



Complexation of whey protein with caffeic acid or (–)-epigallocatechin-3-gallate as a strategy to induce oral tolerance to whey allergenic proteins

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ABSTRACT

Proteins and phenolic compounds can interact and form soluble and insoluble complexes. In this study, the complexation of whey protein isolate (WPI) with caffeic acid (CA) or (–)-epigallocatechin-3-gallate (EGCG) is investigated as a strategy to attenuate oral sensitization in C3H/HeJ mice against WPI. Treatment with WPI-CA reduced the levels of IgE, IgG1, IgG2a and mMCP-1 in serum of mice measured by ELISA. This might be related to CD4⁺LAP⁺Foxp3⁺ T and IL-17A⁺CD4⁺ T (Th17) cell activation, evidenced by flow cytometry of splenocytes. Treatment with WPI-EGCG, in turn, decreased the levels of IgG2a and mMCP-1 in serum of mice, possibly by the modulation of Th1/Th2 response and the increase of CD4⁺ Foxp3⁺ LAP⁻ T and IL-17A⁺ CD4⁺ T (Th17) cell populations. In conclusion, WPI-CA and WPI-EGCG attenuated oral sensitization in C3H/HeJ mice through different mechanisms. We consider that the complexation of whey proteins with CA and EGCG could be a promising strategy to induce oral tolerance.

1. Introduction

Cow's milk (CM) is the most common food allergen to infants, since it is usually the first food protein offered when breastfeeding is not possible, which could lead to an earlier allergic sensitization [1]. Cow's milk allergy (CMA) is an immunologically mediated adverse reaction to one or more CM proteins and mainly manifests against caseins and the two main whey proteins, α -lactoalbumin (α -la) and β -lactoglobulin (β -Lg). It is considered that food allergy is caused by a failure or loss of oral tolerance induction, however, the specific moment when that failure occurs is not yet completely clarified [2]. Tolerance induction is a normal immune phenomenon that occurs in the intestinal mucosa after exposure to allergens. The mechanism through which the oral tolerance is induced is partially related to the ingested antigen dose [3]. In the presence of high doses of antigen, tolerance is mainly caused by the clonal deletion, due to apoptosis or anergy of antigen-specific T cell clones. When the food antigen is at lower concentrations, tolerance is induced by regulatory T cells (Tregs), which is considered to be a central mechanism of tolerance induction by food antigens [4].

Nowadays, it is known that oral tolerance instead of an unresponsive state is an active phenomenon which involves the suppression of specific immune responses to antigens first encountered in the

gastrointestinal tract [5,6]. For instance, Castro-Junior and collaborators [6] have shown that tolerant and immunized mice presented the same number of regulatory and activated T cells in the spleen after immunization to ovalbumin. The authors suggested that, in addition to the immune response suppression mechanisms performed by Tregs, the earlier expression of regulatory cytokines, such as TGF- β and IL-10 and a transitory expression of effector cytokines (IL-2 and IFN- γ), play a role in the induction of tolerance. Lymphocytes with active regulatory properties in the mucosa are crucial to induce tolerance since they could permeate the body and modulate the immune response in a systemic manner [6,7]. CD4⁺ T cells which express TGF- β in the membranes linked to latency-associated peptide (LAP), known as LAP⁺ Treg cells, may be one of the markers of Tregs induced by oral tolerance [7]. Furthermore, a reliable intracellular marker of naturally-occurring Treg cells is the transcription factor forkhead box p3 (Foxp3), since the frequency of CD4⁺ Foxp3⁺ regulatory T cells tend to be lower in allergic individuals [8,9]. The Th17 response also tends to be impaired in food-allergic individuals [9]. Since it has recently been suggested that Th17 cells could transdifferentiate into regulatory T cells depending on the environmental context, IL-17 might be a potential biomarker for tolerance to food allergens as well [10–12].

Due to the lack of clear understanding of the molecular mechanisms

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of food allergies, the widely accepted treatment for this disease has been the avoidance of exposure to the antigen [13]. Medications are available for palliative care of hypersensitivity symptoms, but not for definitive control of allergic disease [14]. Moreover, an alternative treatment for children with high risk or diagnosed with cow's milk allergy is the replacement of milk for hypoallergenic formulas composed of caseins or partially or extensively hydrolyzed whey proteins [15]. However, these formulas may present certain drawbacks, such as residual allergenicity [15,16] or bitter taste and hypertonicity as a result of excessive hydrolysis [17]. Therefore, new nutritional interventions in the prevention and treatment of food allergies are required [14]. More recently, phenolic compounds have been investigated for the treatment of allergic diseases by either avoiding the sensitization phase or relieving the symptoms of allergic reaction [18–20].

The anti-allergic property of phenolic compounds has been related to the inhibitory effect on the activation and degranulation of mast cells [21,22] and on the expression of FCεRI on the surface of those cells [23]. Phenolic compounds could also modify the allergenicity of proteins due to the formation of soluble and insoluble complexes with those macromolecules and consequent epitope modification or reduction of bioavailability [24–28]. For instance, Chung and Champagne [25] reported reduced IgE binding to peanut allergens due to the formation of insoluble complexes with phenolic acids, such as caffeic acid (CA). The (–)-epigallocatechin gallate (EGCG) is a flavonoid mainly found in green tea which possesses anti-allergic properties due to its inhibitory effect on mast cell degranulation, release of histamine and uptake of proteins by monocytes [27,29]. Although CA and EGCG have proven anti-allergic properties, there are still very few studies that use the association of those phenolic compounds with CM proteins to induce oral tolerance.

In the present study, we hypothesize that the complexation of whey protein isolate (WPI) with CA or EGCG reduces the oral sensitizing capacity of WPI, either due to the concomitant administration of anti-allergic compounds - CA and EGCG present in the complexes - or by modifying the WPI structure in a way that it would not be recognized as an antigen by the immune system. Therefore, we analyzed the cellular immune response through the phenotypic characterization of functional lymphocyte subpopulations (Th1, Th2, Tregs and Th17) in response to oral sensitization with WPI-phenolic compounds complexes and challenge with WPI. The antigen-specific immunoglobulin pattern and mast cell activation were also investigated. To the best of our knowledge, this is the first time that complexation of whey protein isolate with phenolic compounds is used for immunomodulatory purposes.

2. Materials and methods

2.1. Materials

Whey protein isolate was obtained from Provon®292 (Glanbia Nutritionals, Kilkenny, Ireland). The total nitrogen content of WPI was determined by the micro-Kjeldahl method [30] and the protein content ($87.5\% \pm 1.4$) was calculated using 6.38 as a conversion factor. Caffeic acid (CAS 331-39-5; 95% purity), EGCG (CAS 989-51-5; 80% purity) and cholera toxin (~95% purity) were purchased from Sigma-Aldrich® (St. Louis, MO, USA).

