
□ CMP$_{sl}$
▨ PBS$_{sl}$+IL-2C
▨ CMPsl+IL-2C

Treatment

days: 0, 42, 76, 110

E

IL-5 pg ml^{-1}

□ RPMI
■ CMP

Control Ag | PBS$_{sl}$ | CMP$_{sl}$ | PBS$_{sl}$+IL-2C | CMP$_{sl}$+IL-2C | Control Ag | PBS$_{sl}$ | CMP$_{sl}$ | PBS$_{sl}$+IL-2C | CMP$_{sl}$+IL-2C

1° Round | 2° Round

A

PBS$_{SI}$ CMP$_{SI}$ PBS$_{SI}$ + IL2C   CMP$_{SI}$ + IL2C

CD11c / MHC II (I-A/I-E)

7.20   4.08   0.94   1.11

Sublingual Mucosa

% MHC II$^+$ CD11c+

B

CD11c / MHC II (I-A/I-E)

10.1   5.13   8.75   8.37

Intestinal Mucosa

% MHC II$^+$ CD11c$^+$

A

CMP/PBS   IL2C

1   2   3   4   5 d

Analysis

B   Lamina Propria

IL-10 pg ml⁻¹

PBS_SI+IL-2C   CMP_SI+IL-2C

TGF-β pg ml⁻¹

PBS_SI + IL-2C   CMP_SI + IL-2C

C

SLN

SSC / FSC   83.0

SSC / CD4   97.7

CD62L / CD25

PBS_SI + IL2C   CMP_SI + IL2C

CD25-CD62L+   CD25+ CD62L+

CD25⁻CD62L⁺   CD25⁺CD62L⁺

CD62L / IL-10   2.08   7.41

% IL-10 in sorted cells

CD25⁻CD62L⁺   CD25⁺CD62L⁺

FoxP3   2.09

% FoxP3 in sorted cells

CD25⁻CD62L⁺   CD25⁺CD62L⁺

CD62L / α4β7   0.82   5.10

% α4β7 in sorted cells

CD25⁻CD62L⁺   CD25⁺CD62L⁺

A

i

ii

iii CFSE 68.9

iv 26.7

v 57.6

CFSE

B

% supression

CD25⁺CD62L⁺
CD25⁻CD62L⁺
CD4 naive T cells

4:1   8:1   16:1   32:1   Teff:Sorted T cell.

C

Sorted cells/PBS | OC

-1 0 7 14 21 28 35 d

CT + CMP

C

PBS   CD25⁻CD62L⁺   CD25⁺CD62L⁺

D

Clinical score

PBS   CD25⁻CD62L⁺   CD25⁺CD62L⁺

Mean increase in footpad thickness (mm)

PBS   CD25⁻CD62L⁺   CD25⁺CD62L⁺