The reagents used in cell culture procedures were: RPMI 1640 medium (Gibco, Invitrogen Corporation, Grand Island, N.Y., USA), HEPES (Mediatech, Inc., Manassas, VA, USA), penicillin (Gibco, Invitrogen Corporation), streptomycin (Gibco, Invitrogen Corporation), Fungizone (Gibco, Invitrogen Corporation), Fetal Bovine Serum (FBS) (Vitrocell, Campinas, SP, Brazil), mouse recombinant IL-2 (Biolegend, San Diego, CA, USA), and concanavalin A (Sigma).

The antibodies used in the ELISA assays were: mouse IgE Balb/c isotype control (Abcam®, Cambridge, MA, USA), purified rat anti-mouse IgE monoclonal antibody (BD Biosciences, San Diego, CA, USA), HRP-conjugated goat anti-Rat IgG whole molecule (Sigma Chemical

Co., St. Louis, MO, USA), purified mouse IgG1 κ isotype control and purified mouse IgG2a κ isotype control (BD Biosciences, San Diego, CA, USA), goat anti-mouse IgG1 heavy chain (HRP) and goat anti-mouse IgG2a heavy chain (HRP) (Abcam®, Cambridge, MA, USA). The antibodies and specific reagents used in flow cytometry assays were: PE anti-mouse LAP (TGF-β1), PE anti-mouse IL-4, APC anti-mouse IL-10, PE anti-mouse IL-2, APC anti-mouse IFN-γ, APC anti-mouse IL-17A, PE anti-mouse Foxp3, intracellular staining permeabilization wash buffer (10×), Foxp3 Fix/Perm buffer set, PerCP/Cy5.5 anti-mouse CD4, monesin, brefeldin A, fixation buffer, flow cytometry cell staining buffer (FCSB), CFSE cell division tracker kit, APC rat IgG2b kappa isotype ctrl, PE mouse IgG1 kappa isotype ctrl, PerCP/Cy5.5 rat IgG2b kappa isotype ctrl, PE rat IgG2b kappa isotype ctrl, FITC rat IgG2a kappa isotype ctrl, APC rat IgG1 kappa isotype ctrl were purchased from BioLegend (San Diego, CA, USA). All other chemicals were of analytical grade.

2.2. Protein-phenolic compounds complexation

The WPI-phenolic compound complexes were obtained by the mixture of stock solutions of WPI and phenolic compound - CA or EGCG. The phenolic compounds stock solutions were prepared by dissolving the compound in deionized water at the concentration of 5 mg/mL (considering the compound purity). Protein stock solution was prepared by suspending WPI in deionized water at the concentration of 5.5 mg/mL (considering the protein content). After mixing the stock solutions in proportions to achieve a molar ratio 1:1 (protein concentration fixed at 5 mg/mL), the complexation was carried out at 25 °C for 60 min at dark, using 0.1 M HCl to adjust pH at 3.5. As WPI contains a mixture of proteins with broad molecular mass (MM) distribution, the MM of this complex was estimated by weighted average of the prevalent proteins (β-Ig - 18.4 kDa, 50–70%; α-La - 14.2 kDa, ~20%; and BSA - 66 kDa, ~5%) [31]. Therefore, the WPI apparent MM assumed was of 20 kDa. Complexes were freeze-dried and stored at –20 °C. The protein content of complexes, determined by micro-Kjeldahl method [30], was considered to prepare the solutions to be administered to the mice.

2.3. Oral sensitization and challenge of mice with WPI

The experimental design of the oral sensitization and challenge of mice is demonstrated in Fig. 1. Female C3H/HeJ mice used in this study were obtained from the Multidisciplinary Center for Biological Research, University of Campinas (Cemib-Unicamp). The experimental and control groups consisted of 3 to 6 mice fed *ad libitum* with cow's milk-free diet (Nuvilab®, Curitiba, Brazil) maintained under specific pathogen-free (SPF) conditions, with controlled light, temperature, and humidity in the Laboratory of Translational Immunology (LTI) facilities. The animal experiments were approved by the Ethics Committee on Animal Experiments of the University of Campinas under the protocol number 4097-1 (Campinas, SP, Brazil).

The animals at 8 weeks of age were divided into four groups (n = 3 to 6 mice per group). The experimental groups were orally sensitized on day 0, 7, 14 and 21 with 1 mg/g weight of WPI, WPI-CA or WPI-EGCG plus 0.3 µg/g weight of cholera toxin (CT) in saline-phosphate buffer (PBS) in a final volume of 0.2 mL/animal. Negative control group (non-sensitized) was administered orally with PBS and CT only. On day 28, all mice were challenged with a 30 mg dose of WPI in PBS (without CT). After 30 min of oral challenge, animals were intraperitoneally anaesthetized with ketamine hydrochloride: 150 mg/kg and xylazine hydrochloride: 10 mg/kg, (both from Vetbrands, Paulínia-SP, Brazil) and peripheral blood were collected by cardiac puncture for serum separation. Blood samples were centrifuged at 25 °C for 15 min at 800 × g and sera were stored at 20 °C for ELISA analysis. The spleen was aseptically removed and used as a source of T cells in lymphocyte proliferation assays, as described below.

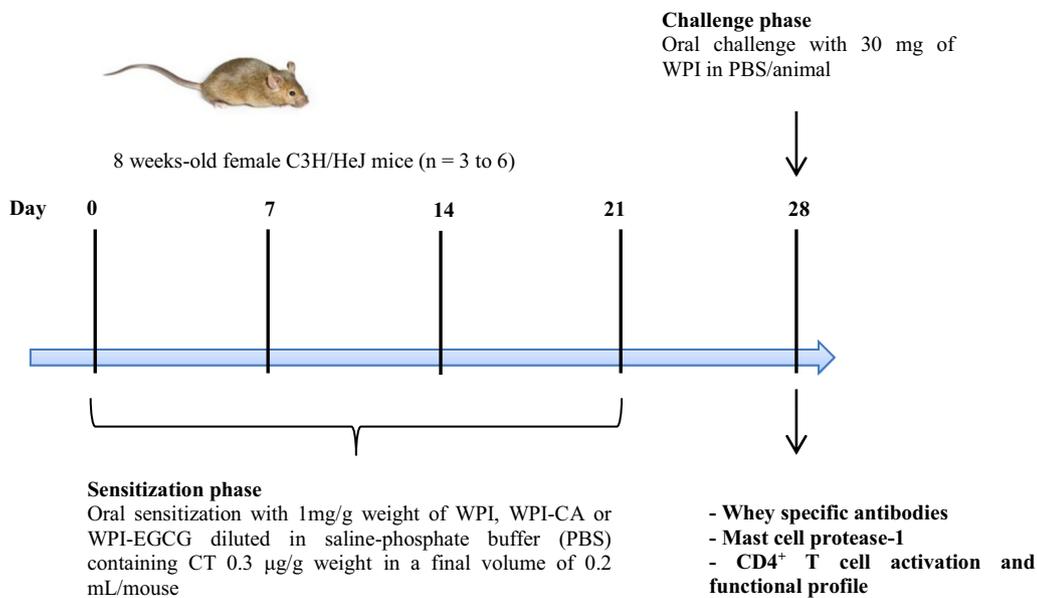


Fig. 1. Experimental design of WPI-induced allergy in a mice model. Female C3H/HeJ mice (8 weeks old) were divided into four groups (n = 3 to 6 mice per group): negative control group (non-sensitized), WPI-CA sensitized group and WPI-EGCG sensitized group. The experimental groups were orally sensitized on day 0, 7, 14 and 21 with 1 mg/g weight of WPI, WPI-CA or WPI-EGCG diluted in saline-phosphate buffer (PBS) containing 0.3 µg/g weight of CT in a final volume of 0.2 mL/mouse. Negative control group (non-sensitized) was applied orally with PBS and CT only. On day 28, all mice were challenged with a 30 mg dose of WPI in PBS (without CT). After 30 min of oral challenge, the animals were anaesthetized and peripheral blood and spleen were collected for the ELISA and flow cytometry analyses, respectively.

2.4. Measurement of serum WPI-specific antibodies

The WPI-specific IgE, IgG1 and IgG2a serum levels were measured by indirect enzyme-linked immunosorbent assay (ELISA), as previously described [32,33], with some modifications. Briefly, high-binding polystyrene microtiter plates (Corning, Cambridge, MA, USA) were coated with 20 µg of WPI overnight at 4 °C. Plates were washed (after each incubation step) and blocked with 50 mM Tris-buffered saline (pH 7.4) containing 0.05% Tween 20 (TBS-T). Serum samples (1:250 for IgE, 1:1000 for IgG1 and 1:500 for IgG2a) were applied and incubated overnight (IgE) or for 2 h at 37 °C (IgG1 and IgG2a). Afterwards, the plates were incubated with goat anti-mouse IgG1 heavy chain (HRP) (1:400,000) or goat anti-mouse IgG2a heavy chain (HRP) (1:500,000) for 1 h at 37 °C. For IgE quantifications, the plates were previously incubated with anti-mouse IgE antibody, followed by incubation with HRP conjugated antibody against anti-mouse IgE antibody. After the washing step, the TMB (3,30,5,50-tetramethylbenzidine) solution (BD optEIA TMB substrate reagent set – BD Bioscience) was added to the plates (100 µL per well) and incubated for 15 min. The reaction was stopped with H₂SO₄ (0.18 mol L⁻¹) and absorbance was measured at 450 nm on an automated spectrophotometer reader (Spectra Max 190, Molecular Devices, Toronto, Canada). The results were expressed as the concentration (ng/mL) of antigen specific antibody using the standard curve of IgE, IgG1 or IgG2a as previously described [29,30].

2.5. Measurement of mouse mast cell protease-1 (mMCP-1)

Serum concentration of mouse mast cell protease-1 (mMCP-1) was determined using a commercial ELISA kit (Thermo Fisher Scientific Inc., Waltham, MA) according to the manufacturer's protocol.

2.6. Lymphocyte proliferation assays

The proliferation of CD4⁺ T lymphocytes, obtained from mice spleen, was analyzed as described by Thomé and co-workers [34] with adaptations. Briefly, the cell suspensions of splenocytes were previously stained with 1.25 µM of CFSE probe according to manufacturer's recommendations. Cells were seeded at 2×10^5 cell/well in the presence of 75 µg/mL of WPI and IL-2 (10 ng/mL), or with the mitogenic stimulus of concanavalin A (ConA - 3 µg/mL) in RPMI 1640 medium supplemented with 25 mM HEPES, 10 U mL⁻¹ of penicillin, 10 µg/mL of streptomycin, and 5 µg/mL of Fungizone and with 10% of FBS. The

plates were incubated for 96 h at 37 °C in a humidified incubator with CO₂ 5%. After this period, cells were washed with PBS and stained with the viability probe Zombie NIR™ fixable viability kit (BioLegend). After staining with flow cytometry cell staining buffer (FCSB), cells were stained with fluorochrome-conjugated monoclonal antibody anti-mouse CD4 PercP-Cy5.5 (Biolegend) and then fixated with Fixation Buffer (Biolegend) according to manufacturer's recommendations. The samples were acquired in FACsVerse™ Flow Cytometer (BD-Bioscience San Jose, CA, USA) and data analyzed with FCS Express V6 software (De Novo Software, Glendale, CA, USA).

2.7. Phenotyping and functional evaluation of CD4⁺ T cells

The functional profile of CD4⁺ T cells was analyzed by the *in vitro* restimulation of splenocytes with WPI (75 µg/mL) in RPMI 1640 medium supplemented with 25 mM HEPES, 10 U mL⁻¹ of penicillin, 10 µg/mL of streptomycin, and 5 µg/mL of Fungizone and with 10% of fetal bovine serum for 18 h at 37 °C in a humidified incubator with 5% CO₂ atmosphere. For the detection of intracellular cytokines, cells were additionally stimulated with ionomycin (20 µg/mL and phorbol 12-myristate 13-acetate (PMA, 200 ng/mL) for 5 h, in the presence of Golgi complex blockers brefeldin A (5 µg/mL) and monensin (5 µg/mL). For Tregs (LAP⁺ Foxp3⁺) frequency analysis, the cultures described above were maintained for 96 h at 37 °C in a humidified incubator with 5% CO₂ atmosphere. After the restimulation period, cells were washed with PBS and stained with the viability probe Zombie NIR™ fixable viability kit (BioLegend) to exclude dead cell population. Then, the staining of the cell surface antigens (CD4 and LAP) with their respective fluorochrome-conjugated antibodies diluted in FCSB was carried out. After wash cycles with FCSB, cells were fixed by incubation for 20 min with fixation buffer, after wash cycle with FCSB, cells were permeabilized with intracellular staining permeabilization wash buffer and stained for the intracellular cytokines (IL-2, IFN-γ, IL-4, IL-10 and IL-17A) with their respective fluorochrome-conjugated antibodies diluted in permeabilization buffer, according to manufacturer recommendations. Foxp3 intracellular staining was carried out using Foxp3 Fix/Perm buffer set (BioLegend) and the anti Foxp3 fluorochrome-conjugated specific antibodies, according to the manufacturer's protocol. The cells were also stained with irrelevant isotype controls for each cell marker analyzed. The samples were analyzed using FACsVerse™ Flow Cytometer (BD-Bioscience San Jose, CA, USA), where 10,000 events gated on CD4⁺ cells for each tube were acquired. The data was

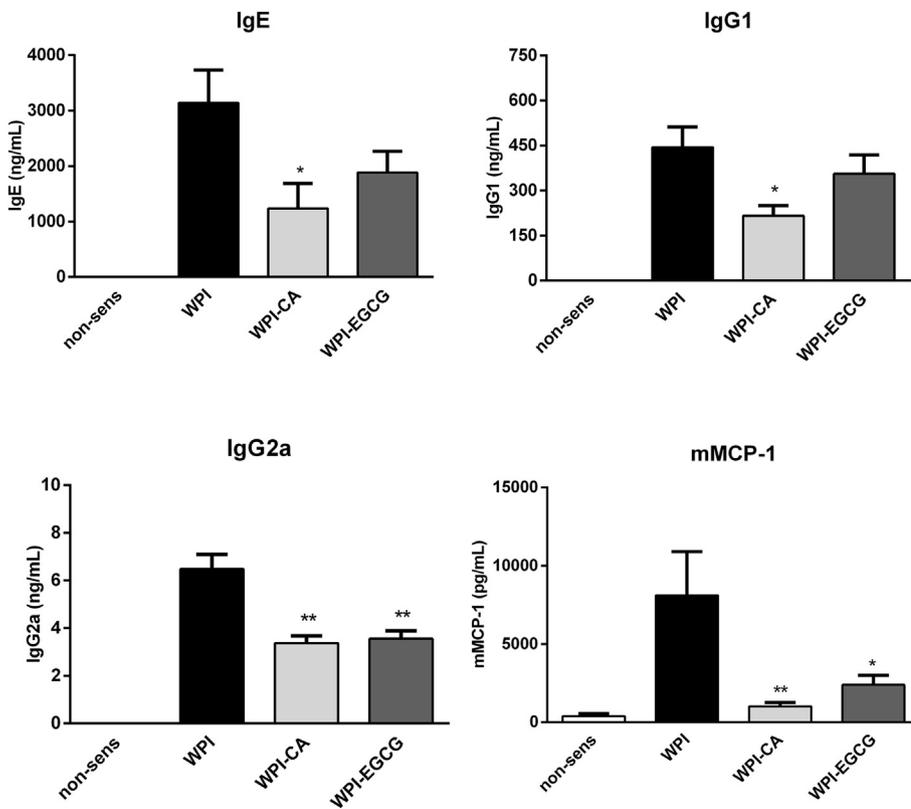


Fig. 2. Oral treatment with WPI-CA and WPI-EGCG decreases WPI-specific antibodies IgE, IgG1 and IgG2a, and mMCP-1 levels in mice serum. Mice were orally sensitized with cholera toxin + PBS only (non-sensitized group), or 1 mg/g weight/animal of WPI, WPI-CA or WPI-EGCG + cholera toxin in PBS for four weeks and after 7 days they were challenged with 30 mg/animal WPI in PBS. WPI-specific antibodies and mMCP-1 levels were measured by ELISA. Columns and error bars represent the mean ± SEM (n = 3 to 6). Asterisk indicates significant differences (*p ≤ 0.05 and **p ≤ 0.001 - Bonferroni's multiple comparisons test) between WPI and WPI-CA or WPI and WPI-EGCG.

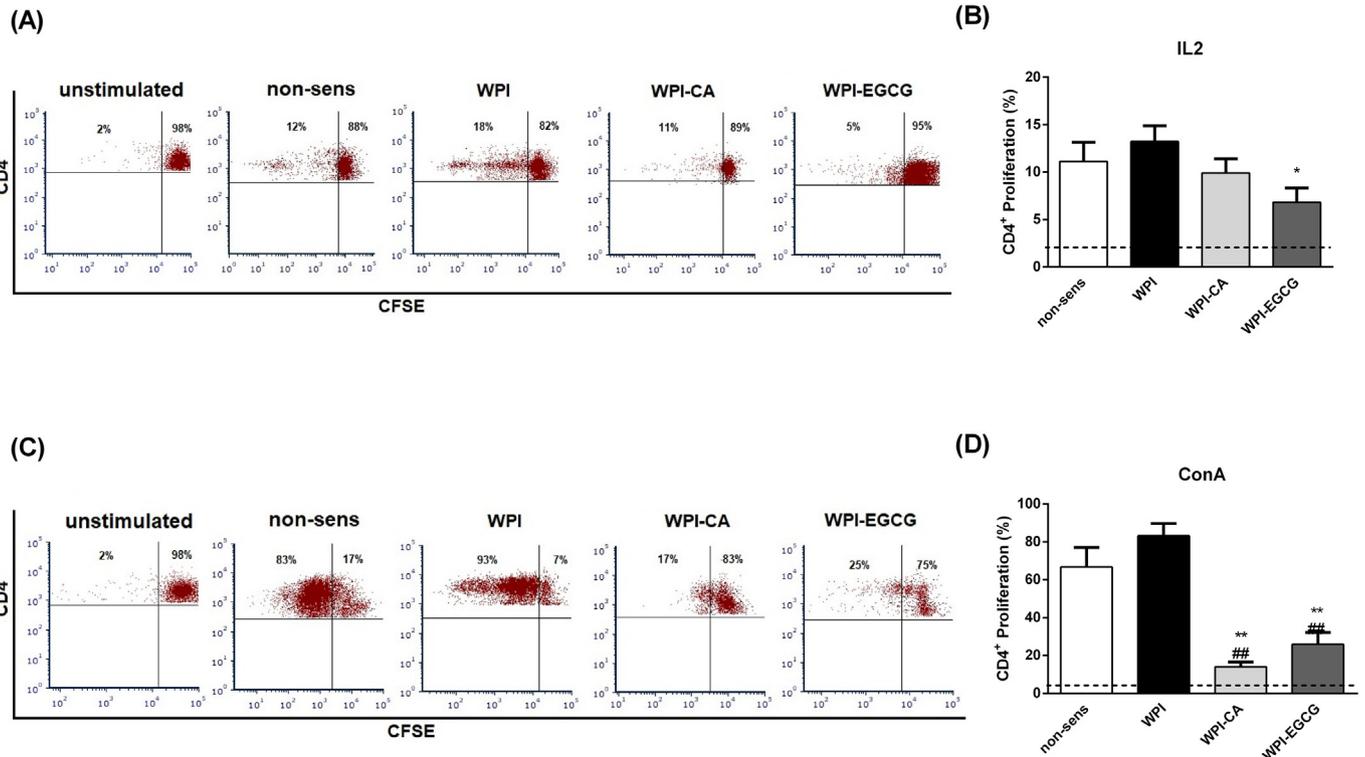


Fig. 3. Oral treatment with WPI-CA and WPI-EGCG complexes reduces proliferation of CD4⁺ T cells restimulated *in vitro* with WPI in the presence of IL-2 (Panels A and B) or ConA (Panels C and D). Panels A and C: representative dot plots obtained from flow cytometry analysis, T cell proliferation was assessed excluding dead cells using the viability probe (Zombie NIR™) and analyzing the frequency of CD4⁺ T cells presenting decay of the probe CFSE (CFSE^{low} - upper left population on dot plots). Panels B and D: Summarized data were expressed as mean ± SEM (n = 3 to 6). Cells were restimulated with 75 µg/mL WPI plus IL-2 (10 ng/mL) (Panel B), or with 3 µg/mL of concanavalin A (ConA) (Panel D) for 96 h. Number sign indicates significant differences (*p ≤ 0.05 and **p ≤ 0.001 - Bonferroni's multiple comparisons test) between non-sensitized and sensitized mice (WPI, WPI-CA and WPI-EGCG). Asterisk indicates significant differences (*p ≤ 0.05 and **p ≤ 0.001 - Bonferroni's multiple comparisons test) between WPI and WPI-CA or WPI-EGCG sensitized mice. The cells which were not restimulated *in vitro* (negative control) are represented as “unstimulated group” in panels A and C, and as dotted lines in panels B and D.

analyzed with FCS Express V6 software (De Novo Software, Glendale, CA, USA).

2.8. Statistical analyses

Results were expressed as mean \pm SEM and were analyzed by the parametric one-way ANOVA and Bonferroni's multiple comparisons test. For all analyses, p values ≤ 0.05 were considered significant.

3. Results

3.1. Effect of complexes on WPI-specific serum antibodies and mMCP-1 levels

The WPI-specific antibodies IgE, IgG1, and IgG2a, as well as mouse mast cell protease-1 levels were measured in the animal serum by ELISA (Fig. 2). The non-sensitized (non-sens) group presented negligible levels of WPI-specific antibodies in the serum (Fig. 2). WPI-CA sensitized group showed a significant decrease of IgE, IgG1 ($p \leq 0.05$), and IgG2a ($p \leq 0.001$) compared to WPI sensitized group. WPI-EGCG sensitized group, in turn, showed a general tendency to reduce specific antibodies compared to WPI sensitized group. However, a significant decrease was observed only in IgG2a levels ($p \leq 0.001$).

The concentration of the mast cell protease-1 (MCP-1), a chymase expressed by intestinal mucosal mast cells, was measured in the animal serum to investigate the mucosal mast cell degranulation. The oral treatment with complexes significantly reduced the concentration of MCP-1 in the serum of WPI-CA group ($p \leq 0.001$) and WPI-EGCG group ($p \leq 0.05$) – Fig. 2.

3.2. Effect of complexes on the CD4⁺ T cell proliferation and their functional profile

In vitro restimulation of CD4⁺ T cells, obtained from the splenocytes cell suspension, with WPI in the presence of IL-2 resulted in a significant reduction of CD4⁺ T cell proliferation ($p \leq 0.05$) in the group sensitized with WPI-EGCG (Fig. 3 - Panel B) compared with WPI group. Since Concanavalin A (Con A) is a mitogen capable of activating T cells in an antigen-independent manner [35], this compound was used as a positive control of CD4⁺ T cell proliferation rate (Fig. 3 - Panel D). Both WPI-CA and WPI-EGCG sensitized groups presented significant reduction of CD4⁺ T cell proliferation compared either to non-sensitized or WPI sensitized group ($p \leq 0.001$) (Fig. 3 - Panel D).

Th1 and Th2 cell activation profiles play an important role in allergy induction [14]. The percentage of CD4⁺ IFN- γ ⁺ Th1 cells was increased in both WPI-CA and WPI-EGCG sensitized groups, however this increase was significant ($p \leq 0.05$) only in the WPI-EGCG group (Fig. 4 - Panel B-1). There were no significant differences in the percentage of CD4⁺ IL-2⁺ Th1 cells among groups (data not shown). Regarding the Th2 cell profile, WPI-CA and WPI-EGCG sensitized groups showed higher CD4⁺ IL-4⁺ Th2 cell and CD4⁺ IL-10⁺ Th2 cell frequencies when compared to WPI sensitized group (Fig. 4 - Panel B-2 and 3). The increase was significant ($p \leq 0.05$) only in the WPI-EGCG sensitized group.

The TGF- β 1 producing T cells in the intestinal mucosal region play a relevant role in the establishment of oral tolerance to dietary antigens [36]. Furthermore, a particular CD4⁺ T regulatory cell expressing the membrane-bound latency-associated peptide (LAP)/TGF- β complex and the transcriptional factor Foxp3 exert important immunomodulatory mechanisms that leads to systemic suppression to a specific antigen [7]. In this sense, we investigated the frequency of CD4⁺LAP⁺Foxp3⁺ T cell and the CD4⁺Foxp3⁺LAP⁻ T cell populations. The mice treated with WPI-EGCG showed significant ($p \leq 0.001$) increased expression of CD4⁺ Foxp3⁺ T cells compared to WPI sensitized mice (Fig. 5 – Panel B-1). Both WPI-CA and WPI-EGCG treated mice presented significant ($p \leq 0.001$ and $p \leq 0.05$, respectively) higher expression of

CD4⁺LAP⁺Foxp3⁺ T cells than the non-sensitized group (Fig. 5 – Panel B-2), and WPI-CA also presented higher expression ($p \leq 0.05$) of this population of cells when compared to WPI (Fig. 5 – Panel B-2).

Another important functional population investigated was the Th17 cell, which presents a plastic functional phenotype in mucosal derived immune responses [10,12]. Here we evaluated IL-17A⁺ CD4⁺ T cells (Th17) *in vitro* restimulated splenocytes, obtained from the different experimental groups. Both WPI-CA and WPI-EGCG showed significant ($p \leq 0.05$) increased expression of IL-17A⁺ CD4⁺ T cells; while sensitization with WPI resulted in even lower frequency ($p \leq 0.05$) of these cells, when compared to non-sensitized mice (Fig. 5 – Panel D).

4. Discussion

Caffeic acid has an anti-allergic property related to the formation of complexes with allergenic proteins and consequent inhibition of IgE-binding [25]. EGCG, in addition to the formation of complexes with allergenic proteins, also presents an inhibitory effect on mast cell degranulation, release of histamine and uptake of proteins by monocytes [27,29]. Herein we investigated the capacity of WPI-CA and WPI-EGCG complexes to induce oral tolerance in mice. Our results suggest that, although WPI-CA did not significantly change the Th1/Th2 response, it did change the regulatory T (Tregs) and Th17 cell profiles when compared to WPI treated mice. This modification should explain the lower levels of WPI-specific IgE, IgG1 and IgG2a, as well as mMCP-1 in serum (Fig. 2) observed in the mice treated with WPI-CA. However, oral treatment with WPI-EGCG significantly modified the functional profile of Th1/Th2 and Treg cells compared to WPI sensitized mice. The mice treated with this complex presented reduced levels of WPI-specific IgG2a and mMCP-1 (Fig. 2) in serum, but not WPI-specific IgE and IgG1. Furthermore, the oral treatment with both complexes was capable of decreasing CD4⁺ T cell proliferation under restimulation with WPI (75 μ g/mL) plus IL-2 or with ConA in culture (Fig. 3), indicating an anti-proliferative effect of complexes. When combined, our results suggest that WPI-CA and WPI-EGCG induce oral tolerance to whey proteins and the tolerance mechanism is differently mediated when one or another complex is administrated.

The production of antigen-specific IgE antibodies by B cells after exposure to an antigen characterizes the first phase of IgE-mediated allergy response [37]. The decreased levels of WPI-specific IgE, as well as IgG1 (Fig. 2), suggest that the oral treatment with WPI-CA attenuated the sensitization phase of allergy. The challenge phase of allergy, in turn, is characterized by the cross-linking of FC ϵ R1 due to antigen binding to IgE on the surface of mast cells, which results in the release of inflammatory mediators [38]. Mast cell protease-1 plays an important role in histamine release by mast cells, eosinophil recruitment, and the induction of allergic response [6]. The reduced serum levels of mMCP-1 (Fig. 2) indicate that oral treatment with both complexes could have avoided the cross-linking of FC ϵ R1 on the surface of mast cells, interfering in the challenge phase of allergy. The reduced levels of mMCP-1 in the serum of animals treated with WPI-CA are in accordance with the lower levels of WPI-specific IgE observed in this group. Oral treatment with WPI-EGCG did not significantly reduce WPI-specific IgE and IgG1, but did cause an important reduction of mMCP-1 release. Thus, we consider that EGCG may have maintained its anti-allergic properties upon complexation with WPI, such as the capacity to suppress FC ϵ R1 expression on the surface of effector cells [23,29].

The anti-proliferative effect of phenolic acids and catechins isolated or in plant extracts is well-known [39]. Caffeic acid reduces cancer growth through the induction of apoptosis of cancer cells [40]. On the other hand, EGCG suppresses either ConA or antigen-induced T cell proliferation, mainly through a direct effect on T cells [41,42]. In a previous study of our research group, polyphenols present in *Passiflora alata* leaves, among them catechin and epicatechin, may have contributed to the anti-proliferative effect of lymphocytes stimulated in culture by Con A [43]. Nevertheless, here we showed the anti-

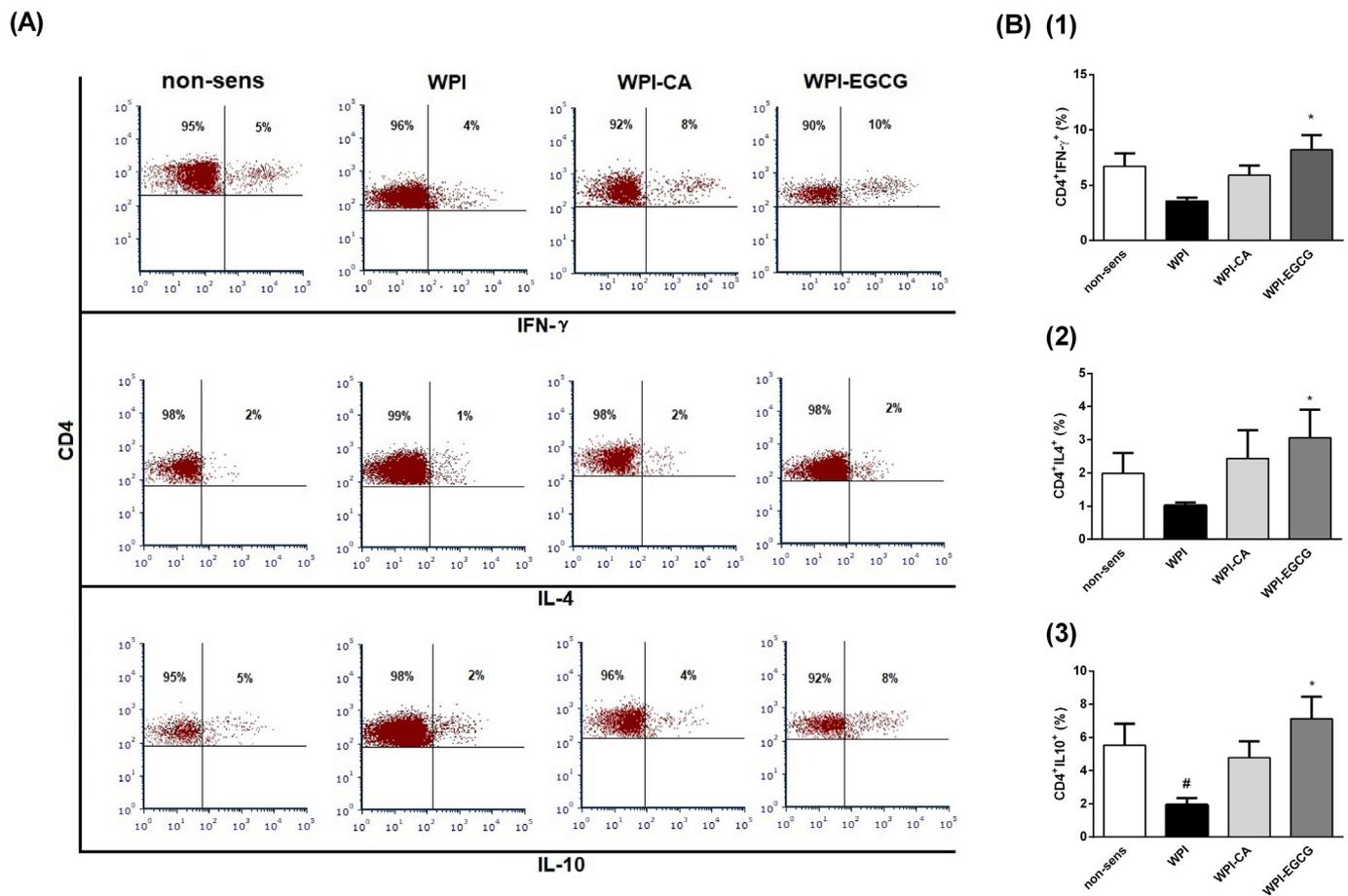


Fig. 4. Effects of WPI-CA and WPI-EGCG complexes oral treatment on the frequency of Th1 and Th2 in CD4⁺ T cells. Panel A: representative dot plots obtained from flow cytometry analysis, CD4⁺ T intracellular cytokines were assessed excluding dead cells using the viability probe (Zombie NIR™) and analyzing the frequencies of CD4⁺ T cells expressing IFN- γ , IL-4 and IL-10 (upper right population on dot plots). Panel B: The frequencies of (1) CD4⁺ IFN- γ ⁺ (Th1); (2) CD4⁺ IL-4⁺ (Th2) and (3) CD4⁺ IL-10⁺ (Th2) producing-cells were expressed as mean \pm SEM (n = 3 to 6). Number sign indicates significant differences ($^{\#}p \leq 0.05$ - Bonferroni's multiple comparisons test) between non-sensitized and sensitized mice (WPI, WPI-CA and WPI-EGCG). Asterisk indicates significant differences ($^*p \leq 0.05$ - Bonferroni's multiple comparisons test) between WPI and WPI-CA or WPI-EGCG sensitized mice.

proliferative effect of WPI-phenolic compounds complexes rather than isolated phenolic compounds. This result suggests that complexation with proteins likely protected the bioactive properties of phenolic compounds during gastrointestinal digestion or even that a synergic anti-proliferative effect of proteins and phenolic compounds are taking place. Moreover, considering that the animals were orally sensitized with WPI or complexes, and CD4⁺ T cell proliferation was investigated *in vitro* by restimulation of splenocytes with WPI (but not with WPI-phenolic compounds complexes), IL-2 or Con A, we consider that WPI-phenolic compound complexes showed an *in vivo* anti-proliferative effect. This effect could be related to the clonal anergy induction on CD4⁺ T cells, or other suppressive tolerogenic mechanisms induced in oral tolerance [5,44].

Allergy is generally considered a disease related to an imbalance of Th1/Th2 immune response toward Th2 profile, which results in the production of IgE [45]. In this sense, we investigated the Th1/Th2 cell functional profile by flow cytometry. WPI-CA did not significantly affect the Th1/Th2 functional profile of splenocytes restimulated with WPI (Fig. 4). Castro-Junior and co-workers showed no differences in the frequency of IL-2-producing CD4⁺ T cells and similar higher levels of IL-2 and IL-10 cytokines between immunized and tolerant mice to ovalbumin (OVA) [6]. The authors suggested that instead of specific inhibition of lymphocyte activity, tolerant mice also showed similar levels of global lymphocyte activity [6]. However, the outcome of T cell activation was qualitatively different [6]. Our results support this idea since WPI-CA oral treatment resulted in a similar functional profile of T

cells. Nonetheless, the animals presented lower levels of WPI-specific antibodies and mMCP-1 than the WPI sensitized group (Fig. 2). Additionally, our results could suggest that the inhibitory effect of WPI-CA on WPI-induced food allergy does not arise from a modulation of Th1/Th2 response, but rather because of Tregs activation.

WPI-EGCG oral treatment, in turn, apart from reducing CD4⁺ T cell proliferation (Fig. 3), also affected Th1/Th2 functional profile through an increase of the CD4⁺ IFN- γ ⁺ Th1, CD4⁺ IL-4⁺ Th2, and CD4⁺ IL-10⁺ Th2 cells (Fig. 4). The high frequency of CD4⁺ IL-4⁺ Th2 cells in this group of mice (Fig. 4) could be the reason why this complex did not significantly drop the specific-WPI IgE and IgG1 levels. During the sensitization phase of allergic diseases, the differentiation and clonal expansion of allergen-specific CD4⁺ Th2 cells producing IL-4 is essential for the induction of B-cell class-switch to the ϵ -immunoglobulin heavy chain and the production of allergen-specific IgE antibody [46,47]. However, limitations in the detection of IL-4 cytokine have to be considered in the analysis of Th2 populations, as previously described [48]. On the other hand, the raised frequency of CD4⁺ IFN- γ ⁺ Th1 and CD4⁺ IL-10⁺ Th2 cells may be protective against allergic reaction and cooperate in the induction of tolerance to allergen [45,49]. Lee and co-workers demonstrated that IFN- γ , when produced during oral tolerance induction, down-regulates migration of T cells to peripheral sites of inflammation [49]. IL-10, in turn, is considered a regulatory cytokine, especially in the presence of TGF- β , and increased secretion of this cytokine has been related to successful allergen desensitization therapies [46,50]. Moreover, IL-10 could reduce the

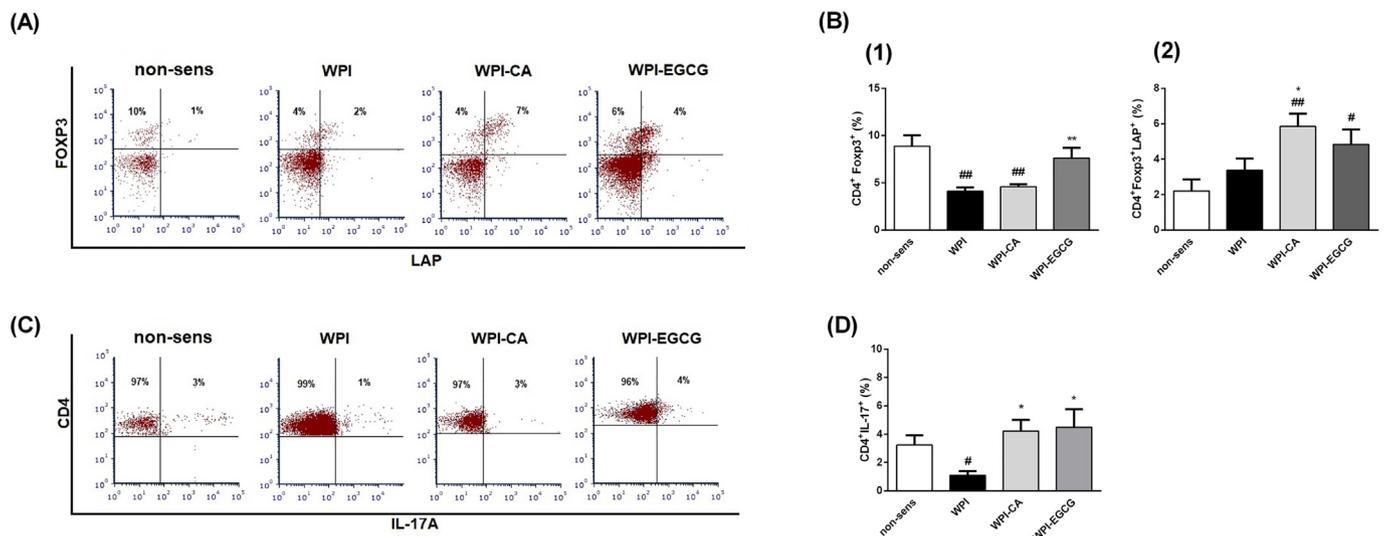


Fig. 5. Effects of WPI-CA and WPI-EGCG complexes on the percentage of Treg and Th17 cells among CD4⁺ T cells in spleens of oral sensitized mice. Panel A: representative dot plots obtained from flow cytometry analysis, CD4⁺ FOPX3⁺ T and CD4⁺ LAP⁺ FOPX3⁺ T cell populations were assessed excluding dead cells using the viability probe (Zombie NIR™) and analyzing the frequencies of CD4⁺ T cells positive to FOPX3 marker only (lower right population on dot plots), and CD4⁺ T cells positive to FOPX3 and LAP⁺ (TGF-β1) (upper right population on dot plots), respectively. Panel C: representative dot plots obtained from flow cytometry analysis of IL17A⁺ CD4⁺ T cell population. Panel B: The frequencies of (1) CD4⁺ FOPX3⁺ T cells, (2) CD4⁺ LAP⁺ FOPX3⁺ T cells, and Panel D: IL-17A⁺ CD4⁺ T cells (Th17) were expressed as mean ± SEM (n = 3 to 6). Number sign indicates significant differences (*p ≤ 0.05 and **p ≤ 0.001 – Bonferroni's multiple comparisons test) between non-sensitized and sensitized mice (WPI, WPI-CA and WPI-EGCG). Asterisk indicates significant differences (*p ≤ 0.05 and **p ≤ 0.001 – Bonferroni's multiple comparisons test) between WPI and WPI-CA or WPI-EGCG sensitized mice.

expression of MHC class II molecules and various co-stimulatory molecules in macrophages and dendritic cells (DC) [51]. Lee and co-workers recently demonstrated that oral administration of *Aloe vera* gel increased the production of IL-10 in mice [45]. The authors associated the increased release of this cytokine to the suppression of OVA-induced food allergic symptoms [45].

Regulatory T cells (Treg) constitute an essential mechanism for the establishment and maintenance of tolerance to self-antigens since these cells suppress both the sensitization and effector phases through different modes of action [46,52]. In the present work, oral treatment of mice with WPI-CA resulted in significant differences in the frequency of CD4⁺ LAP⁺ FOPX3⁺ T cells, while WPI-EGCG orally treated mice presented significant higher frequency of CD4⁺ FOPX3⁺ Treg cells compared to WPI sensitized mice (Fig. 5). These results indicate that both complexes induce oral tolerance by activation of different populations of Tregs. Treg cells suppress immune response directly by cell-cell contact or indirectly by releasing anti-inflammatory cytokines, such as IL-10 and transforming growth factor-β1 (TGF-β1) [6,40]. Tregs can inhibit the antigen-induced activation and degranulation of mast cells and basophils, as well as suppress mast cell FcεRI expression [53]. This could explain the expressive reduction of mMCP-1 in the serum of mice treated with WPI-EGCG without significant reduction of WPI-specific IgE and IgG1 (Fig. 2). Treg cells can still promote the generation of tolerogenic DC cells and avoid DC maturation [54]; impair the infiltration of eosinophils and other effector T cells into inflamed tissues by the release of IL-10 and TGF-β rather than by cell-cell contact-dependent mechanisms [55]. These mechanisms could also be involved in the anti-allergic effect of WPI-CA and WPI-EGCG shown herein.

Recently, it has been suggested that Th17 subset of T cells may play a role in the prevention and management of food allergy, which makes IL-17 a potential biomarker for tolerance to food allergens [9,11]. Dhuban and co-workers reported that food-allergic children presented an impaired Th17 response to antigen stimulation *in vitro* [9]. The raised frequency of IL-17A⁺ CD4⁺ T cells (Th17) in splenocytes of mice treated with both WPI-CA and WPI-EGCG (Fig. 5) could indicate that Th17 cells, at least partially, were involved in the attenuation of allergic reaction. Furthermore, it has recently been suggested that Th17 cells

have plasticity in their functions, *i.e.*, these cells could transdifferentiate into regulatory T cell profile depending on the environmental context [12]. For instance, TGF-β is a key inducer for both FOPX3⁺ Treg and Th17 development [10]. On the other hand, all-trans retinoic acid - a vitamin A metabolite - induces T cell differentiation toward Treg, however, acts as an inhibitor for Th17 skewing [10].

It is important to emphasize that when a new component is added to a food matrix, the digestion process could be modified [56–58]. The complexation with phenolic compounds may have changed the WPI digestion pattern in a way that different peptides were released and absorbed. If different peptides are formed, the immune system may not recognize those peptides as allergens due to the destruction of antigenic regions (epitopes). Moreover, the different peptides released may even work as immunomodulatory compounds. The capacity of whey protein hydrolysates to induce oral tolerance has already been demonstrated [4]. WPI could still have acted as a carrier for CA and EGCG, protecting them from degradation during the gastrointestinal digestion, which might have favored the maintenance of phenolic compounds anti-allergic property, such as the inhibitory effect on mast cell degranulation, observed in the present work [56–58]. Therefore, the possible immunomodulatory properties of digested complexes associated with the anti-allergic properties of phenolic compounds could yield a synergic anti-allergic effect due to the complexation. This might be related to the reduced WPI-specific antibodies and mast cell degranulation without any significant change in Th1/Th2 balance in WPI-CA treated animals. Nevertheless, a detailed investigation of the effect of complexation on the digestion, physicochemical characteristics and bioactive properties of the peptides released, must be carried out to confirm this hypothesis. In addition, it is important to highlight that morphological studies on the intestine were not performed in the present study mainly because of the short time protocol of the animal experiment. The main goal of the present work was to demonstrate that complexation with phenolic compounds can change the oral sensitizing capacity of WPI. However, our study opened a new window of opportunity for further investigations regarding the effect of WPI-phenolic compounds complexes on the allergic response in the intestine.

5. Conclusions

Oral sensitization of mice with WPI-CA attenuated allergic reaction by decreasing levels of WPI-specific IgE and IgG1 in serum, antibodies known for their association to inducing allergic symptoms in mice. Moreover, both WPI-CA and WPI-EGCG were capable of decreasing the levels of mMCP-1 in serum, indicating that the mucosal mast cell activation and degranulation were reduced. Our results suggest that oral treatment with WPI-phenolic compound complexes tends to induce tolerance to WPI through different mechanisms. The WPI-EGCG complex possibly modulates the immune response through the increase of CD4⁺ IFN- γ ⁺ Th1, CD4⁺ IL-10⁺ Th2, CD4⁺ Foxp3⁺ Tregs, and IL-17A⁺ CD4⁺ T cells (Th17). On the other hand, WPI-CA might modulate the allergic reaction by the activation of CD4⁺ LAP⁺ Foxp3⁺ T and IL-17A⁺ CD4⁺ T cells. In conclusion, we suggest that complexation with CA or EGCG can be a promising strategy to reduce the oral sensitization capacity of WPI.

Declarations of interest

None.

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